Abnormalities in T cell lineages from patients with Langerhans cell histiocytosis

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ABSTRACT

Langerhans cell histiocytosis (LCH) is a rare inflammatory disease characterised by lesions containing $CD1a^+$ myeloid lineage 'LCH' cells. Other immune cells such as T cells are also present within LCH lesions and the cytokine milieu suggests T cell activation. T cells have an established role in regulating cellular immunity and there is already evidence that multiple T cell lineages are enriched in LCH lesions, implying that they may have a role in LCH pathogenesis.

$Foxp3^+$ regulatory T cells (Tregs) and the immune suppressive cytokine, transforming growth factor beta (TGF-β), are enriched in lesions and peripheral blood from patients with LCH when compared to healthy individuals. Whilst LCH cells are reported to produce TGF-β, this cytokine can be produced by $Foxp3^+$ Tregs from healthy individuals as a mechanism of cell suppression. Suppression of immune cell activation is a distinctive function of $Foxp3^+$ Tregs, although the precise role of Tregs and their implication in LCH pathogenesis is not established. In the first results chapter, LCH cells, conventional T cells and $Foxp3^+$ Tregs from LCH patients were characterised. The functional competency of LCH lesion Tregs was confirmed by demonstrating that Tregs produced suppressive (TGF-β) rather than inflammatory (tumour necrosis factor and interferon gamma) cytokines *in vitro*. Unusual expression of CD56 by a considerable proportion of Tregs from LCH lesions was identified, alongside a reduction in the proportion of $CD8^+CD56^+$ T cells in total T cells from LCH patients. There was a negative correlation between these T cell populations and overall, the results provide preliminary evidence that Tregs may inhibit important cytotoxic T cell subsets, including unconventional T cells, which often display CD56 on their cell surface.

Similar to $Foxp3^+$ Tregs, unconventional T cells are other examples of T cell lineages with powerful regulatory abilities. They have the ability to enhance inflammatory responses as well as inhibiting them. Unconventional T cell lineages include mucosal-associated invariant T (MAIT) cells, type I natural killer T (NKT) cells and gamma-delta (γδ) T cells. These lineages are associated with inflammatory conditions, although

their importance has not been studied in LCH. In the second results chapter, unconventional T cell lineages from LCH patients were characterised and their role was investigated. Results identified a deficiency in the relative MAIT cell frequency and abnormalities in the subset distributions of each lineage in LCH patients. Abnormalities in unconventional T cell frequencies are associated with immune dysregulation in other disease settings and results from this study highlight that immune dysregulation of MAIT cells in particular may be important in LCH pathogenesis. The lineages investigated in this study could elicit typical inflammatory responses *in vitro* when challenged, suggesting that increasing MAIT cell frequency in LCH could potentially be beneficial to patients.

CD1a is a lipid binding protein that can prime T cells to elicit immune responses. It is almost ubiquitously expressed on the surface of LCH cells and it is a diagnostic indicator for LCH, however its role in the disease is unknown. CD1a is not classically expressed on the surface of mature T cells, however a study by the Kannourakis group identified $CD1a^+$ T cells unique to LCH lesions. The function of this population is currently unknown. Additionally, since LCH cells express CD1a, it is conceivable that they are interacting directly with CD1a-restricted T cells. In the final results chapter, the impact of CD1a expression in LCH was explored. The results of this study indicate that CD1a can be induced on the surface of T cells by activation and this induction is associated with TNF production by those T cells. Furthermore, CD1a-restricted T cells were detectable in LCH lesions and a considerable proportion of these cells displayed the activation marker CD25, indicating that they may be interacting directly with LCH cells and therefore could be involved in LCH pathogenesis.

In conclusion, the results from this study provide a preliminary understanding of how the immune environment of patients with LCH is different to that of healthy individuals. This study is the first to recognise CD1a-restricted T cells, alterations to $CD56^+$ T cell and MAIT cell proportions, as well as the expression of CD56 and the production of TGF-β by $Foxp3^+$ Tregs, from LCH patients. These findings could potentially influence the progression of LCH, thereby highlighting potential targets for new immune based therapies.

STATEMENT OF AUTHORSHIP

This thesis comprises only my original work towards the PhD except where indicated in the **Preface**. Due acknowledgment was given in the main text to all other materials used.

Jenée Mitchell
14/12/18

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Special mention is deserved for my colleagues/friends who took the time to read through my thesis. Thanks **Sharon Olsen**, **Megan Earls** and **Melissa Brown** for showing interest in my project and giving me feedback to polish this document.

Part of this work was supported by a grant funded by the **Histiocytosis Association**, which is a wonderful cause and I am grateful for this opportunity. I am also appreciative of the participation of patients and healthy donors in this study. Likewise, I am thankful for the generous provisions of CD1d and MR1 monomers from the **Godfrey** and **McCluskey laboratories**, respectively. Thank you to **Professor Jan-Inge Henter**, **Dr Egle Kvedaraite** and **Dr Tatiana von Bahr Greenwood**, our collaborators at the Karolinska Institutet (Childhood Cancer Research Unit, Department of Women's and Children's Health) and the Karolinska University Hospital in Stockholm, Sweden, for their provision of LCH patient samples to complete this study, and for their expert knowledge in the LCH field. Likewise, I am delighted that the **Children's Cancer Centre Tissue Bank** at the Murdoch Children's Research Institute and The Royal Children's Hospital (www.mcri.edu.au/childrenscancercentretissuebank) could contribute to our LCH patient sample base and it is noteworthy that the Children's Cancer Centre Tissue Bank is made possible thanks to the support of Cancer In Kids @ RCH (www.cika.org.au), Leukaemia Auxiliary at RCH (LARCH), the Murdoch Children's Research Institute and The Royal Children's Hospital Foundation. The histology team (thanks again **Mel**!) at **Australian Clinical Labs**, St John of God Hospital, Ballarat were also supportive of this study in preparing microscope slides with tissue sections.

Finally, and importantly, I am grateful for my fantastic support network of family and friends, especially **Mum** and **Nana**, who have always encouraged me to pursue my interests. To **Casey** – thanks for sticking by me and always helping me through. This thesis is dedicated to **Stanley Thomas Mitchell** (1927-2016). Thank you for teaching me generosity and determination.

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LIST OF ABBREVIATIONS

° C	Degrees Celsius
%	Percent
2-ME	2-mercaptoethanol
5-OP-RU	5-(2-oxopropylideneamino)-6-D-ribitylaminouracil
7-AAD	7-aminoactinomycin
AD	Active LCH (disease)
ANPEP	Alanyl aminopeptidase, membrane
APC	Antigen presenting cell
BCS	Bovine calf serum
BRAF	B-raf proto-oncogene, serine/threonine kinase
BSA	Bovine serum albumin
BV	Brilliant violet
C	Carbon
CCL	Chemokine ligand
CCR	Chemokine receptor
CD	Cluster of differentiation
CD300LF	CD300 molecule like family member F
CD40LG	CD40 ligand
cDNA	Complimentary deoxyribonucleic acid
CFSE	Carboxyfluorescein succinimidyl ester
CHO	Chinese hamster ovary
Cl	Chloride
CLEC2D	C-type lectin domain family 2 member D
CNS	Central nervous system
Ct	Cycle threshold
CTCM	Complete tissue culture medium
CO_2	Carbon dioxide
CTLA4/CTLA-4	Cytotoxic T lymphocyte-associated antigen-4
CXCL	Chemokine C-X-C motif ligand

CXCR	Chemokine C-X-C motif receptor
DAPI	4',6-Diamidine-2'-phenylindole dihydrochloride
DC	Dendritic cell
DC-LAMP	Dendritic cell-lysosomal associated membrane protein
DDM	Dideoxymycobactin
DN	Double negative
DNA	Deoxyribonucleic acid
E	Glutamic acid
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
Fc	Fragment crystallisable
FITC	Fluorescein isothiocyanate
FOXP3/Foxp3	Forkhead box p3
g	Gravitational force (g-force)
GATA-3	GATA binding protein 3
GM-CSF	Granulocyte-macrophage colony stimulating factor
GOI	Gene of interest
H	Hydrogen
HD	Healthy donor
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HLA	Human leukocyte antigen
HLA-DR	Human leukocyte antigen – DR isotype
HLA-DRA	Major histocompatibility complex, class II, DR alpha
HREC	Human Research Ethics Committee
I-TAC	Interferon-inducible T-cell alpha chemoattractant
ICAM1	Intracellular adhesion molecule 1
ICOS	Inducible costimulatory factor
IFN-γ	Interferon gamma
IL	Interleukin
IL2RA	Interleukin 2 receptor subunit alpha

ITGA4	Integrin subunit alpha 4
ITGAM	Integrin subunit alpha M
ITGAX	Integrin subunit alpha X
IU	International units
JAG2	Jagged 2
K	Potassium
KIR2DL4	Killer cell immunoglobulin-like receptor, 2 domains, long cytoplasmic tail, 4
KRAS	KRAS proto-oncogene, GTPase
LC	Langerhans cell
LCH	Langerhans cell histiocytosis
LIF	Leukaemia inhibitory factor
LIFR	Leukaemia inhibitory factor receptor
LPC	Lysophosphatidylcholine
M	Molar
MAIT cell	Mucosal associated invariant T cell
MAP2K1	Mitogen-activated protein kinase kinase 1
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MHC I	Major histocompatibility complex class I
MHC II	Major histocompatibility complex class II
min	Minutes
MIP-3α	Macrophage inflammatory protein-3 alpha
mL	Millilitres
mM	Millimolar
MR1	Major histocompatibility complex related protein 1
mRNA	Messenger ribonucleic acid
Na	Sodium
NAD	Non-active LCH (disease)
NCAM1	Neural cell adhesion molecule 1

ng	Nanograms
NK cell	Natural killer cell
NKT cell	Natural killer T cell
nM	Nanomolar
NRAS	NRAS proto-oncogene, GTPase
O	Oxygen
P	Phosphate
PB	Peripheral blood
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death protein-1
PDCD1LG2	Programmed cell death-1 ligand-2
PD-L1	Programmed cell death-1 ligand-1
PE	Phycoerythrin
pg	Picograms
PHA	Phytohaemagglutinin
PLZF	Promyelocytic leukaemia zinc finger
PMA	Phorbol 12-myristate 13-acetate
qPCR	Real-time quantitative polymerase chain reaction
RAF	Proto-oncogene, serine/threonine kinase
RANTES	Regulated on activation, normal T cell expressed and secreted
RAS	Proto-oncogene, GTPase
RBC	Red blood cell
RNA	Ribonucleic acid
RPLP0	Ribosomal protein lateral stalk subunit P0
RPMI	Roswell Park Memorial Institute
SIGLEC1	Sialic acid binding immunoglobulin like lectin 1
TCR	T cell receptor
TGFB1	Transforming growth factor beta 1
TGF-β	Transforming growth factor beta
Th	T helper
TNF	Tumour necrosis factor

Treg	Regulatory T cell (Foxp3^{+})
Tresp	T responder
TSA	Tyramide signal amplification
V	Valine
v/v	Volume per volume
WBC	White blood cell
WTA	Whole transcriptome amplification
α	Alpha
β	Beta
γ	Gamma
Δ/δ	Delta
µg	Micrograms
µL	Microlitres
µM	Micromolar

PREFACE

This thesis is original, independent work by the author, Jenee M. Mitchell.

A version of **Chapter 1** was published as 'A potentially important role for T cells and regulatory T cells in Langerhans cell histiocytosis' (Mitchell, J. M., Berzins, S. P. & Kannourakis, G) in *Clinical Immunology* (for manuscript see **Appendix B** or https://doi.org/10.1016/j.clim.2018.06.004).

A version of **Chapter 4** was prepared as a manuscript, 'Foxp3^{+} regulatory T cells from Langerhans cell histiocytosis lesions co-express CD56 and produce transforming growth factor beta' to be submitted for publication (for current version of manuscript see **Appendix B**).

A version of **Chapter 5** was published as 'Altered populations of unconventional T cell lineages in patients with Langerhans cell histiocytosis' (Mitchell, J.M., Kvedaraite, E., Bahr Greenwood, von, T., Henter, J.-I., Pellicci, D. G., Berzins, S. P. & Kannourakis, G) in *Scientific Reports* (for manuscript see **Appendix B** or http://doi.org/10.1038/s41598-018-34873-y).

My contribution to the review article was writing the manuscript and creating the figures, which were critically reviewed by Stuart P. Berzins and George Kannourakis.
My contribution to the research manuscripts was performing the experiments, analysing the results, preparing the figures and writing the manuscripts. I also contributed to designing the experiments alongside Stuart P. Berzins and George Kannourakis. Tatiana von Bahr Greenwood, Egle Kvedaraite, Jan-Inge Henter and George Kannourakis recruited patients and provided tissue samples and clinical information. Stuart P. Berzins and George Kannourakis critically revised the manuscripts and Tatiana von Bahr Greenwood, Egle Kvedaraite, Daniel G. Pellicci and Jan-Inge Henter reviewed the manuscripts. Stuart P. Berzins and George Kannourakis led the investigations.

PUBLICATIONS RELATED TO THESIS

1. Mitchell, J.M., Kvedaraite, E., Bahr Greenwood, von, T., Henter, J.-I., Pellicci, D. G., Berzins, S. P. & Kannourakis, G. Altered populations of unconventional T cell lineages in patients with Langerhans cell histiocytosis. *Sci. Rep.* **8,** 16506 (2018).
2. Mitchell, J. M., Berzins, S. P. & Kannourakis, G. A potentially important role for T cells and regulatory T cells in Langerhans cell histiocytosis. *Clinical Immunology* **194,** 19–25 (2018).
3. West, J. A., Olsen, S. L., Mitchell, J. M., Priddle, R. E., Luke, J. M., Åkefeldt, S. O., Henter, J.-I., Turville, C. & Kannourakis, G. Polyclonal T-cells express CD1a in Langerhans cell histiocytosis (LCH) lesions. *PLoS ONE* **9,** e109586 (2014).

PRESENTATIONS

60th American Society of Hematology Annual Meeting, San Diego (2018)

Co-expression of CD56 and production of transforming growth factor beta by Foxp3 regulatory T cells from Langerhans cell histiocytosis lesions. Abstract published in *Blood*.

Mitchell, J.M., Kvedaraite, E., von Bahr Greenwood, T., Henter, J-I., Pellicci, D.G., Berzins, S.P. & Kannourakis, G.

33rd Annual Meeting of the Histiocyte Society, Singapore (2017)

The role of regulatory T cells in the immune regulation of Langerhans cell histiocytosis (poster). Abstract published in *Pediatric Blood & Cancer*.

Mitchell, J.M., Kvedaraite, E., von Bahr Greenwood, T., Henter, J-I., Pellicci, D.G., Berzins, S.P. & Kannourakis, G.

Federation University Australia Higher Degrees by Research Conference, Ballarat, Victoria (2017)

Immune regulation by T cells in Langerhans cell histiocytosis (oral presentation).

Mitchell, J.M.

International Congress of Immunology, Melbourne, Victoria (2016)

The role of T cells in the immune regulation of Langerhans cell histiocytosis (poster). Abstract published in *European Journal of Immunology*.

Mitchell, J.M., Pellicci, D.G., Berzins, S.P. & Kannourakis, G.

Federation University Australia Higher Degrees by Research Conference, Ballarat, Victoria (2016)

The role of T cells in the immune regulation of Langerhans cell histiocytosis (oral presentation).

Mitchell, J.M.

CD1-MR1 Conference, Lorne, Victoria (2015)

CD1 expression on activated T cells (poster).

Mitchell, J.M., Godfrey, D.I., Pellicci, D.G., Berzins, S.P. & Kannourakis, G.

31st Annual Meeting of the Histiocyte Society, Athens, Greece (2015)

The role of T cells in the immune regulation of Langerhans cell histiocytosis (poster).

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1.0. LITERATURE REVIEW

1.1. General classification of cellular immune responses

Immune homeostasis is regulated by a complex arrangement of cells, tissues and molecules, which have the collective ability to combat disease (**Figure 1.1**). In humans, the immune system essentially comprises two distinct arms that work together to prevent pathogenesis and the development of disease[1,2]. Innate immunity is an ever present and immediate defence against disease causing agents, namely, pathogens. Specific mechanisms of innate immunity include physical and chemical barriers such as the skin and mucosa[3]. Innate immunity also includes cells such as phagocytes, whose role is to engulf and degrade pathogens[4], and natural killer (NK) cells, which are able to secrete perforins and granzymes to perforate and lyse infected target cells[5,6]. These cells discriminate between self and non-self by non-specific pathogen recognition mechanisms such as microbial pattern recognition receptors, or altered molecules on infected cells[2]. Innate immune cell activation prompts the recruitment of adaptive immune cells that can in turn further enhance the immune response[1,2]. T cells and B cells, which are lymphocytes of the adaptive immune system, are collectively able to recognise a vast array of antigens presented by specialised antigen presenting cells (APCs)[1]. They are able to do so via expression of highly specific T cell and B cell antigen receptors which are almost unique to each individual cell prior to antigenic exposure[1]. Following antigen exposure, the responding T cell and/or B cell clones that recognise the specific antigen will proliferate in order to coordinate the immune response and eventually establish a memory pool, that will provide a rapid immune response upon repeat exposure to the same antigen[1]. This project focusses heavily on T cells (see **Section 1.3**) and also investigates their interactions with APCs, which are elaborated upon in **Section 1.2**.

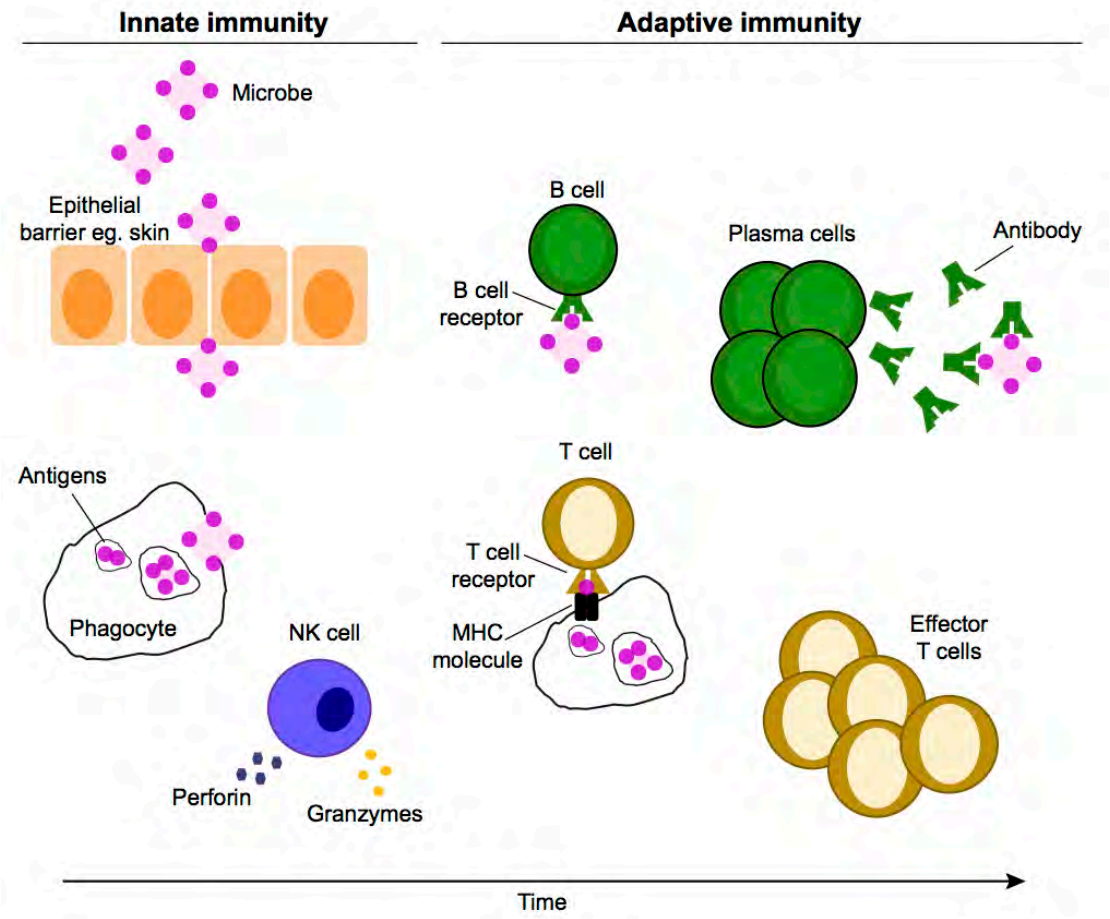


Figure 1.1. Summary of cellular immune responses

Tissue resident cells of the innate immune system such as phagocytes and NK cells are the first lines of defence against pathogens such as microbes if the initial epithelial barrier is breached. Phagocytes engulf and degrade microbes and display microbial antigens on their cell surface on major histocompatibility complex (MHC) molecules. NK cells can perforate and lyse infected target cells by secreting perforin and granzymes. Following innate immunity, B cell and T cell receptor recognition of microbial antigens is a major constituent of the signalling necessary to elicit clonal T cell and B cell proliferation, induce effector functions and clear infections.

1.2. The role of DCs in immunity

Many cell types have the ability to present antigen to T cells. Professional APCs include, but are not limited to macrophages, monocytes and dendritic cells (DCs), all of which are phagocytic in nature and major constituents of the innate immune system[7-9]. These cell lineages typically express major histocompatibility complex class II (MHC II) molecules, which bind to and present captured peptide antigens to T cells via the T cell receptor (TCR)[10]. Importantly, DCs are regarded as the most efficient of

all APCs for promoting T cell activation and have a key role in linking innate and adaptive immunity[11] by presenting foreign antigens to T cells via their TCRs.

DCs comprise a heterogeneous group of immune cells found in all tissues, however they are particularly concentrated at interface tissues such as the skin and gut, and within lymphoid organs[12,13]. Human lineage DCs can be defined by their expression of the cell adhesion molecule, integrin CD11c and the MHC II receptor, human leukocyte antigen-DR (HLA-DR)[14].

DCs can be classified into subsets including myeloid lineage DCs (otherwise known as classical DCs) and Langerhans cells (LCs). Myeloid DCs are found in all lymphoid, connective and interface tissues and they sample and process tissue antigens and subsequently migrate to the lymph nodes to present these antigens, potentially eliciting a T cell mediated response toward the specific pathogen or tolerance toward a self-antigen[15,16]. LCs are a highly specialised subset of DCs, usually located in the epidermis, which is one of the first points of contact for pathogens that breach the skin barrier[12,17]. Similar to DCs, LCs can be characterised by their expression of CD11c and MHC II. Additionally, they express CD1a, which is an MHC-like molecule[18] (structure discussed in detail in **Chapter 6**) that presents lipid antigens to T cells. LCs also express CD207, otherwise known as langerin, which is a calcium-dependent glycan-binding protein suggested to regulate non-conventional antigen internalisation[17]. LCs and classical DCs are alike in that they both maintain tissue homeostasis by local proliferation and cell death[12]. Murine classical DCs can however be replenished by blood derived precursors[19], although murine LCs maintain tissue homeostasis unaided by circulating precursors, but upon severe skin inflammation they can be repopulated by circulating precursors[20].

1.3. The role of T cells in immunity

T cells collectively have a central role in cell-mediated immunity and can be broadly categorised into the major subsets of $CD8^+$/cytotoxic T cells and $CD4^+$/T helper (Th) cells. $CD8^+$ T cells are typically activated by antigens presented via MHC I molecules

and upon exposure (**Figure 1.2**), $CD8^+$ T cells can directly kill infected cells[21]. In contrast, $CD4^+$ T cells are activated via MHC II antigen presentation (**Figure 1.2**), which will subsequently induce signalling and recruitment of other inflammatory or anti-inflammatory immune cells to the location[22].

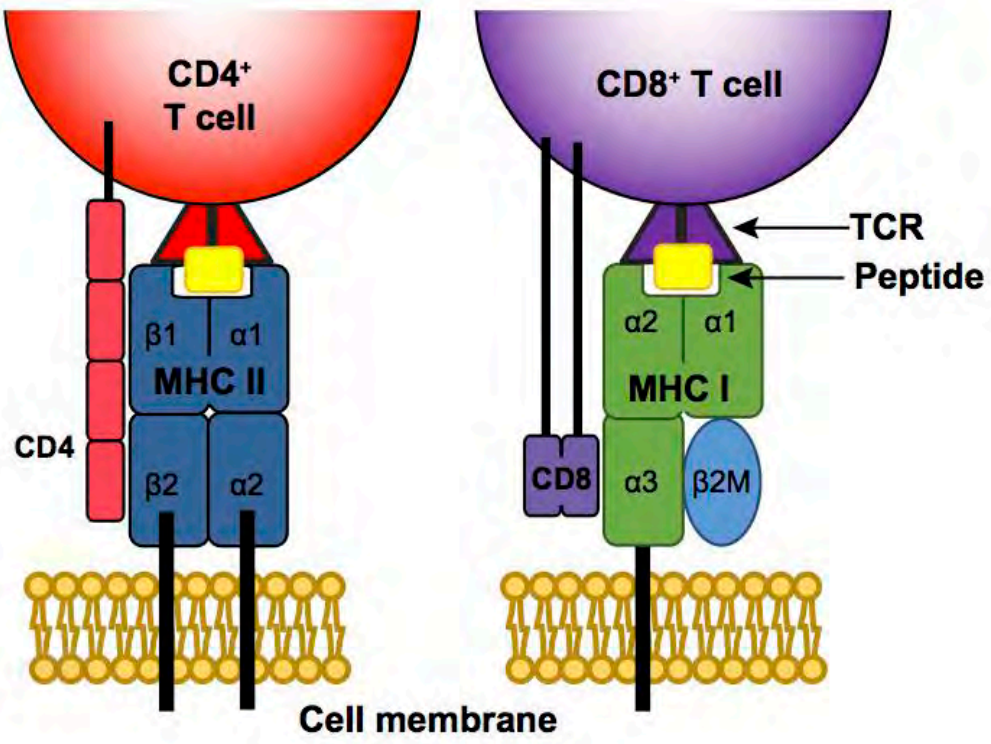


Figure 1.2. Antigen presentation by MHC molecules to TCRs

MHC structures are membrane bound complexes that present peptide antigens to T cells via the TCR. CD8 associates with MHC I, while CD4 associates with MHC II.

There are many functionally distinct subsets of T cells with specific cytokine and transcription factor expression profiles. Th1 cells express the transcription factor T-bet along with cytokines interleukin-2 (IL-2) and interferon gamma (IFN-γ), whereas Th2 cells express the transcription factor GATA-3 and cytokines IL-4, IL-5 and IL-13[22]. A Th1 response is a pro-inflammatory response that induces cell mediated immunity and phagocyte-dependent inflammation[22,23]. IL-2 expression enables T cell survival and proliferation[24], while IFN-γ activates macrophages to destroy pathogens[25]. Th1 cells can also regulate tissue injury by release of these cytokines and recruit cytotoxic T cells to kill using cytolytic molecules such as perforin to attack cell membranes and granzymes to induce programmed cell death[22]. In contrast, a Th2 response is a

somewhat anti-inflammatory response that occurs in infection with parasites, allergic responses and asthma[22,23]. IL-4 produced by Th2 cells inhibits pro-inflammatory responses[23,26], while IL-5 activates eosinophils, which are specialised leukocytes that assist in parasite protection, and are also implicated with allergy[23,27]. Additionally, IL-13 modulates macrophage activity[28]. There are more categories of Th immune responses, each with a varied role in immunity that include (but are not limited to) Th9 cells, Th17 cells, Th22 cells and follicular Th cells.

T cells can be further characterised as naïve, activated or memory cells depending on their cell surface protein expression[22]. Naïve T cells are mature circulating cells with a highly diverse TCR repertoire and the collective potential to recognise an extraordinary array of pathogens, though they have not yet encountered their specific antigen[22,23]. Activated or effector T cells have been stimulated and once the stimulus is gone, most of these cells die, although some survive and become circulating or tissue resident memory cells ready to quickly counteract a secondary infection[21-23].

Finally, there are specialised T cell subsets that have a governing role in cell-mediated immunity and these encompass several subsets such as $Foxp3^+$ regulatory T cells (Tregs) along with a group of unconventional or 'innate-like' T cells including non-MHC restricted mucosal associated invariant T (MAIT) cells, natural killer T (NKT) cells and gamma-delta (γδ) T cells. Interest in the role of unconventional T cells in immune homeostasis is growing because these cells are considered to act at the boundaries of innate and adaptive immunity. These and other regulatory T cell lineages will be discussed further and in the context of Langerhans cell histiocytosis in **Section 1.4** and beyond.

1.4. Introduction to Langerhans cell histiocytosis

Langerhans cell histiocytosis (LCH) is commonly characterised by lesions containing myeloid lineage cells that co-express CD1a and CD207 that are referred to as LCH cells. Studies on these presumably pathogenic LCH cells have produced a reasonable understanding of the basic biology and origins of this disease and led to improved

treatment options for many patients, although morbidity, mortality and associated sequelae still persist. In addition to LCH cells, the lesional microenvironment contains an inflammatory infiltrate of T cells, macrophages, eosinophils, neutrophils, B cells, plasma cells and multinucleated giant cells[29,30], whose importance is not well defined (**Figure 1.3** and **Figure 1.4**). The LCH lesion cellular infiltrate and cytokine milieu is collectively central to the inflammatory microenvironment and resultant organ damage, so while the LCH cells are important, it is likely that other immune cells within LCH lesions contribute to the progression of LCH and may be potential targets for new immune based treatments.

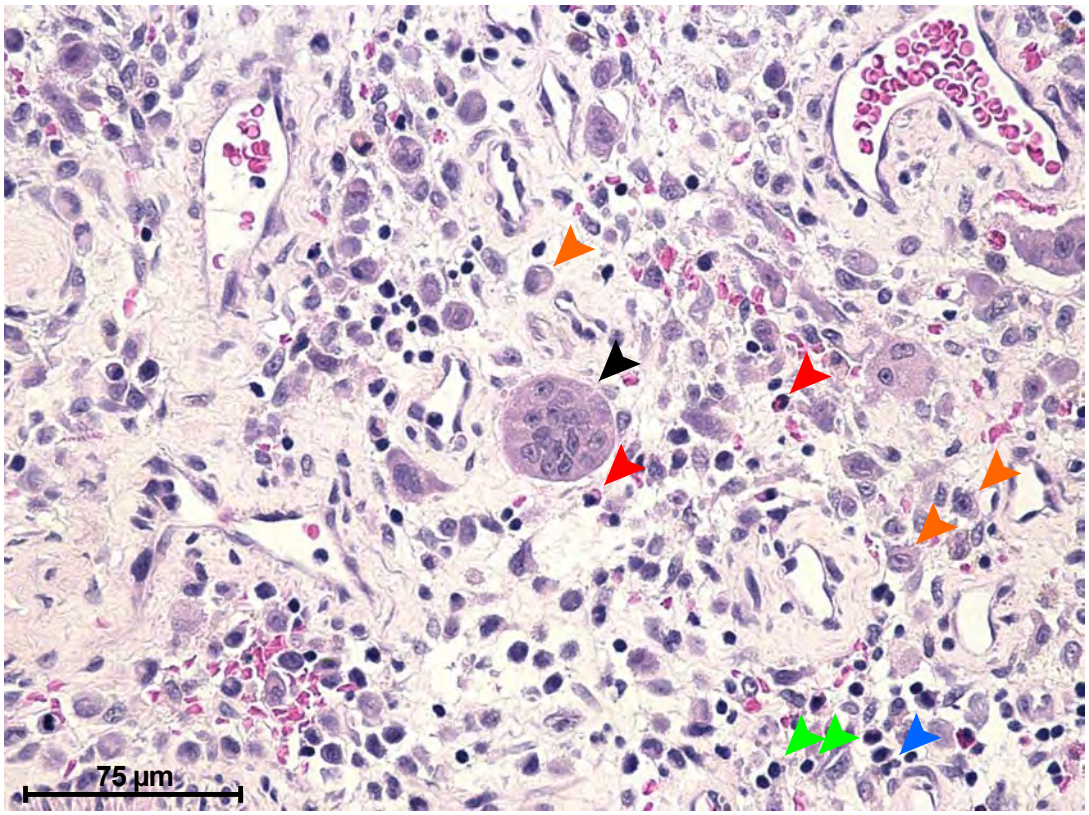


Figure 1.3. Histology of LCH lesions.

A haematoxylin and eosin stained formalin-fixed paraffin-embedded tissue section of a characteristic LCH lesion, containing a variety of immune cells including histiocytes (orange arrows), lymphocytes (green arrows), neutrophils (blue arrow), eosinophils (red arrows) and multinucleated giant cells (black arrow). The term histiocyte encompasses LCH cells, monocytes, macrophages, DCs and LCs.

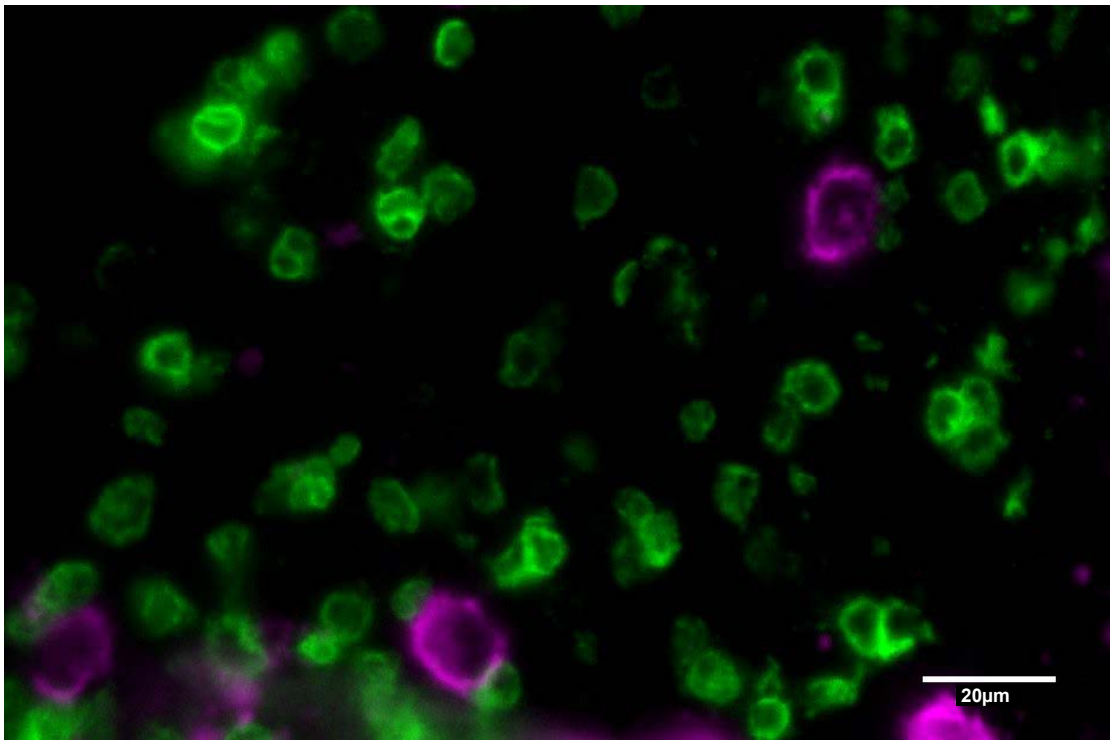


Figure 1.4. LCH cells and T cells in LCH lesions.

An immunofluorescent stained formalin-fixed paraffin-embedded tissue section of an LCH lesion containing CD1a$^+$ LCH cells (magenta), and CD3$^+$ T cells (green).

The inflammatory microenvironment of lesions suggests that failure of immune regulation could be an important factor in LCH development and progression. T cell subsets with a regulatory function including Foxp3$^+$ Tregs and γδ T cells have already been identified as having abnormal frequencies in LCH patients, and other unconventional T cells with regulatory roles, including type I NKT cells, MAIT cells and CD1a-restricted T cells have not been studied at all. These so-called immuno-regulatory cells influence other immune cells through potent cytokine release and cell-cell contact, including regulating the recruitment, activation or function of immune cells such as cytotoxic and/or Th cells (**Figure 1.5**). They are therefore prime candidates for influencing the inflammatory setting of LCH lesions. There are several specialised lineages of T cells with regulatory functions that can promote or inhibit antigen-specific and antigen-independent immune responses in cancer, infection and autoimmunity, and their influential roles make them appealing immunotherapy targets in diseases where immune dysregulation is a factor. The precise role of these

cells in LCH remains poorly understood, but they could conceivably promote or inhibit the development of lesions. A better understanding of the role of regulatory T cell subsets in LCH will improve our understanding of the causes and progression of inflammatory lesions in LCH.

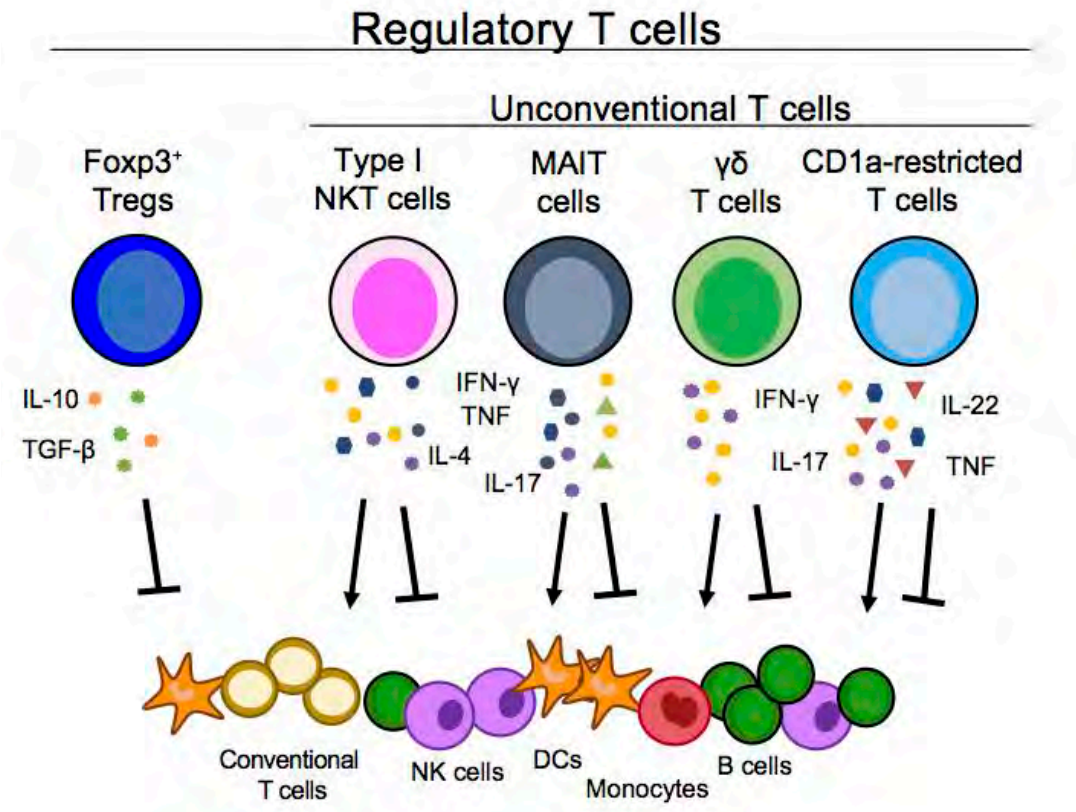


Figure 1.5. Regulatory T cells can inhibit or promote the response of other immune cells.

Regulatory T cells including Foxp3$^+$ Tregs and unconventional T cells such as type I NKT cells, MAIT cells, γδ T cells and CD1a-restricted T cells can influence the recruitment, activation and functional status of other immune cells such as conventional T cells, NK cells, DCs, monocytes and B cells through cytokine release.

1.5. What we currently know about LCH

LCH is a rare and multifaceted disease of unknown cause[31,32]. The disease occurs mostly in children with an incidence of 4-5 cases per million per year in those under 15 years with more frequent detection in the 0-3 year age group[32], although it has also been suggested that the incidence is higher (8.9/10^6 cases)[33]. LCH also affects 1-2 adults per million each year (most commonly young adults) but may be underdiagnosed in this population[32,34]. LCH lesions are caused by an inflammatory

infiltrate, which includes (amongst other immune cells) presumably pathogenic LCH cells, and T cells[35], which are the focus of this project.

LCH lesions can be unifocal or multifocal involving one or more organs. The clinical outcome for LCH is diverse, ranging from a single lesion where there is usually a good prognosis, to disseminated disease and mortality, with many intermediate stages. The liver, spleen and bone marrow are considered high risk organs[36], as lesional infiltration of these sites can result in organ dysfunction. For patients with risk organ involvement, the mortality rate is approximately 15%[37]. Other commonly occurring sites for lesions are within osseous, cutaneous, pulmonary and pituitary tissue, and infiltration of bone and skin has a better prognosis than infiltration of the 'risk organs'. Pituitary lesions often cause central diabetes insipidus, and neurological problems such as neurodegenerative central nervous system (CNS) disease are also common with CNS infiltration[32,38]. Orthopaedic abnormalities and hearing loss are other commonly acquired sequelae[39].

1.5.1. Treatment options for LCH

Treatment of LCH varies depending on the severity and location of lesions. Patients with low risk lesions may only require observation, while those with high risk lesions usually undergo surgical removal of lesions and/or systemic or topical administration of steroids in conjunction with chemotherapy[40]. A combination of prednisolone and vinblastine[41] has been used to treat LCH since 1966 with an overall response rate of 60%[42]. The mechanism of this treatment is not completely understood, but could be due to an immunosuppressive impact on the LCH cells within the microenvironment. Radiation is typically a last resort treatment, particularly for children[41], and while it can improve or stabilise lesion-related symptoms in most LCH patients, short- and long-term morbidity is often observed[40]. Etoposide chemotherapy has been used for systemic disease but has high toxicity and may contribute to the development of myelodysplastic syndrome[41,43]. A trial of cladribine and cytosine-arabinoside salvage therapy for 'risk-organ' LCH patients with sub-optimal responses to standard treatment showed an excellent response rate, although all patients experienced

severe toxicity[44]. Most recently BRAF and MEK inhibitors have been trialled in LCH and are discussed further in **Section 1.5.3**. Better treatment options are still required, along with the need for earlier detection to reduce life-long sequelae in LCH patients.

1.5.2. Aetiology of LCH

LCH is a histiocytic disease, meaning it is one of a group of rare and varied proliferative disorders derived from DCs and macrophages[45]. Cells of the histiocyte lineage include professional antigen-processing and presenting cells such as monocytes, macrophages, DCs and LCs[29]. LCH was first reported around 1900 and was originally referred to as Hand-Schüller-Christian disease and Letterer-Siwe disease[36]. Hand-Schüller-Christian disease was described and diagnosed in young children as eosinophilic granulomatous lytic bone lesions, diabetes insipidus and exophthalmos or abnormal eyeball protrusion[46], while Letterer-Siwe disease was a more systemic and invasive disease often involving the liver, spleen, bone marrow or skin, and commonly resulting in mortality[46]. Due to the pathological similarities in the two diseases, they were unified as histiocytosis X by Farber and Lichtenstein in 1953, with the 'X' representing a cell of unknown origin[29,46-49]. Decades on langerin-dependent Birbeck granules, which are unusual rod or 'tennis racket' shaped organelles that are characteristic of LCs[50], were identified in LCH lesions[51]. Consequently, LCH cells were described as the progeny of migrating mature epidermal LCs and histiocytosis X became 'LCH'[51]. Most recently, however, it was hypothesised that LCH cells instead arise from an early myeloid precursor cell[49].

LCH is diagnostically defined by immunohistochemical detection of $S100^+$ and $CD1a^+$ (or $CD207^+$) LCH cells (histiocytes)[29,36,45]. Additionally, the cells can be recognised by their unique reniform or 'coffee-bean' shaped nuclei[46]. Whether the LCH cells are neoplastic or the result of an immune dysfunction is still somewhat contentious. Historically LCH cells were identified as clonal using X-inactivation studies[52,53], however several groups have highlighted the shortcomings of using this approach to confirm clonality due to skewed X-inactivation[54,55]. Immune dysregulation has also been suggested as a potential cause for LCH because of the inflammatory nature of

lesions[30,56-58]. The most recent etiologically relevant finding is the association of RAS/RAF/MEK/ERK cell signalling pathway mutations by the detection of somatic *BRAF V600E* and *MAP2K1* protein kinase gene mutations within LCH cells[48,59-64]. This association is pertinent because it gives weight to the 'misguided myeloid DC precursor' hypothesis that describes LCH cells as immature DCs derived from early myeloid precursors[13,65].

1.5.3. The significance of mutations in LCH

BRAF V600E, a mutation in the RAS/RAF/MEK/ERK pathway, accounts for 90% of all *BRAF* mutations in human cancers[66,67] and is also the most common *BRAF* mutation identified to date in LCH lesions[48,59-61]. Approximately 50% of LCH lesions reportedly contain the *BRAF V600E* mutation within LCH cells[68]. *BRAF V600E* substitutes amino acid valine in position 600 with glutamic acid, stimulating downstream signal transduction of MEK and ERK protein kinases constitutively, which can in turn activate transcription factors to promote uncontrolled cell division[59,60,63,66,69]. The detection of *BRAF V600E* in LCH may be helpful in the treatment of this disease. Patients treated with targeted therapy to inhibit the BRAF protein show encouraging early results[70], although the long-term effects of these drugs, which could conceivably result in severe consequences when administered to children, is currently unknown[71].

Mutations in other protein kinase genes affecting the RAS/RAF/MEK/ERK signalling pathway were subsequently discovered in LCH cells. *MAP2K1* mutations were detected in *BRAF* wild type LCH lesions, meaning that the *BRAF* and *MAP2K1* mutations were found in mutually exclusive lesions[62-64] (**Figure 1.6**). These account for a further 27.5% of LCH lesions harbouring MAPK pathway mutations and suggests that MAPK pathway mutations are reported in almost 80% of LCH cases. All mutations in the *MAP2K1* gene result in a similar outcome to *BRAF V600E*, and are associated with constitutive signal transduction and oncogenesis[62,63]. Other RAS/RAF/MEK/ERK pathway mutations that have been detected infrequently in LCH lesions include *KRAS* and *NRAS* mutations, a *BRAF* gene fusion and small in-frame *BRAF* deletions in lesions lacking *BRAF V600E* and *MAP2K1* mutations[48,59,63,72-75]. This suggests that there may

be other MAPK pathway mutations in wild type lesions that are yet to be detected. These findings could potentially account for the constitutive activation of ERK that is seen in LCH cells[60] and the activation of LCH cells could in turn affect immune regulation in LCH lesions. RAS/RAF/MEK/ERK cell signalling pathway mutations are known to affect DC differentiation[76,77] and maturation[77] and if this occurs within LCH lesions, T cell responses may be directly affected within the microenvironment. Given this, treatment with BRAF or MEK inhibitors, which target LCH cells, could also have a considerable effect on the T cells within LCH lesions.

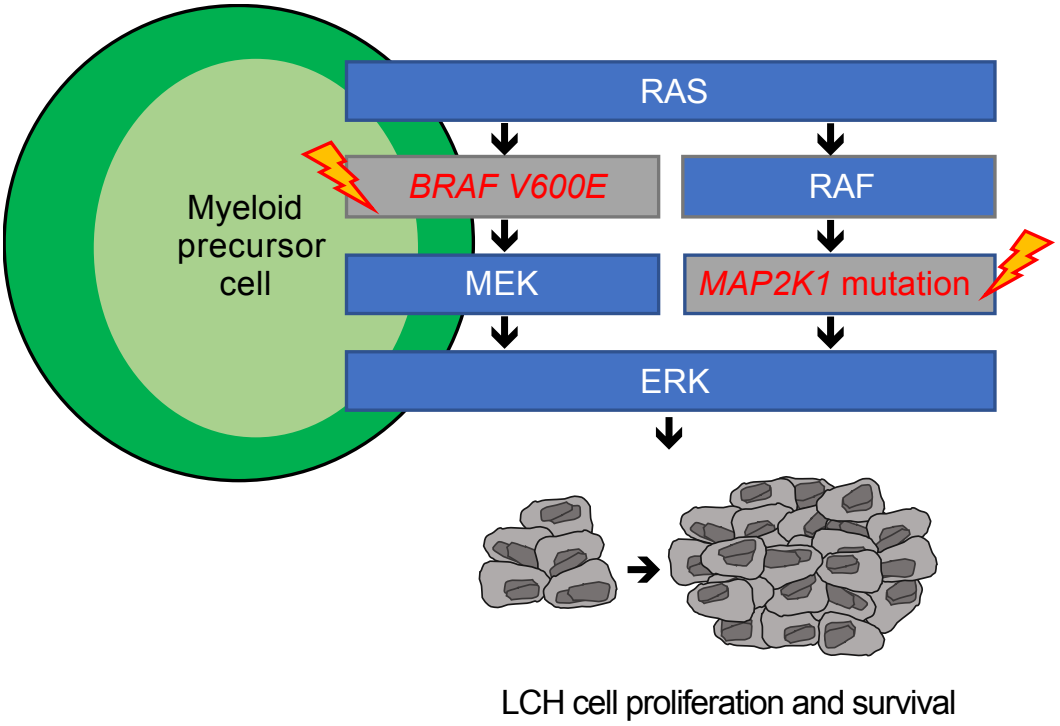


Figure 1.6. RAS/RAF/MEK/ERK cell signalling pathway mutations in LCH.

Myeloid precursor cells with various mutations in the MAPK pathway may result in increased LCH cell proliferation and/or survival.

The detection of RAS/RAF/MEK/ERK cell signalling mutations in LCH cells suggests that LCH is an inflammatory myeloid neoplasm and aligns with the 'misguided myeloid DC precursor' hypothesis[32,45,47,78,79], although opinions vary in the LCH field[80]. The hypothesis supports the idea that mutations arise at different stages of DC lineage commitment. In patients with low risk disease, mutations are contained within lesions, whereas in patients with high risk disease mutations have been identified in the blood

and bone marrow[47]. This suggests that low risk disease occurs as a result of a mutation in a tissue localised committed DC precursor or monocyte, whereas high risk disease is the result of a mutation in either a bone marrow derived $CD34^+$ stem cell or progenitor, or a committed DC precursor or monocyte in the blood or bone marrow[47,78]. Additionally, it is thought that in self-healing, congenital skin-limited LCH, mutations occur within the lineage pathway of epidermal LCs[46,81]. There is also a CNS/neurodegenerative component of LCH, which is difficult to study and diagnose pathologically because of its location and because typical $CD1a^+$ and/or $CD207^+$ LCH cells can be absent from the inflammatory sites[82]. *BRAF V600E* was detected in myeloid/monocytic cells from brain biopsies and also in peripheral blood mononuclear cells (PBMCs) from patients with CNS-LCH very recently[83]. Similarly, late-onset neurodegenerative LCH was shown to harbor mutations in microglial or erythro-myeloid progenitor lineage cells[84].

1.6. T cells potentially involved in LCH immune regulation

LCH cells make up 36-58% of lesional cells[29] and share common features with epidermal LCs and activated LCs in their expression of CD1a, CD207 and T cell costimulatory molecules, however a distinguishing feature is their lower surface expression of the MHC II molecule, HLA-DR[85,86]. The LCH microenvironment typically contains pro-inflammatory cytokines consisting of high levels of tumour necrosis factor (TNF), IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1beta (IL-1β), IL-2 and leukaemia inhibitory factor (LIF)[30,45,56,65,87]. Kannourakis and Abbas[30] reported that cytokines detected within LCH lesions reflect local T cell activation. In line with this, Egeler et al.[56] demonstrated that both T cells and LCH cells within LCH lesions produced high levels of cytokines. Locally produced chemokine ligands (CCLs) suspected to be produced by LCH cells, such as CCL20, CCL5 (RANTES) and chemokine C-X-C motif ligand (CXCL) 11 (I-TAC) have also been suggested to contribute to the accumulation of T cells within lesions (unpublished data reported in study by Annels et al.[88]).

The *in vitro* addition of T cell costimulatory molecule CD40 ligand-transfected fibroblasts to LCH cells was shown to induce surface expression of HLA-DR and CD86 on LCH cells and it also improved the allogeneic response of T cells towards LCH cells[85]. This suggests that the presence of CD40 ligand and presumably the resultant engagement of CD40 on LCH cells, induces LCH cell maturation, however T cells within lesions have been shown to express CD40 ligand[89]. This indicates that the LCH lesions may facilitate a microenvironment in which immune regulatory cell responses such as T cell responses are inhibited.

T cells are heavily involved in anti-tumour immune responses, however tumours can evade the immune system by expressing inhibitory molecules. If we consider LCH to be neoplastic disease, we can speculate whether LCH cells make adaptations that result in an immune system evasion. T cells have a central role in cell-mediated immunity and their interactions with APCs are an important component of immune regulation. Antibodies to T cell costimulatory molecules such as cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and programmed cell death protein-1 (PD-1) have been used as immunotherapy drugs and have shown a good clinical response in the treatment of various cancers[90-92]. LCH cells have low surface expression of HLA-DR, which is important for antigen-dependent $CD4^+$ T cell activation[85,86]. Although immune cells such as T cells are being recruited to the site and contributing to the inflammation, they may be unable to completely recognise and destroy LCH cells within the microenvironment because of reduced receptor-ligand interactions or inhibitory signalling such as PD-1-PD-L1 signalling. Investigating T cells with a regulatory function may identify how inflammation is established and maintained in LCH lesions and how LCH cells are evading the immune system. Defining this process is likely to lead to new possibilities for using targeted therapies to manipulate the immune system into tumour immunity.

1.6.1. $CD1a^+$ T cells

The Kannourakis group identified a previously unreported subset of T cells expressing CD1a in LCH, although the function of these cells was not described[31]. CD1a is not

normally expressed by mature T cells, however it is expressed by immature thymocytes and downregulated in the latter stages of T cell development[93]. More recently, a similar finding of CD1a expression by human skin-derived NK-like cells called group 2 innate lymphoid cells was reported[94]. Abnormal CD1a expression on immature T cells in malignancies[95,96] and on extrathymic T cells in tonsil tissue[97] have been reported, however the presence of polyclonal $CD1a^+$ T cells within LCH lesions is the only published example (to the best of my knowledge) of CD1a expression by mature T cells[31]. These cells were not detected in the peripheral blood from LCH patients or in blood or tonsil tissue from healthy donors, but instead appeared restricted to the lesions[31] where inflammation may have induced them. The functional significance of $CD1a^+$ T cells is unknown and their functional capacity has not been studied. They are intriguing because there is already good evidence of specialised T cells subsets having an important role in LCH. For example, there are several reports of enriched $Foxp3^+$ Tregs in the peripheral blood and lesions from patients with LCH[57,65,98-100], and unconventional γδ T cells were also detected in high numbers in cutaneous LCH[101]. The significance of these cells and the potential involvement of other unconventional T cell subsets is discussed next.

1.6.2. $Foxp3^+$ Tregs

$Foxp3^+$ Tregs are a regulatory T cell subset defined by expression of the forkhead box p3 (Foxp3) transcription factor. $Foxp3^+$ Tregs are identified by their expression of CD3, CD4, CD25 and Foxp3, although targeting Foxp3 for identification results in cell death and prevents functional analysis. A surrogate definition of $CD3^+CD4^+CD25^+CD127^{low}$ lymphocytes can be used to identify Tregs, which allows for functional analysis of this subset[102,103]. $Foxp3^+$ Tregs help to maintain immunologic tolerance[104] by suppressing T cell activation through direct cell-cell contact and by secreting the inhibitory cytokines TGF-β and IL-10[105-107]. This is associated with tolerogenic activity in areas such as the gut and respiratory surfaces but can also lead to inhibition of anti-tumour immune responses[22,105].

Increased frequencies of Foxp3$^+$ Tregs were identified in LCH lesions in several studies[65,98-100]. Senechal et al.[57] found that 18% of lesional T cells in children's LCH tissue samples were CD4$^+$CD25$^+$ and confirmed in 9 of 11 samples that these cells strongly expressed Foxp3. They also observed that the Tregs were in close proximity to the LCH cells[57]. A supporting study identified Foxp3$^+$ Tregs in high numbers in LCH lesions (22-30% of T cells) and determined a positive correlation between the number of Foxp3$^+$ Tregs within lesions and the total lymphocyte infiltration density[98]. Allen et al.[65] reported an increase in the expression of *FOXP3* in T cells from LCH lesions compared with T cells isolated from patient matched peripheral blood along with an increase in the expression of *CTLA4* in T cells, which can promote the suppressive function of Foxp3$^+$ Tregs[65]. The presence of high numbers of Foxp3$^+$ Tregs within LCH lesions suggests a potential role for these cells in the pathogenesis of the disease.

Immature DCs have previously been shown to promote Foxp3$^+$ Treg production and immune tolerance[16]. As LCH cells are considered myeloid cells and perhaps functionally immature DCs[32], Tong et al.[98] proposed that LCH cells could potentially promote Foxp3$^+$ Treg production and immune tolerance. This could explain the enrichment of Foxp3$^+$ Tregs in lesions and the subsequent suppression of T cell and other immune cell responses towards LCH cells, which would account for LCH cell survival, and immune cell infiltration in lesions (**Figure 1.7**). Quispel et al.[100] showed that activated conventional T cells within LCH lesions correlated with Foxp3$^+$ Tregs and suggested that that Foxp3$^+$ Tregs might increase to counteract the immune response of activated conventional T cells. It is important to determine whether Foxp3$^+$ Tregs have a suppressive role in LCH to increase our understanding of immune regulation in LCH.

Senechal et al.[57] also detected a significant increase in the absolute number and frequency of Foxp3$^+$ Tregs (CD3$^+$CD4$^+$CD25$^+$Foxp3$^+$) in the blood from patients with newly diagnosed LCH compared with age-matched controls. The presence of elevated levels of Foxp3$^+$ Tregs in the blood from patients with active LCH indicates that the lesion microenvironment alone may not be a complete representation of this disease

and there may be more widespread, potentially systemic causes or effects taking place.

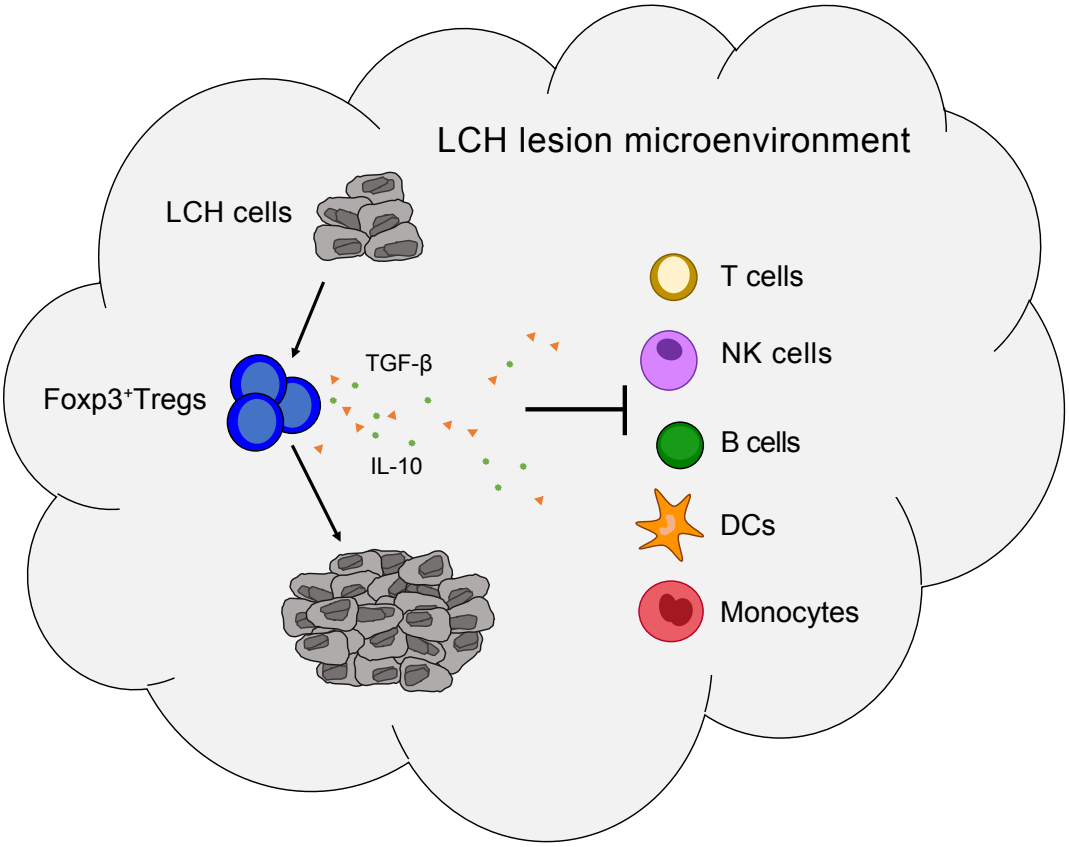


Figure 1.7. LCH cells may promote Foxp3⁺ Tregs.

LCH cells are potentially responsible for promoting Foxp3⁺ Tregs within LCH lesions, accounting for the enrichment of Foxp3⁺ Tregs in LCH. Foxp3⁺ Tregs may subsequently inhibit the immune response of other cells within the microenvironment, thus promoting LCH cell survival.

1.6.3. Unconventional T cells

A number of unconventional T cell subsets such as γδ T cells, type I NKT cells (referred to herein as NKT cells) and MAIT cells are involved in tumour immunity[108-113]. While conventional T cell subsets make up a large and important component of the immune system, the interest in unconventional T cells is growing (reviewed by Godfrey et al.[108]). It is well established that conventional T cells recognise and respond to peptide antigens presented by MHC molecules, however unconventional T cells recognise and respond to a variety of non-peptide antigens such as lipids and vitamin B metabolites presented by MHC-like molecules such as CD1 and MHC-related protein 1 (MR1)

respectively (reviewed by Rossjohn et al.[18]) (**Figure 1.8**). Some subsets of unconventional T cells have limited TCR diversity and are capable of rapid antigen-specific and non-specific immune responses. Unconventional T cells are associated with the immune regulation of cancer, infection, and autoimmunity, and could be involved in LCH.

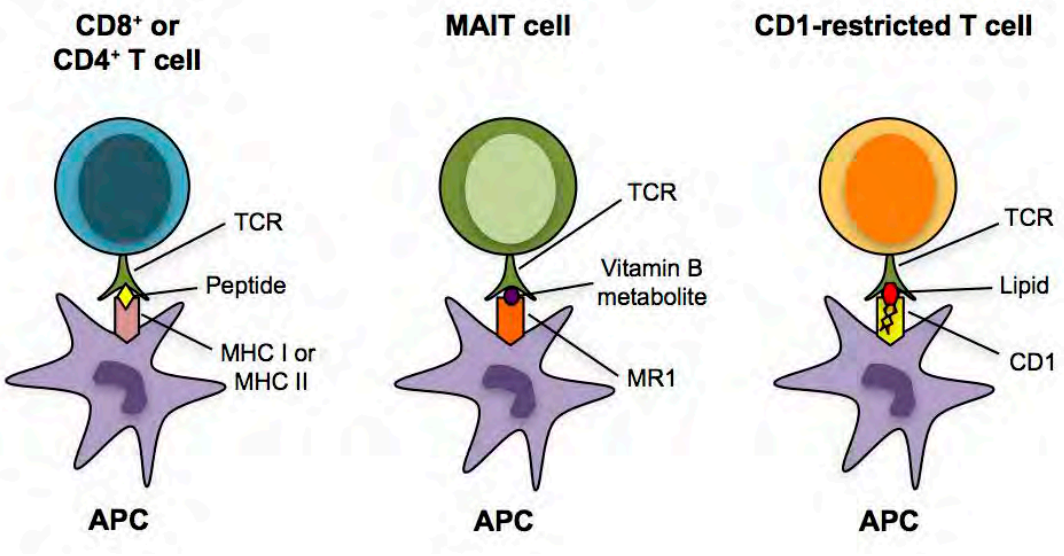


Figure 1.8. MHC, MR1 and CD1.

Illustration shows the different types of interactions between antigen presenting molecules, antigens and TCRs for MHC I and MHC II, MR1 and CD1. MHC molecules present peptide antigens to T cells via the TCR. MR1 presents vitamin B metabolites to MAIT cells. CD1 presents lipid antigens to CD1-restricted T cells.

One research study discovered a high frequency of γδ T cells (30% of total T cells) in three of six cutaneous LCH lesions[101]. A caveat to that study is that the proportions of γδ T cells within LCH lesions were not compared to any control such as healthy peripheral blood or skin, therefore it is difficult to assess if the frequency is unusual. A separate case study found a single LCH patient with increased circulating γδ T cells (25%) in comparison to 50 healthy controls (<10%) although whether this represented a proportion of lymphocytes or total T cells is unclear[114]. γδ T cells usually make up around 5% of peripheral blood T cells and they can provide tumour immunity through direct killing and indirectly through IFN-γ release[115]. For example, cytotoxic γδ T cells isolated from patients with various types of cancer were expanded and shown to kill tumour cell lines and primary cancers *in vitro*[116]. In contrast, IL-17-producing γδ T cells

were suggested to promote tumour development[115], therefore γδ T cells have both tumour-promoting and -protective properties, thus it is important to better define their role in LCH. Reports of increased circulating and lesional γδ T cells associated with LCH are certainly of interest but need further investigation to determine whether they are definitely associated with LCH.

It is surprising that CD1d-restricted NKT cells and MR1-restricted MAIT cells have never been examined in the context of LCH, especially because immune dysregulation is a suggested cause for the disease. NKT cells and MAIT cells are increasingly associated with diseases characterised by immune dysregulation[112,113,117-119] and it is feasible that deficiencies could result in failures of immune regulation that contribute to LCH progression. Indeed there are already many examples where NKT cell and MAIT cell defects are associated with localised tissue inflammation, including psoriasis, allergies and tumours[109,111-113,118,120,121].

NKT cells make up 0.01-0.5% of human T cells[122-125] and produce a wide variety of cytokines with the ability to bias the immune response towards Th1 (IFN-γ), Th2 (IL-4), Th10 (IL-10) or Th17 (IL-17) type responses[122,124,126]. NKT cells therefore have the potential to modify an immune microenvironment[122,127,128], suggesting that they could have a powerful role in the context of tumour immunity. Reduced NKT cell frequency correlates with poor survival in acute myeloid leukaemia[129-131] and head and neck cancer[110,132], while increased frequencies in colon cancer and haematological malignancies are linked to better prognoses[129,131,133].

MAIT cells, like NKT cells, are capable of powerful and rapid immune responses. MAIT cells respond to infection by secreting inflammatory cytokines IFN-γ and TNF[134,135], both of which are present in LCH lesions. MAIT cells also upregulate CD40 ligand upon activation, which can in turn mature DCs and was also shown to mature LCH cells[85,136]. To date, most studies on MAIT cells have focussed on microbial infection, although it is increasingly apparent that they are also present in some tumours[112,113] and other sites of inflammation[137,138] and thus should be examined in the context of LCH. Like NKT cells, MAIT cells have not been studied in LCH, but expression of CD161 is one of

the defining characteristics of MAIT cells and it is noteworthy that the C-type lectin receptor for CD161, *CLEC2D*, is overexpressed by LCH cells compared with skin-resident LCs[65].

Given the presence of high levels of CD1a expression in LCH lesions, there is a capacity to activate or inhibit CD1a-restricted T cells in LCH. To date little is known about the relatively newly discovered CD1a-restricted T cells, although CD1a expression is altered in several disease models[139], including aberrant expression in cancers such as hairy cell leukaemia[140] and some T lymphoblastic lymphomas[141]. CD1a is also linked with autoimmune skin diseases such as atopic eczema[142,143] and psoriasis[144], thus CD1a-restricted T cells may be implicated in the pathology of many disease states, including cancer and autoimmunity. CD1a-restricted T cells have been detected in the blood from healthy individuals using a variety of endogenous lipid ligands and exogenous lipid antigens[145-147]. CD1a-restricted T cells can express the skin homing receptors CCR4, CCR6 and CCR10, and produce IL-22[145], IFN-γ and TNF[145-147]. These cells may play a vital role in skin immunity, however the circulatory presence of these cells also infers a potential role for CD1a-restricted T cells more systemically.

1.7. Summary and perspective

Foxp3$^+$ Tregs, unconventional T cells and CD1a$^+$ T cells are important avenues of investigation with respect to LCH because they or subsets thereof have already been identified as being abnormal in or unique to LCH. There is scope to further determine the role of those already implicated, as well as other T cell subsets in LCH. To date these immunoregulatory subsets have not been well studied, likely due to the rarity of LCH tissue samples and recent advances within the unconventional T cell/Treg fields. Additionally, there is an under-representation of functional studies in the LCH field. While many of the current LCH studies are focussed on mutations within LCH cells, it is important to look at the role of immune cells such as T cells because of their presence in lesions and the potential ligand-receptor interactions between LCH cells and T cells. Studies on Foxp3$^+$ Tregs, unconventional T cells and CD1a$^+$ T cells could

therefore help us to better understand the nature of the perplexing microenvironment that characterises LCH.

T cells with a regulatory function are already implicated in a wide variety of diseases such as cancer and autoimmunity, although some have not been studied at all in LCH. They are a vital component of the immune system and play a key role in immune cell homeostasis, mostly through the production of a diverse range of cytokines. By better understanding the role of T cells with a regulatory function in LCH we may be able to determine whether LCH lesions result from an immune system imbalance or evasion, and improve treatment options for LCH.

2.0. RESEARCH QUESTIONS

This project will conduct a comprehensive analysis of unconventional T cells in patients with LCH along with a specific investigation into the role of $Foxp3^+$ Tregs and $CD1a^+$ T cells from these patients. The relative frequency and phenotype of various T cell subsets, along with their *in vitro* functional capabilities will be investigated. The overarching hypothesis for this study is that LCH progression is associated with immune dysregulation and that some T cell subsets interact with LCH cells and secrete cytokines that contribute to the dysregulation.

In this research project, experiments were conducted in alignment with the following questions central to LCH and more broadly, immunotherapeutic studies:

1. Do T cells both within the LCH microenvironment and systemically play a role in the progression of LCH?
2. Can we identify T cell specific targets for future immunotherapeutic benefits in patients with LCH?

2.1. Hypothesis 1

LCH cells recruit $Foxp3^+$ Tregs to the microenvironment and $Foxp3^+$ Tregs suppress the activation of conventional T cells.

In LCH lesions $Foxp3^+$ Tregs are present in high numbers, although their role is poorly defined. It is hypothesised that LCH cells are immature myeloid cells[32,65] that recruit $Foxp3^+$ Tregs to the lesional microenvironment, and $Foxp3^+$ Tregs provide protection of LCH cells by suppressing other immune cells[98] such as conventional T cells. This theory does not account for the local cytokine milieu including IFN-γ, TNF and IL-2, all of which reflect local T cell activation[30,56] and this calls into question whether $Foxp3^+$ Tregs within the microenvironment have a fully functional suppressive capacity over conventional T cells. The classification of LCH cells is not well understood but it is important with respect to establishing whether there are LCH cell-T cell interactions.

LCH-like cells[148,149] and increased numbers of Foxp3$^+$ Tregs[57] were also reported in the circulation of LCH patients, thus effects of LCH can also be investigated outside of lesions. **Chapter 4** explores the phenotype of lesional conventional T cells, Foxp3$^+$ Tregs and myeloid cells, including LCH cells, comparing their characteristics and functional abilities to those from the circulation from LCH patients and healthy donors. **The aim of this study is to characterise LCH cells, conventional T cells and Foxp3$^+$ Tregs within the LCH microenvironment, and also to better understand whether the effects of LCH extend to the peripheral blood.**

2.2. Hypothesis 2

LCH progression is caused by immune dysregulation and given their potential to interact with LCH cells and their known regulatory nature, unconventional T cells contribute to the dysregulation.

High numbers of γδ T cells were reported in cutaneous LCH lesions[101], although further studies are warranted to determine the importance of this finding. NKT cells and MAIT cells have not been researched in the context of LCH, however they are becoming increasingly associated with the immune regulation of cancer, infection and autoimmunity[109,112,113,118]. There is evidence of T cell activation within lesions[30,56] and despite low surface MHC II expression by LCH cells[85,86], there is expression of *CD1D* and *CLEC2D*[65], genes encoding for proteins that have the potential to directly interact with NKT cells and MAIT cells, respectively. Collectively, these are indicators that unconventional T cells could be involved in the progression of LCH. **Chapter 5** investigates the phenotype of various unconventional T cell lineages in LCH patients, comparing their characteristics and functional abilities to those from healthy donors. **The aim of this study is to investigate the role of unconventional T cells in LCH.**

2.3. Hypothesis 3

A. *CD1a is upregulated on T cells due to activation.*

B. *CD1a-restricted T cells can recognise LCH cells via TCR-CD1a interactions and these interactions have a direct role in the composition of LCH lesions.*

CD1a binds to a variety of lipids that can prime T cells for specific immune responses[150]. CD1a expression by LCH cells is well established as a hallmark of LCH, although the role of this molecule in LCH is unknown. More recently, mature T cells within lesions were shown to express CD1a[31], although the function of these cells and the significance of their presence in lesions is not yet determined. Human CD1d is upregulated on activated T cells[151,152] and although each CD1 isoform has a distinct trafficking route in APCs[153], CD1a could similarly be expressed by T cells in LCH due to activation. There is also a potential capacity for CD1a-restricted T cells specifically to be associated with LCH given the high expression of CD1a in lesions. Firstly, **Chapter 6** explores the function and importance of $CD1a^+$ T cells in LCH and secondly, the role of CD1a-restricted T cells in LCH is investigated. **The aim of this study is to explore what impact CD1a expression has in LCH.**

3.0. MATERIALS AND METHODS

3.1. Ethics statement for the use of human blood and tissue

Healthy donor blood buffy coats and serum specimens were obtained from the Australian Red Cross Blood Service under agreement 14-02VIC-07 and ethics approval was granted by Ballarat Health Services and St John of God Ballarat Hospital Human Research Ethics Committee (HREC; HREC/15/BHSSJOG/5). Peripheral blood and LCH lesion tissue samples were obtained with written informed consent by patients (and/or parents of children where appropriate) with LCH. Ethics approval was granted by Federation University Australia HREC (A08-100) and Ballarat Health Services and St John of God Ballarat Hospital HREC (HREC/10/BHSSJOG/57).

3.2. Clinical details of patients with LCH

The cohort included 13 male and 7 female patients with LCH whose age at diagnosis ranged from 8 months to 68 years of age, while specimens for analysis were collected from patients between the ages of 22 months and 68 years (**Table 3.1**). The most commonly affected tissues within the cohort were bone and skin, with lung, lymph node, CNS and liver tissues affected in other instances. The lesional tissue specimens for analysis were mostly bone derived lesions, although there was also a pulmonary lesion and a lesion from an involved lymph node. Of the 20 blood specimens available, 12 were from patients with active LCH and 8 were from patients with non-active disease. With the exception of one individual, patients with active disease had no known prior treatment with steroids or chemotherapy at the time of specimen collection, but most of those with non-active disease had received treatments (**Table 3.1**).

Patient Code	Specimen description	Sex	Age at diagnosis	Tissues affected	Treatment prior to specimen	Age at specimen	Disease status at specimen	Sequelae, other comments
1	Bone lesion	M	68	Bone	Previous irradiation at site of and excision of hip lesion	68	Active disease (AD)	Diabetes insipidus from age 55
1	Matched blood							
2	Pulmonary lesion	M	40	Lung	Nil	40	AD	Mild pulmonary fibrosis, smoker
2	Matched blood							
3	Involved lymph node	M	9	Bone, lymph node	Nil	9	AD	Skull defect
4	Blood	M	18 months	Skin	Nil	13	Non-active disease (NAD)	
5	Blood	M	53	Bone, lung, bone marrow	Vinblastine/prednisolone	64	NAD	Mild pulmonary fibrosis
6	Blood	M	39	Lung	Vinblastine/prednisolone	52	NAD	
7	Blood	F	60	Skin	Methotrexate/prednisolone	64	NAD	Leg scarring
8	Blood	F	36	Bone	Lesion excision	37	NAD	
9	Blood	F	25	Bone	Vinblastine/prednisolone	42	NAD	Ataxia at time of specimen
10	Blood	M	41	Skin	Nil	41	AD	
11	Blood	F	39	Bone, lung	Nil	39	AD	
12	Blood	F	34	Lung	Biopsy and vinblastine/prednisolone	54	NAD	Long term pulmonary fibrosis
13	Bone lesion	M	2	Bone	Nil	2	AD	Mutation in BRAFV600, CNS-risk lesion
13	Matched blood							

14	Bone lesion	M	7	Bone	Nil	7	AD	Mutation in BRAF600
14	Matched blood							
15	Bone lesion	M	2	Bone, skin, CNS	Nil	2	AD	Mutation in BRAFV600, at the time of the specimen, the patient had only a unifocal skull lesion
15	Matched blood							
16	Blood	F	2	Bone	Nil	2	AD	
17	Blood	F	14	Bone	Nil	14	AD	
18	Blood	M	8 months	Skin, lymph node, liver	Nil	22 months	AD	No detectable mutation in BRAFV600
19	Blood	M	10	Bone, skin	Cytarabine, prednisolone and vinblastine (ceased 6 months prior to specimen)	12	AD	Diabetes insipidus, mutation in BRAFV600
20	Blood	M	67	Bone, skin	Nil	67	NAD	

Table 3.1. Clinical details of study participants with LCH.

3.3. Isolation of PBMCs from healthy donors

Buffy coats from healthy donors were diluted 1:1 with in house phosphate buffered saline (PBS; described in **Section 3.5**) and PBMCs were isolated by standard density gradient using Histopaque-1077 (Sigma-Aldrich) as per manufacturer's instructions using a Hettich Rotina 380 R or 420 R centrifuge, then cryopreserved (described in **Section 3.7**).

3.4. Isolation of immune cells and serum from LCH patients

Peripheral blood samples from LCH patients were diluted 1:1 with PBS and PBMCs were isolated by standard density gradient using Histopaque-1077 (Sigma-Aldrich) or Lymphoprep (Axis-Shield PoC AS, Oslo, Norway). Alternatively, white blood cells (WBCs) were isolated using an in house red blood cell (RBC) lysis buffer (described in **Section 3.6**) then cryopreserved. LCH lesional tissue was processed into a single cell suspension using MACS tumour dissociation kit for humans (Miltenyi Biotec) as per manufacturer's instructions, then cryopreserved. Serum specimens were snap frozen at -80° Celsius (C).

3.5. PBS

A 10x solution of PBS was made comprising 1.37 M sodium chloride (NaCl; Chem-Supply), 27 mM potassium chloride (KCl; Sigma-Aldrich), 100 mM sodium phosphate dibasic (Na_2HPO_4; Chem-Supply) and 20 mM potassium phosphate monobasic (KH_2PO_4; Sigma-Aldrich) in Milli-Q water (Merk Millipore) with the pH adjusted to 7.4 with sodium hydroxide (NaOH; Sigma-Aldrich). The stock solution was then filtered through a 0.45-micron filter membrane (Merk Millipore) using a vacuum pump, then autoclaved (Tuttnauer). A 1x solution was prepared by diluting 10x stock with Milli-Q water (1:9), then filtering and autoclaving as per 10x stock preparation.

3.6. RBC lysis

RBC lysis buffer was made in house by dissolving 155mM ammonium chloride (NH_4Cl; Sigma-Aldrich), 10mM potassium bicarbonate ($KHCO_3$; Sigma-Aldrich) and 0.1mM

ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich) into Milli-Q water and then adjusting the pH to between 7.2 and 7.4. The buffer was subsequently filtered through a 0.45-micron filter membrane (Merk Millipore) using a vacuum pump, then autoclaved (Tuttnauer).

Blood was diluted with 5-10 times RBC lysis buffer solution and incubated in lukewarm water with occasional mixing and inversion until solution became darker indicating RBC lysis (approximately 10 minutes (min)). Cells were centrifuged at 300 x g (Hettich Rotina 380 R or 420 R) for 5 min at room temperature and supernatant was discarded. Cells were incubated with fresh RBC lysis buffer for a further 5 min where necessary, then centrifuged at 300 x g for 5 min at room temperature and supernatant was discarded. Cell pellet was suspended in ice-cold PBS to wash, then centrifuged at 300 x g for 5 min at 4° C and supernatant was discarded. This step was repeated for a second wash, then the WBC pellet was cryopreserved.

3.7. Cryopreservation and thawing of cells

Cells were suspended in freezing mix containing 50% (v/v) bovine calf serum (BCS; Sigma-Aldrich), 40% (v/v) Roswell Park Memorial Institute (RPMI) medium 1640 (Gibco) and 10% (v/v) dimethyl sulfoxide (Sigma-Aldrich), and transferred to cryogenic vials (ThermoFisher Scientific). Vials were transferred to controlled rate-freezing containers (Nalgene cryogenic freezing containers containing 100% isopropanol *or* BioCision CoolCell® cell freezing containers) and stored at -80° C overnight, and then placed into liquid nitrogen for long term storage. To thaw cells cryogenic vials were placed in warm water or a 37° C bead bath (Lab Armor) to partially thaw, then contents were added to ice-cold FACS buffer (PBS + 1% BCS (v/v) + 2 mM EDTA) to wash.

3.8. Cell counts

Cells were counted using the Countess™ automatic cell counter (Invitrogen) using trypan blue (Invitrogen) exclusion.

3.9. Flow cytometry

3.9.1. Viability dyes

Viability dyes were used for all experiments. Cells were stained with either 7-aminoactinomycin D (7-AAD; BD Pharmingen), fixable viability stain 510 (BD Horizon), or fixable viability stain 700 (BD Horizon) alongside other cell staining reagents.

3.9.2. Monoclonal antibodies

Cell surface antibodies used were specific for human antigens and are as follows: CD1a-BV421 (HI149; BD Horizon), CD1a-BV605 (SK9; BD Biosciences), CD1b-FITC (M-T101; BD Biosciences), CD1c-APC (L161; BioLegend), CD1d-PE (51.1; eBioscience), CD3-PE-Cy7 (UCHT1; BD Pharmingen), CD3-Pacific Blue (HIT3a; BioLegend), CD3-BV605 (HIT3a; BD Horizon), CD3-BV650 (UCHT1; BD Horizon), CD4-PE (RPA-T4; BioLegend), CD4-PE-CF594 (RPA-T4; BD Horizon), CD4-PE-Cy7 (SK3; BD Pharmingen), CD4-APC-Cy7 (RPA-T4; BD Pharmingen), CD4-BV650 or -BV711 (SK3; BD Horizon), CD8-APC-Cy7 (SK1; BD Pharmingen), CD8-BV510 (RPA-T8; BD Horizon), CD8-BV605 (SK1; BD Horizon), CD11c-PE-CF594 or -BV605 (B-ly6; BD Horizon), CD14-PerCP-Cy5.5 (M5E2; BD Pharmingen), CD14-APC (MϕP9; BD Biosciences), CD14-AlexaFluor700 (TÜK4; AbDSerotec), CD19-BV510 (SJ25C1; BD Horizon), CD19-PerCP/Cy5.5 (SJ25C1; BioLegend), CD25-PE-Cy7, (M-A251; BD Pharmingen), CD25-BV711 (2A3; BD Horizon), CD56-BV786 (NCAM16.2; BD Horizon), CD69-BV650 (FN50; BD Horizon), CD127-BV421 or -BV786 (HIL-7R-M21; BD Horizon), CD161-APC or -PE/Dazzle 594 (HP-3G10; BioLegend), HLA-DR-BV605 (G46-6; BD Horizon), TCR Vα7.2-FITC (3C10; BioLegend), and TCRγ/δ-1-PE-Cy7 (11F2; BD Biosciences).

3.9.3. CD1 and MR1 monomers and tetramers

Biotinylated human CD1d monomers, PBS44 lipids and CD1a monomers loaded with endogenous lipids were kindly provided by the Godfrey laboratory (Department of Microbiology and Immunology, Peter Doherty Institute, University of Melbourne, Parkville, Australia) and 5-OP-RU (5-(2-oxopropylideneamino)-6-D-ribitylaminouracil)-loaded MR1 monomers were generously provided by the McCluskey laboratory

(Department of Microbiology and Immunology, Peter Doherty Institute, University of Melbourne, Parkville, Australia).

For CD1d tetramer production, PBS44 was sonicated for 30 min and then loaded into CD1d (5.7 μg: 100 μg) overnight at room temperature in the dark. 20 μg of PE streptavidin (BD Pharmingen) was then added to PBS44 loaded-CD1d on ice by adding 2 μg every 15 min.

For MR1 tetramer production, PE streptavidin was added to 5-OP-RU-loaded MR1 on ice at 3.4 μg per 5 μg MR1, 1/10 at a time every 10 min.

For CD1a tetramer production, 20 μg PE streptavidin was added to endogenous lipid-loaded CD1a on ice by adding 2 μg every 15 min.

3.9.4. Cell surface labelling protocol

Following cell thawing and cell counting, cells were transferred to 5 mL polystyrene round-bottom tubes and incubated with 50 μL of antibody/tetramer/viability dye/s at their optimal titre with human Fc Block™ (BD Pharmingen) in FACS buffer on ice in the dark for 15 min. Cells were washed in FACS buffer and centrifuged at 300 x g for 5 min at 4° C, then supernatant was discarded and cells were suspended in fresh FACS buffer for analysis.

Analyses were performed using either a BD FACS Aria II cell sorter or BD LSR Fortessa (BD Biosciences). Data were analysed using FlowJo software (Treestar) unless otherwise specified. For all flow cytometry analyses, cells were primarily gated to identify live, single lymphocytes (**Figure 3.1**) unless otherwise described. Additionally, unless otherwise described, all comparisons between different groups were conducted relative to the $CD3^+$ (T cell) population or subpopulations thereof due to the varied immune cell isolation methods used.

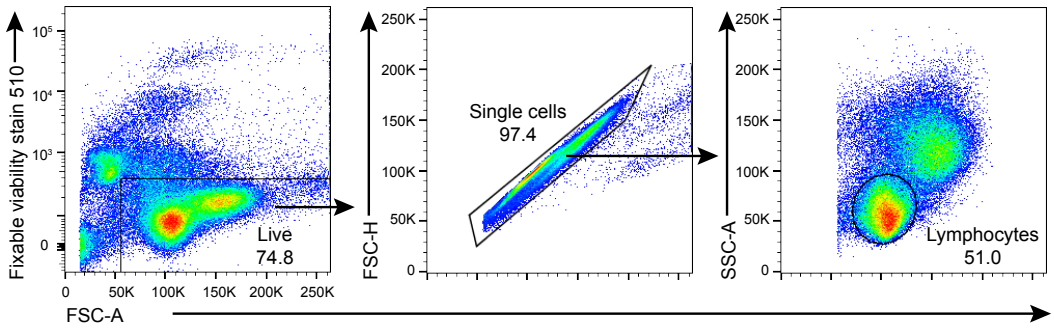


Figure 3.1. Gating strategy for identifying live, single lymphocytes.

Representative plots from healthy donor derived PBMCs demonstrating the hierarchical gating strategy used to identify viable lymphocytes. Live cells are identified (left) using fixable viability stain (or 7-AAD) to exclude dead cells, then doublets are excluded based on forward scatter parameters (centre), then lymphocytes are gated on based on forward and side scatter parameters (right).

3.9.4. Foxp3 transcription factor staining

Following surface staining the eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher Scientific) was used to fix and permeabilise cells and detect the transcription factor Foxp3, as per manufacturer's instructions.

3.9.5. Cytokine staining

Following cell stimulation (described in **Section 3.11**) cells were cultured in GolgiPlug (BD Biosciences) at 1/1,000 µL for 4-5 hours at 37° C. Cells were then labelled with surface antibodies, then the BD Cytofix/Cytoperm Plus Fixation/Permeabilisation Kit (BD Biosciences) was used to fix and permeabilise cells and detect intracellular cytokines, as per manufacturer's instructions.

Intracellular antibodies specific for the following cytokines were used: IL-4-APC (BD Pharmingen), TNFa-FITC (Mab11; eBioscience), TNF-PE-CF594 (Mab11; BD Horizon), TNFα-APC-Cy7 (MAb11; BioLegend), IFNγ-BV711 (B27; BD Horizon) IL-17A-BV786 (N49-653; BD Horizon).

3.9.6. Fluorescence-activated cell sorting

All fluorescence-activated cell sorting (FACS) was performed using a BD FACS Aria II cell sorter (BD Biosciences) with FACSDiva™ software (BD Biosciences). For purification of cell populations, cell suspensions were labelled with the appropriate antibodies, then desired populations were sorted into individual tubes containing 500 µL fetal bovine serum (FBS; Sigma-Aldrich). Isolated populations were then assessed for purity by flow cytometry analysis.

3.9.7. Bead based cytokine detection immunoassays

LEGENDplex (BioLegend) bead based cytokine detection immunoassays were used to identify secreted cytokines following *in vitro* cell culture and stimulation. Cell culture supernatants were collected and stored at -80° C before use. Both the Human Th Cytokine Panel (13-plex; BioLegend) and the Human Free Active/Total TGF-β1 Assay (BioLegend) were used to detect secreted cytokines as per manufacturer's instructions. Analysis was performed using LEGENDplex™ Data Analysis Software (BioLegend) with cytokines quantified by comparing samples to a set of standard curves that were prepared in parallel with supernatant samples.

3.10. Multiplex immunohistochemistry

Sections of archival formalin-fixed paraffin-embedded tissue blocks from LCH patients were mounted onto positively coated adhesive glass microscope slides by Australian Clinical Labs, St John of God Hospital, Ballarat.

Primary antibodies used for immunofluorescent staining were polyclonal rabbit anti-human CD3 (Dako), monoclonal mouse anti-human FOXP3 [236A/E7] (Abcam) and polyclonal rabbit anti-human TGF-β (Abcam). CD3 and FOXP3 were labelled with HRP conjugated secondary antibodies followed by tyramide signal amplification (TSA) as described below (**Section 3.10.2**) and Alexa Fluor 568 goat anti-rabbit IgG (H + L; ThermoFisher Scientific) was used as a secondary antibody to identify TGF-β in tissues as described below (**Section 3.10.3**).

Multiplex combinations were as follows:

1. CD3-Cy5 (TSA), FOXP3-fluorescein (TSA), TGF-β-Alexa Fluor 568 (standard immunofluorescent labelling), DAPI nuclear stain (ThermoFisher Scientific)
2. CD3-fluorescein (TSA), FOXP3-Cy5 (TSA), TGF-β-Alexa Fluor 568 (standard immunofluorescent labelling), DAPI nuclear stain

Each incubation step took place in an agitated humid chamber with protection from light where necessary. For each wash step, slides were rinsed with EnVision™ FLEX wash buffer (Dako) then immersed in wash buffer and agitated for 3 min, then placed into a second wash buffer and agitated for further 3 min.

All immunohistochemistry images were captured using an Evos FL Auto 2 cell imaging system (ThermoFisher Scientific) and analyses were completed using Image J software (National Institutes of Health).

3.10.1. Antigen retrieval and blocking

Slides were boiled by microwaving in EnVision™ FLEX target retrieval solution, high pH (Dako) antigen retrieval buffer for 10 min. Slides were placed in wash buffer for 5 min, then blocked for 5 min with a PBS solution containing 0.25% casein, 2% goat serum, 0.1% Triton X-100 (Sigma-Aldrich), 0.5% TWEEN 20 (Sigma-Aldrich) and 0.05% sodium azide (Sigma-Aldrich).

3.10.2. TSA

Following each antigen retrieval and block, slides were rinsed with wash buffer and stained with primary antibody and incubated for 15 min. Slides were subsequently washed as previously described, then blocked for 8 min using EnVision™ FLEX peroxidase-blocking reagent (Dako). Following the incubation, slides were washed again then stained with EnVision™ FLEX HRP (Dako) for 15 min. After secondary antibody labelling, slides were washed then labelled with reagents from the TSA™ - plus fluorescein system (Perkin-Elmer) and incubated for 10 min.

To multiplex, all antigen retrieval, blocking and TSA steps were repeated on the same slide with alternate antibodies in the listed combinations.

3.10.3. Standard immunofluorescent labelling

Following TSA stains above, antigen retrieval and blocking was conducted as previously described. Slides were subsequently rinsed with wash buffer and stained with primary antibody and incubated for 1 hour. Following the incubation, slides were washed and then stained with species specific secondary antibody and incubated for 30 min.

3.10.4. Nuclear labelling and coverslip mounting

Following the above multiplexing steps, slides were washed and then incubated with DAPI for 5 min. Slides were then washed and coverslips were added using ProLong Gold™ Antifade Mountant (ThermoFisher Scientific) and allowed to dry in the dark.

3.11. *In vitro* cell culture conditions for stimulation assays

All cell culture procedures were carried out under sterile conditions within a class II biosafety cabinet (BH2000 Series Clean Air by Clyde-Apac). Cells were housed in a 37° C incubator with 5% CO_2 in T cell medium or complete tissue culture medium (CTCM) unless otherwise indicated.

3.11.1. T cell medium

T cell medium consisted of TexMACS Medium (Miltenyi Biotec) supplemented with 10% FBS, 1x penicillin-streptomycin (Sigma-Aldrich) and 50 µM 2-mercaptoethanol (2-ME; ICN Biomedicals).

3.11.2. CTCM

CTCM consisted of RPMI 1640 + GlutaMAX™ (-l) (Gibco) supplemented with 10% FBS, 15 mM HEPES (Gibco), 0.1 mM non-essential amino acids (Gibco), 1 mM sodium pyruvate (Gibco), 1x penicillin-streptomycin and 50 µM 2-ME.

3.11.3. Purified T cell stimulation assay using PMA and ionomycin

$CD3^+$ T cells were purified by FACS with a purity >95%, except from 2 LCH peripheral blood samples, which were >92%, then rested overnight at 37° C in T cell medium and subsequently stimulated for 5 hours with 10 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma) and 1 μg/mL ionomycin (Sigma) in the immediate presence of GolgiPlug (1/1,000 μL) before harvesting cells for the analysis of intracellular cytokines.

3.11.4. $Foxp3^+$ Treg suppression assay

96-well, round bottom plates (Sigma) were coated with anti-CD3 by incubating wells with a 50 μL solution of PBS with suboptimal (1 μg/mL) purified mouse anti-human CD3 (UCHT1; BD Biosciences) at 37° C for at least 2 hours. Wells were then washed twice with PBS prior to seeding cells. Purified populations of $CD3^+CD4^+CD25^+CD127^{low}$ cells (Tregs) and $CD3^+CD4^+$ T responder cells (Tregs excluded) were isolated by FACS. For carboxyfluorescein succinimidyl ester (CFSE) labelling, isolated T responder cells were suspended in 1 mL pre-warmed PBS with 0.1% bovine serum albumin (BSA; Sigma), then 1 μL of 1 mM CFSE proliferation dye (eBioscience) was added and rapidly mixed, then incubated for 8 min at 37° C in the dark. Reactions were quenched by the addition of 1 mL ice-cold FBS. Cells were then centrifuged at 300 x g for 5 min at 4° C, then supernatants were discarded and cell pellets were resuspended in T cell medium. Cultures of 10,000 T responder cells with varying ratios of Tregs (10:1, 5:1 and 2:1) were incubated at 37° C for 4 days, then harvested to assess proliferation.

3.11.5. Stimulation assay using purified T cell and Treg subsets

Purified cell populations (described in **Section 4.2.10.3**) were isolated by FACS with a purity >95% and rested overnight at 37° C in T cell medium. Cell populations were subsequently stimulated for 12 hours with 10 ng/mL PMA and 1 μg/mL ionomycin then cultured with GolgiPlug (1/1,000 μL) for a further 4 hours before collecting supernatants and harvesting cells for the analysis of cytokines.

3.11.6. MAIT cell stimulation with 5-OP-RU and TGF-β

PBMCs derived from healthy donors were cultured for a total of 22 hours in T cell medium with concentrations of recombinant human TGF-β1 (CHO derived; PeproTech) ranging from 0.1-10 ng/mL. Cell cultures were stimulated for 6 hours prior to harvesting by adding 5-OP-RU to achieve a 2 nM solution, with GolgiPlug (1/1,000 µL) added for the final 5 hours of culture.

3.11.7. Assay for induction of cell surface CD1 expression by T cells

PBMCs derived from healthy donors were cultured in a variety of cell culture medium conditions at 37° C for 72 hours with 1.25 µg/mL phytohaemagglutinin (PHA)-L solution (eBioscience). Cell culture medium conditions were as follows:

C1. CTCM (10% FBS)
C2. CTCM (minus 10% FBS) + 10% human serum
C3. CTCM (minus 5% FBS) + 5% human serum
C4. TexMACS serum-free media + 1x penicillin-streptomycin + 50 µM 2-ME

Cells were harvested at 24, 48 and 72 hour time points for analyses following a prior 4 hour incubation with GolgiPlug.

3.12. Gene expression studies

3.12.1. RNA extraction, cDNA synthesis and transcriptome amplification

All procedures were carried out under sterile conditions within a laminar flow work station (PCR series by Clyde-Apac). Prior to and during transcriptome amplification procedures, all equipment was treated with RNaseZap (Sigma).

Cell populations were purified by FACS, then the REPLI-g WTA (whole transcriptome amplification) Single Cell Kit (QIAGEN) for transcriptome amplification of small cell populations (<1000) was used to extract and reverse transcribe total RNA and amplify complimentary DNA (cDNA) directly from cells, as per manufacturer's instructions

using a thermal cycler (Applied Biosystems Gene Amp® PCR System 2700). Because there was no intermediate step where RNA could be quantified, successful real-time quantitative polymerase chain reaction (qPCR) amplification using several different primers was deemed a measure of successful transcriptome amplification (**Appendix A, Tables A1-A3**).

3.12.2. qPCR

RT_2 SYBR® Green qPCR Mastermix (QIAGEN), nuclease-free water (QIAGEN) and RT_2 qPCR primer assays were combined with cDNA as per manufacturer's instructions. RT_2 qPCR primer assays (QIAGEN) used for gene expression studies were as follows: *IL2RA*, *TNF*, *CD40LG*, *LIF*, *LIFR*, *FOXP3*, *CTLA4*, *TGFB1*, *IL10*, *NCAM1*, *CD1A*, *CD1B*, *CD1C*, *CD1D*, *MR1*, *CLEC2D*, *CD80*, *CD86*, *CD274*, *PDCD1LG2*, *CD40*, *HLA-DRA* and reference gene *RPLP0*. No-template control samples were included for each primer assay, substituting cDNA volumes with nuclease free water. Aliquots of each component were added to 0.1 mL strip tubes (QIAGEN) in triplicate and capped then placed in the Rotor-Disk 72 rotor (QIAGEN) and placed into the Rotor-Gene Q (QIAGEN) for qPCR cycling. Conditions for amplification, annealing, extension and melt curve analysis were as per QIAGEN RT_2 qPCR primer assay instructions. Melt curve analysis was performed to confirm that specific product was amplified as indicated by the data sheet for each individual primer assay. Results for qPCR were exported to Microsoft Excel (Microsoft Corporation, US) for data analysis. For each sample, Ct values were derived by calculating means from sample triplicates. These values were then used to calculate delta Ct (ΔCt), the difference between the gene of interest and the reference gene Ct values (ΔCt =ΔCt (gene of interest) - ΔCt (reference gene)).

3.12.3. Analysis of publicly available microarray data

Microarray data produced and described by Allen et al.[65] was reanalysed using the Gene Ontology PANTHER overrepresentation test (released 20171205; available at http://www.geneontology.org). The annotations were derived using PANTHER version 13.1, released 2018-02-03. The lists analysed were genes names (available at http://www.jimmunol.org/content/184/8/4557/tab-figures-data) that were

upregulated by >2-fold in (**1**) $CD207^+$ LCH cells compared to control $CD207^+$ skin LCs, and in (**2**) $CD3^+$ T cells from LCH lesions compared to control LCH patient peripheral blood $CD3^+$ T cells. Each group of upregulated genes was compared against the *Homo sapiens* reference list (all genes in database). Annotation data sets used in the enrichment analyses were the 'GO biological process complete' and 'Reactome pathway'. Fisher's exact test with false discovery rate (FDR) multiple test correction was used to determine overrepresented processes and pathways. Raw and FDR adjusted p-values for all processes and pathways listed were <0.05. The results presented display only the most specific terms from each biological process or pathway that was enriched.

3.13. Statistical analyses

Unless otherwise stated, data were analysed using GraphPad Prism (GraphPad Software). If all data sets being compared or correlated were normally distributed based on D'Agostino and Pearson omnibus normality tests (alpha =0.05), parametric tests were chosen. If any data set did not pass normality testing, non-parametric tests were chosen. The appropriate choice of statistical test was also based on whether the data being compared were matched/paired or unmatched/unpaired. Statistical tests conducted are described in the text and/or figure legend. Differences were considered statistically significant at an alpha level of 0.05. Lesions and matched peripheral blood samples are colour coded on graphs for tracking across all chapters.

4.0. CHARACTERISATION OF T CELLS AND LCH CELLS IN LCH PATIENTS

4.1. Introduction

LCH lesions consist of a range of immune cells including conventional T cells, Foxp3$^+$ Tregs and LCH cells, while 'LCH-like cells' and increased numbers of Foxp3$^+$ Tregs have also been reported in the periphery of patients with active disease[57,148,149]. Since both T cells and LCH cells are suspected contributors to LCH lesion inflammation[30,56], and Foxp3$^+$ Tregs are enriched in the lesions[57,65,98-100], it is important to determine the role of each of these cell populations and elucidate whether they are beneficial or detrimental to resolving LCH. LCH cells are presumed to be pathogenic, although immune dysfunction is also a likely contributor to the progression of LCH. Understanding whether LCH cells are pathogenic, whether conventional T cells from LCH patients are normally distributed and properly functioning, and whether the enrichment of Foxp3$^+$ Tregs contributes to LCH progression, will potentially assist us in identifying and applying new immune based therapies to treat patients.

4.1.1. LCH cells

The origin and phenotype of LCH cells likely dictates the type of T cells recruited and activated within the microenvironment through mechanisms such as antigen presentation, immune checkpoint inhibition and cytokine production. Defining this is important with respect to LCH pathogenesis, however the origin of LCH has been a somewhat confusing journey. Nezelof et al.[51]. was first to describe an origin for LCH cells, suggesting that they were mature epidermal LCs that had migrated to the sites of lesional inflammation. Despite this, the migration history of LCH cells remains unclear. LCs are known to express chemokine receptor (CCR) 6, and are likely stabilised in the epidermis by CCL20 (MIP-3α; macrophage inflammatory protein-3α), the ligand for CCR6[154-156]. Comparable to LCs and the epidermal environment, LCH cells were shown to express CCR6[88,157], and CCL20 was overexpressed in lesions, including by LCH cells (unpublished data reported in study by Annels et al.[88]). Chemokine C-X-C motif

receptor (CXCR) 4 was also shown to be expressed by LCH cells in lesions from most patients (69%)[149], and CXCR4 is necessary for migration of LCs out of the epidermis[158]. Likewise CXCL12, the ligand for CXCR4 was shown to be abundantly expressed within lesions[149]. Fleming et al.[157] found that LCH cells expressed CCR7 which might suggest the ability to migrate to lymph nodes[159-162], however Annels et al.[88] found contrastingly that CCR7 expression was absent on LCH cells, suggesting a more immature phenotype. More recent studies have shown that *BRAF V600E* induced ERK activity induces CCR7 expression and consequently DC migration[163], and this may occur to LCH cells in some settings, given they can harbour MAPK pathway mutations such as *BRAF V600E*.

Since the first description of LCH cell origin, a better understanding of the ontogeny of LCs[12,17,20] has led to questioning whether LCH cells are actually pathogenic migrating LCs at all. Ginhoux and Merad[12] recognised that the origin of LCs could be distinct from classical DCs and they hypothesised that yolk-sac derived primitive macrophages, which are present prior to bone marrow haematopoiesis, migrate to the skin during embryogenesis, where they proliferate and differentiate into LCs. If LCs develop and colonise the epidermis during embryogenesis and differentiate and self-renew within the epidermis rather than relocating from the bone marrow as DCs do, it is unlikely that LCH cells in tissues other than the skin would be migratory LCs[12,17,20]. In parallel with these studies, Allen et al.[65] compared the gene expression profile of LCH cells to $CD207^+$ skin LCs. Several genes associated with early myeloid cells were overexpressed in LCH ($CD207^+$) cells, including *CD300LF*, *ITGAX*, *ITGAM*, *ICAM1*, *SIGLEC1*, *CD33*, *CD1D*, *ITGA4*, and *ANPEP*. These results suggest that LCH cells align more closely with an immature DC phenotype rather than a migrating mature LC. Together these studies support the concept that LCH cells arise from early bone marrow derived myeloid precursors rather than yolk-sac derived mature LCs[65]. LC identifying markers CD1a and CD207 are, however, strongly co-expressed by LCH cells[85].

Further phenotypical classification of LCH cells is important with respect to establishing their level of maturation and defining whether this plays a role in the pathogenesis of LCH, and whether it has downstream consequences for T cell

recruitment and activation. LCH cells from bone and chronic lesions were shown to co-express CD68 and CD14[85], both of which are monocyte/macrophage immunophenotypic markers, with CD14 downregulated upon monocyte to macrophage differentiation[85,164]. Interestingly LCH cells displayed very little surface expression of DC maturation markers CD83, CD86, and MHC II, and maturation and activation marker DC-LAMP (DC-lysosomal associated membrane protein)[85,165,166]. LCH cells from patients with self-healing disease or isolated cutaneous disease often expressed CD86 but not CD14, suggesting a more mature DC phenotype than that seen in bone and chronic lesions[85]. CD80, another DC maturation marker and T cell costimulatory molecule was however, frequently detected on LCH cells in the study[85]. CD40 was also co-expressed by LCH cells from the aforementioned bone and chronic lesions[85].

4.1.2. Conventional T cells

Antigen presenting molecule-TCR interactions are crucial in immune regulation[18] and therefore the level of maturity of LCH cells could affect immune regulation by T cells within lesions. It is especially important to know the specific characteristics and phenotype of T cells within LCH patients. For example, whether there is an imbalance between Th cells and cytotoxic T cells, or a bias towards a Th1, Th2 or Th17 response within the microenvironment, and whether T cells from LCH patients display an activated phenotype. A better understanding of these characteristics may aid us in understanding how LCH progresses and could help to develop future treatment strategies for LCH patients.

4.1.3. Foxp3$^+$ Tregs

T cell function can also be inhibited by Foxp3$^+$ Tregs, which were reported to be increased in the blood and lesions from patients with LCH by several groups[57,65,98-100]. It is not established whether Foxp3$^+$ Tregs from LCH patients have an immunosuppressive capacity like those from healthy individuals, or whether Foxp3$^+$ Tregs are beneficial or unfavourable to LCH pathogenesis. Foxp3$^+$ Tregs can maintain tolerance in immune environments in a contact-dependent manner or by secreting

the inhibitory cytokines TGF-β and/or IL-10, and it is by these mechanisms that they are able to inhibit anti-tumour immune responses and promote the development of tumours (reviewed by Sakaguchi et al.[167] and Chaudhary and Elkord[168]).

Foxp3$^+$ Tregs in LCH lesions express inducible costimulatory factor (ICOS) and are in close proximity to LCH cells, which express ICOS ligand[57,100]. These observations suggest that there might be specific Treg-LCH cell interactions within the lesional environment, and given the enrichment of Foxp3$^+$ Tregs within lesions they are potentially involved in LCH pathogenesis. Additionally, Allen et al.[65] identified an upregulation of *CTLA4* in T cells from LCH lesions, and CTLA-4 may be expressed by the Tregs. Furthermore, Foxp3$^+$ Tregs were reported to be increased in the blood from patients with active LCH[57], which strengthens their potential importance in LCH pathogenesis. Expression of the Foxp3 transcription factor is synonymous with low CD127 expression and typically defines Foxp3$^+$ Tregs along with their expression of CD3, CD4 and the IL-2 receptor CD25[102,103], although not all studies investigating Tregs in LCH have taken the stringent approach of gating on CD3$^+$CD4$^+$CD25$^+$Foxp3$^+$ or CD127^{low} lymphocytes in concert.

The immune suppressive cytokines TGF-β and IL-10, which are commonly produced by Tregs have been detected within lesions[30,56,57,99,100,169] and blood[148,170] from patients with active LCH. Studies have suggested that LCH cells are a source of TGF-β[65,169] and that TGF-β is one of the drivers of the LCH cell phenotype[148,171,172], whilst IL-10 was described as being produced largely by macrophages[85] and LCH cells[99] within lesions. Since LCH lesions are enriched with Foxp3$^+$ Tregs and the cytokines they are known to produce are also present, it is conceivable that lesional Tregs too are a source of TGF-β and/or IL-10 production. Historically the identification of Foxp3$^+$ Tregs has not allowed for functional studies because staining for the Foxp3 transcription factor requires cell permeabilisation. Consequently, the function of Foxp3$^+$ Tregs in LCH lesions is unknown, but an effective surrogate gating strategy to detect CD3$^+$CD4$^+$CD25$^+$CD127^{low} lymphocytes (Tregs)[102,103] has been developed that allows for downstream functional assays on the Treg population.

4.1.4. Introduction overview

This chapter aims to understand whether T cells within the lesions and the circulation of LCH patients play a role in LCH pathogenesis by defining their relative frequencies, characteristics and functional abilities. This study set out to stringently quantify the relative frequency of Tregs in LCH patients and to test their functional ability to better define their role in LCH. This study also intends to better define the immunophenotype of LCH cells to understand whether they could be responsible for the recruitment of $Foxp3^+$ Tregs.

4.2. Results

4.2.1. Identification of conventional T cell subsets in patients with LCH

To understand whether immune dysregulation is a factor in LCH progression this study investigated the relative frequencies of conventional T cell subsets in LCH patients in comparison to healthy donors. Increases or decreases in the proportions of cytotoxic T cell ($CD8^+$) and Th cell ($CD4^+$) subsets could indicate immune dysregulation. For example, reduced numbers of $CD8^+$ T cells could reduce the ability of this population to lyse and clear pathogenic target cells such as LCH cells, and alterations to the number of $CD4^+$ T cells might create excessive or inadequate inflammation and recruitment of other immune cells.

T cell subsets were identified according to the following criteria: $CD4^+$ T cells were classified as $CD3^+CD4^+CD8^-$ lymphocytes, $CD8^+$ T cells as $CD3^+CD8^+CD4^-$ lymphocytes, and double negative (DN) T cells as $CD3^+CD4^-CD8^-$ lymphocytes. Specimens analysed were from the peripheral blood from healthy donors, patients with active and non-active disease, and from lesions from LCH patients (**Figure 4.1A**).

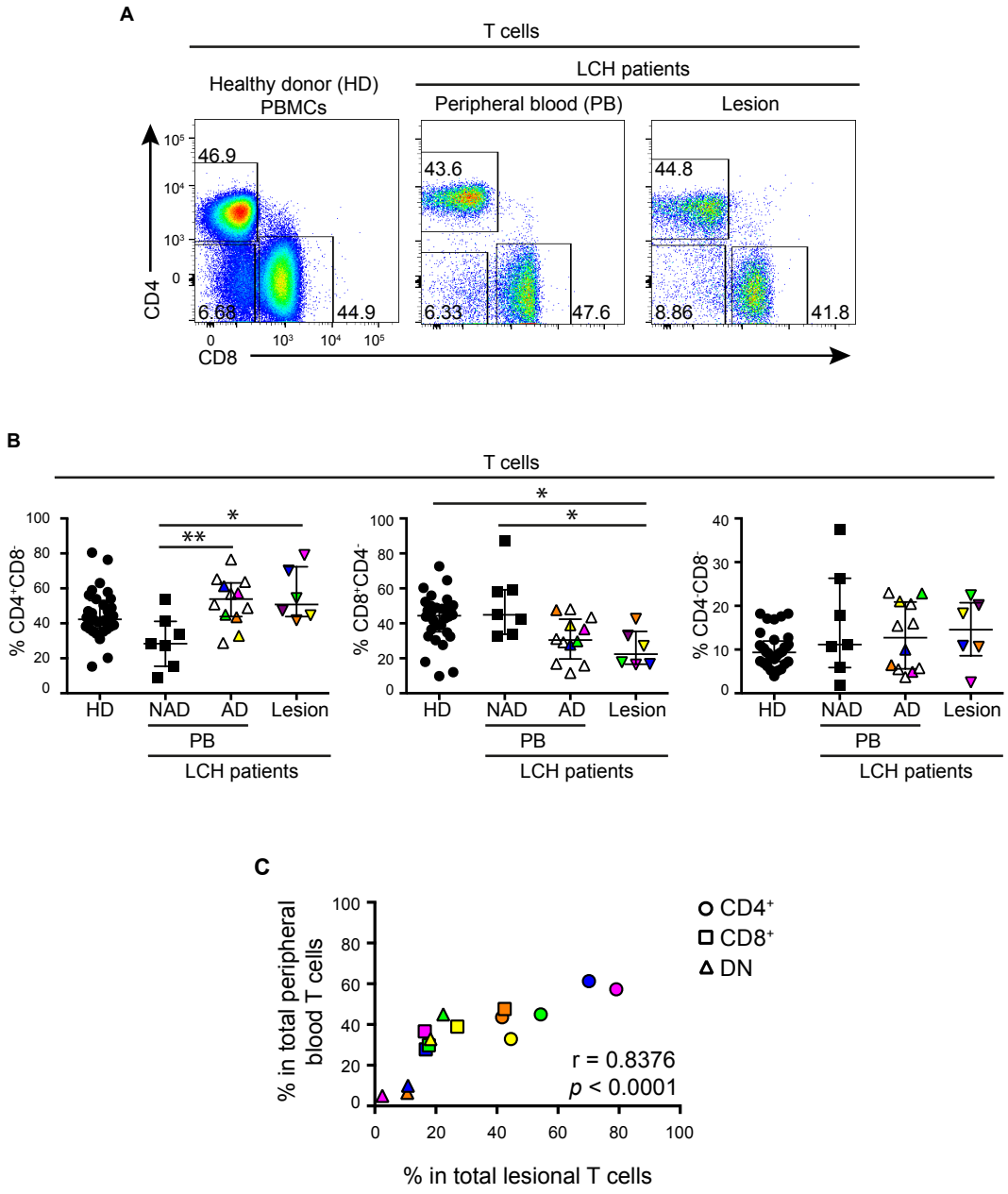


Figure 4.1. Circulatory and lesional T cell proportions in LCH.

*(**A**) Flow cytometry gating strategy for identifying $CD4^+$ T cells, $CD8^+$ T cells and $CD4^-CD8^-$ T cells in T cells from the peripheral blood from healthy donors (left) and from blood (centre) and lesions (right) from LCH patients. (**B**) Proportions of $CD4^+$ T cells (left), $CD8^+$ T cells (centre) and $CD4^-CD8^-$ T cells (right) in T cells from blood from healthy donors and patients with non-active and active LCH, and from lesions from LCH patients. (**C**) Proportions of $CD4^+$ T cells, $CD8^+$ T cells and $CD4^-CD8^-$ T cells in the total T cell population from lesions versus matched blood from LCH patients. Kruskal-Wallis tests with Dunn's multiple comparisons were conducted for (**B**) and a Pearson's two-tailed correlation test was conducted for (**C**). *p <0.05, **p <0.01, error bars indicate median + interquartile range. Lesions and matched peripheral blood samples are colour coded on graphs.* ***HD =healthy donor, PB =peripheral blood, NAD =non-active LCH, AD =active LCH, DN =$CD4^-CD8^-$ T cells.***

4.2.1.1. Circulatory and lesional T cell proportions in LCH

The frequency of $CD4^+$ T cells expressed as a proportion of total T cells was significantly higher in the blood (*p =0.0085, median =53.90%*) and lesions (*p =0.0163, median =50.82%*) from LCH patients compared to blood from patients with non-active disease (median *=28.34%*) (**Figure 4.1B**). The proportion of $CD8^+$ T cells was also significantly decreased in lesions (*median =22.36%*) compared to the proportion in peripheral blood from healthy donors (*p =0.0358, median =44.36%*) and patients with non-active LCH (*p =0.0391, median =44.92%*) (**Figure 4.1B**). No significant difference was observed in the proportions of DN T cells across the groups although interestingly, there was greater variability in the proportion of DN T cells from the peripheral blood from patients with active (*range =3.72-32.91%*) and non-active LCH (*range =1.92-37.50%*) compared to controls (*range =3.92-18.22%*) (**Figure 4.1B**). Because the proportion of each subset in the total T cell population appeared consistent within the lesions and blood from patients with active LCH, this study tested for a correlation between the subset proportions in blood from patients with active disease and matched lesions (**Figure 4.1C**). The subset proportions ($CD4^+$, $CD8^+$ and DN T cells) in the lesional T cell population showed a significant positive correlation (*r =0.8376, p <0.0001*) with the proportions found in the blood from patients with active LCH. The correlation indicates that if any differences are identified in T cell subset proportions in blood from patients with active disease compared to those seen in LCH lesions, this subset could be important in LCH pathogenesis. For example, an alteration to the subset frequency might suggest increased proliferation, migration, inhibition, or apoptosis in the population of interest.

4.2.1.2. Foxp3 is expressed by $CD4^+CD25^+CD127^{low}$ T cells

It is reported that patients with LCH have an enrichment of $Foxp3^+$ Tregs in both their peripheral blood and lesions[57,65,98-100]. For this study Tregs were identified as $CD4^+CD25^+CD127^{low}$ T cells and the results presented here confirm that Foxp3 was expressed by this population (**Figure 4.2A**). From herein $CD4^+CD25^+CD127^{low}$ T cells will be referred to as Tregs, and all other T cells as conventional T cells. Since the frequency of $Foxp3^+$ Tregs is reportedly increased in LCH patients, it is important to identify whether the differences observed in this study in the total $CD4^+$ T cell

population are due to increased Foxp3$^+$ Tregs. Data were therefore reanalysed to test for differences in the proportions of CD4$^+$ T cells, CD8$^+$ T cells and DN T cells in the conventional T cell population excluding Tregs. There remained a trend towards an increase in the proportion of CD4$^+$ T cells in total conventional T cells from the blood from patients with active LCH (*median =51.67%*) compared to those with non-active disease (*median =26.78%*), but this was no longer significant (*p =0.0588*) (**Figure 4.2B**). By excluding Tregs from this analysis the sample size and thus the statistical power of the test was reduced, therefore it is difficult to make a conclusive assessment about whether conventional CD4$^+$ T cells (excluding Tregs) are increased in patients with LCH. After excluding Tregs from the analysis of CD8$^+$ T cells, a significant (*p =0.0454*) decrease was identified in the proportion of CD8$^+$ T cells in T cells from the blood from patients with active LCH (*median =34.21%*) compared to healthy donors (*median =47.67%*) (**Figure 4.2B**).

4.2.2. Functional capability of T cells from patients with LCH

To establish whether T cells from LCH patients can mount a cytokine response to stimulation, the *in vitro* functional capacity of T cells from LCH patients was investigated. T cells were stimulated with PMA and ionomycin (for methods see **Section 3.11.3**), and it was determined that T cells from LCH patients can produce the inflammatory cytokines TNF and IFN-γ when challenged, although there was little spontaneous cytokine production in the unstimulated controls (**Figure 4.3A**). There was no striking difference in the proportions of T cells that could produce Th1 cytokines when comparing T cells in the peripheral blood from healthy donors and LCH patients, and LCH lesion T cells (**Figure 4.3B**). When examining the TNF$^+$ (or) IFN-γ$^+$ population for CD8$^+$ and CD8$^-$ T cell subset distributions, the groups also appeared similarly distributed (**Figure 4.3C**). The CD8$^-$ T cell population comprised both CD4$^+$ T cells and DN T cells because these populations were indistinguishable following stimulation and subsequent downregulation of CD4. Production of Th2 and Th17 cytokines IL-4 and IL-17A, respectively, were measured in T cells from LCH patients, however these cytokines were produced at negligible levels (**Figure 4.3D)**.

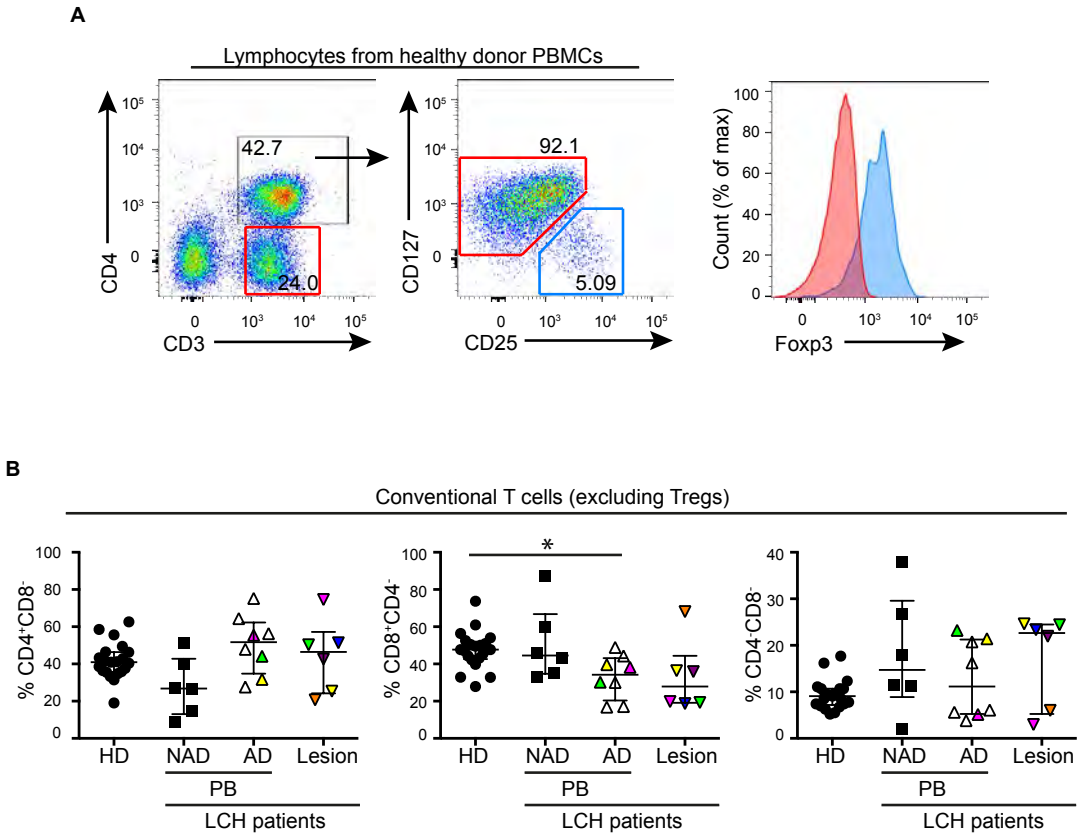


Figure 4.2. Gating on CD4$^+$CD25$^+$CD127^{low} T cells enriches for Foxp3$^+$ Tregs.

*(**A**) Flow cytometry gating strategy (left) for identifying CD4$^+$CD25$^+$CD127^{low} T cells (blue gate) and 'conventional' (all other) T cells (red gates). Histogram (right) displaying high Foxp3 expression in CD4$^+$CD25$^+$CD127^{low} T cells (blue) and low expression in conventional T cells (red). Data are representative of 2 individual experiments. (**B**) Proportions of CD4$^+$ T cells (left), CD8$^+$ T cells (centre) and CD4$^-$CD8$^-$ T cells (right) in conventional T cells (excluding Tregs) from the peripheral blood from healthy donors and patients with non-active and active LCH, and from lesions from LCH patients. Kruskal-Wallis tests with Dunn's multiple comparisons were conducted for (**B**). *p <0.05, error bars indicate median + interquartile range. Lesions and matched peripheral blood samples are colour coded on graphs.* ***HD =healthy donor, PB =peripheral blood, NAD =non-active LCH, AD =active LCH.***

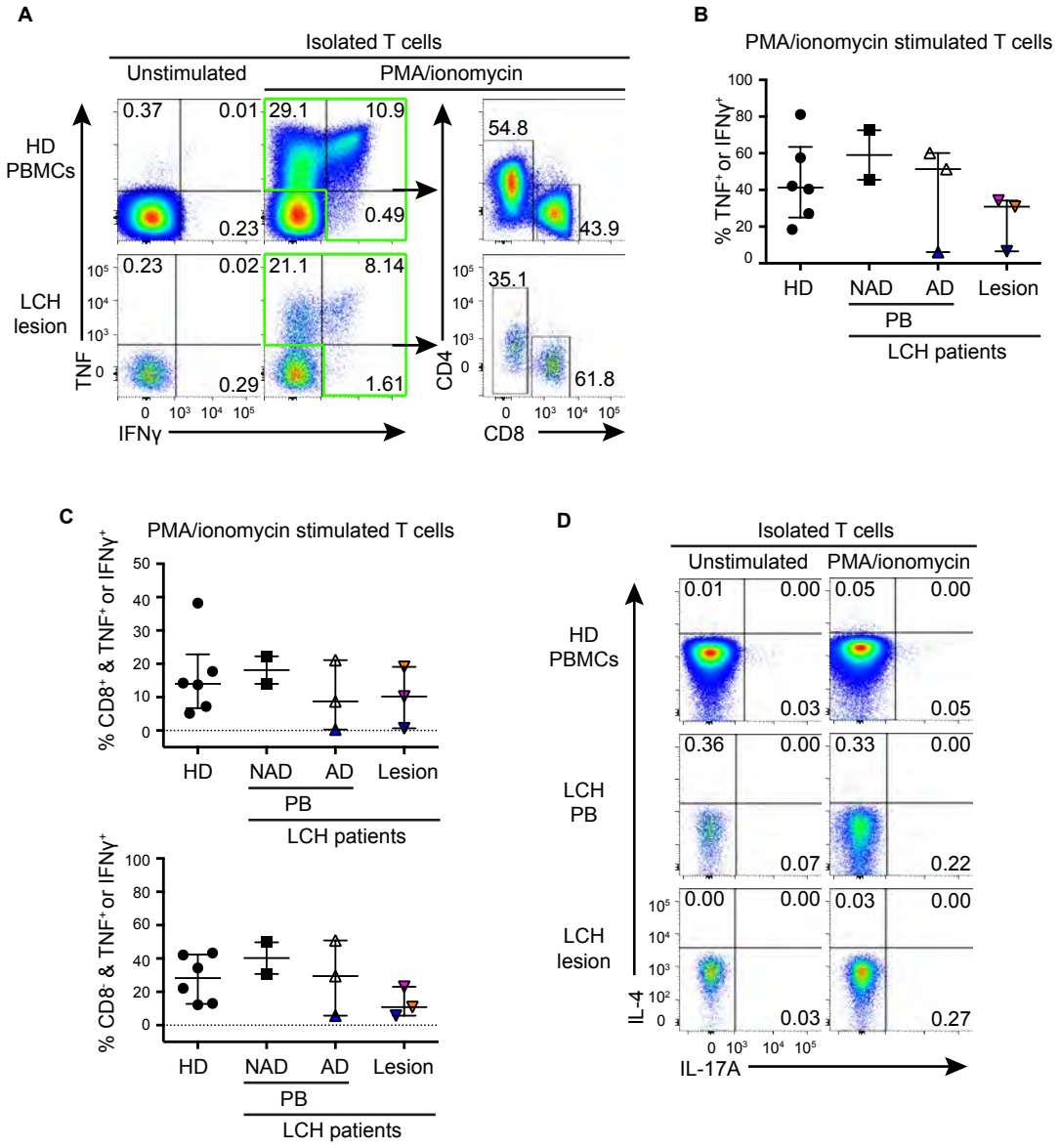


Figure 4.3. Cytokine production by T cells from patients with LCH.

*(**A**) Flow cytometry gating strategy for post-stimulation identification of TNF and IFN-γ producing T cells and identification of CD8$^+$ and CD8$^-$ T cells within the cytokine producing population. (**B**) Proportions of TNF or IFN-γ $^+$ T cells in total T cells from the peripheral blood from healthy donors and patients with non-active and active LCH, and from lesions from LCH patients. (**C**) Proportions of cytokine producing CD8$^+$ T cells and CD8$^-$ T cells in T cells from blood from healthy donors and patients with non-active and active LCH, and from lesions from LCH patients. (**D**) Gating strategy and representative plots for post-stimulation identification of IL-4 and IL-17A producing T cells in T cells from blood from healthy donors and from blood and lesions from LCH patients. Kruskal-Wallis tests with Dunn's multiple comparisons were conducted for (**B**) and (**C**). Error bars indicate median + interquartile range. Lesions and matched peripheral blood samples are colour coded on graphs.* ***HD =healthy donor, PB =peripheral blood, NAD =non-active LCH, AD =active LCH.***

4.2.3. Activation status of T cells from patients with LCH

This study also investigated whether T cells from patients with LCH were active without *in vitro* manipulation. Expression of the IL-2 receptor CD25 was examined in T cells from lesions (**Figure 4.4A**) and peripheral blood (**Figure 4.4B**) from LCH patients, however there was little expression of CD25 on cells outside of the gate that was established to identify Tregs ($CD4^+CD25^+CD127^{low}$ T cells). LCH lesion T cells were also analysed for HLA-DR expression and this was low similar to that seen in T cells from healthy donor PBMCs (**Figure 4.4C**).

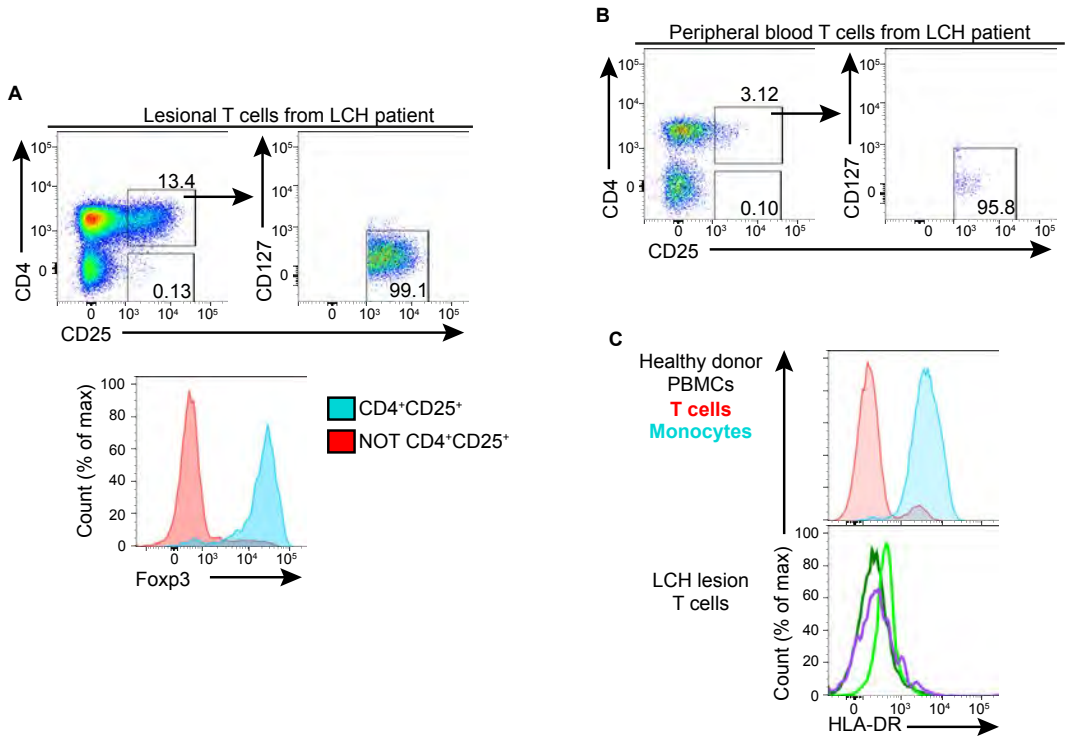


Figure 4.4. Expression of activation marker CD25 in patients with LCH.

*(**A**) Representative flow cytometry plots demonstrating that CD25 expressing T cells from LCH lesions are $CD4^+$, $CD127^{low}$ and $Foxp3^+$. (**B**) Representative plots demonstrating that CD25 expressing T cells from LCH patient peripheral blood are $CD4^+$ and $CD127^{low}$. (**C**) Histogram demonstrates that LCH lesion T cells from three individual patients express low HLA-DR similarly to T cells from healthy donor derived PBMCs. Plots are representative of six individual donors for (**A**), 14 for (**B**).*

4.2.3.1. Expression of activation associated genes in conventional T cells

Conventional T cells (excluding Tregs) were purified by FACS from the peripheral blood from healthy donors and from blood and lesions from patients with active LCH (**Figure 4.5A**) and post-sort purity was >96% (**Appendix A, Table A1**). Expression of a range of genes associated with T cell activation were investigated in the purified population (**Figure 4.5B**). Because only small numbers of purified conventional T cells were able to be collected from LCH patient specimens, it was necessary to conduct whole transcriptome amplification via a 'cells to cDNA' approach (for methods see **Section 3.12.1**). Gene amplification using a range of primers was successful in four of six samples from healthy donors, one of five samples from the peripheral blood from patients with active LCH and two of five lesional samples as determined by SYBR based qPCR (**Appendix A, Table A1**). Amplification occurred in a lower than desirable number of samples isolated from the blood and lesions from LCH patients, therefore due to sample size it is difficult to establish whether there are any differences in the expression levels of the genes measured in LCH patients when compared to healthy donors (**Figure 4.5B**). Importantly however, the presence of activation-associated genes (*IL2RA*, *TNF*, *CD40LG*, *LIF* and *LIFR*) was confirmed in conventional T cells from the peripheral blood and lesions from patients with active LCH (**Figure 4.5B** and **Appendix A, Table A1**).

4.2.3.2. Analysis of publicly available data on T cells from LCH lesions

To understand the activation status of T cells from LCH lesions and to establish which biological processes and pathways are associated with them, this study conducted a reanalysis of publicly available microarray data on isolated T cells from LCH lesions[65] (available at http://www.jimmunol.org/content/184/8/4557/tab-figures-data). Genes considered for the enrichment analysis were increased in expression by a minimum of two-fold in LCH lesion T cells compared to peripheral blood T cells and all enrichment analysis data presented were selected based on Fisher's exact test with FDR multiple test correction. Raw and FDR adjusted p-values for all processes and pathways listed were <0.05.

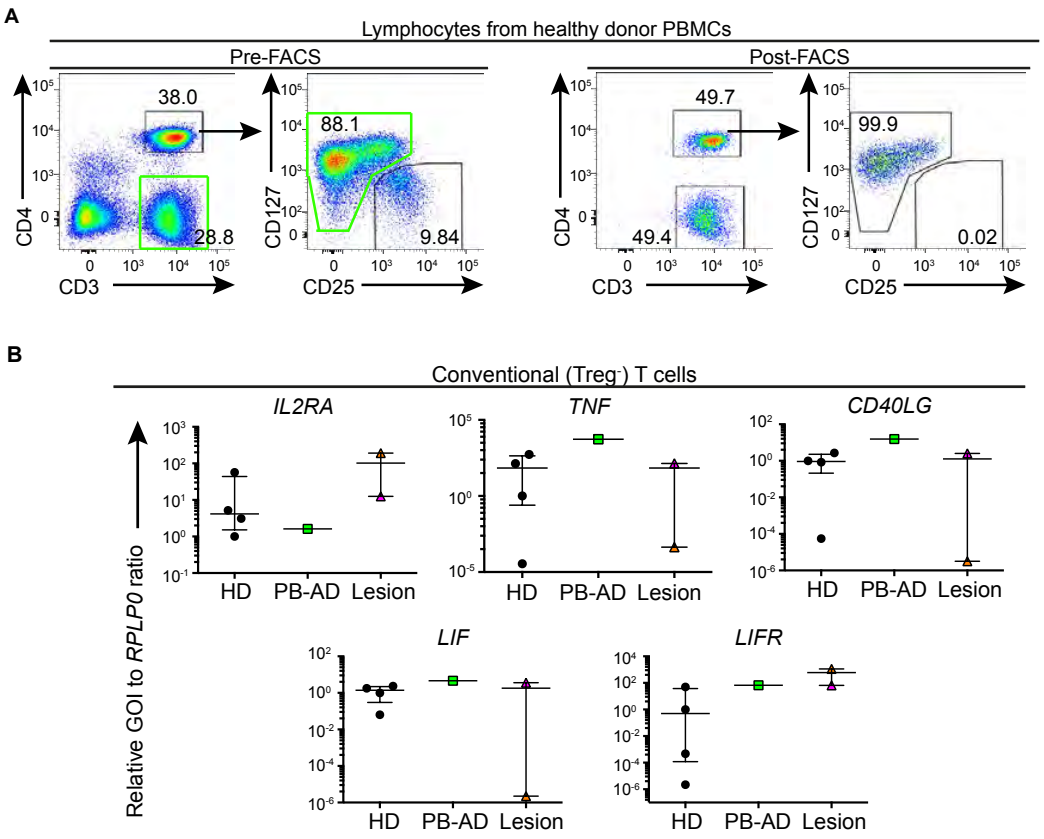


Figure 4.5. Expression of activation associated genes in conventional T cells.

*(**A**) Flow cytometry gating strategy for identifying an isolating conventional T cells excluding Tregs ($CD4^{+}CD25^{+}CD127^{low}$ T cells) (left) and post-FACS analysis of conventional T cells (excluding Tregs) (right). Green gates denote conventional T cell sort parameters. (**B**) Relative gene of interest to reference gene (RPLP0) copy number ratio for IL2RA, TNF, CD40LG, LIF and LIFR in conventional T cells from the peripheral blood from healthy donors and from blood and lesions from patients with active LCH. Error bars indicate median + interquartile range. Lesions and matched peripheral blood samples are colour coded on graphs.* ***GOI =gene of interest, HD =healthy donor, PB-AD =active LCH peripheral blood.***

Gene Ontology biological process complete analysis (**Table 4.1**) identified the upregulation of genes involved in 'regulation of tolerance induction dependent on immune response' (GO: 0002652; *HAVCR2* and *FOXP3*), 'negative regulation of Th1 type immune response' (GO: 0002826, *IL1RL1* and *HAVCR2*), 'regulation of IL-10 secretion (GO:2001179, *HLA-DRB1* and *TNFRSF9*), 'positive regulation of TNF-mediated signalling pathway' (GO:1903265, *HSPA1A* and *HSPA1B*), 'negative regulation of IFN-γ production' (GO:0032689, *HLA-DRB1*, *IL1RL1*, *HAVCR2* and *FOXP3*), 'negative regulation of T cell proliferation' (GO:0042130, *SDC4*, *HLA-DRB1*, *HAVCR2*, *FOXP3*, *CTLA4*, *GPNMB*), 'negative regulation of T cell differentiation (GO:0045581,

FOXP3, *CD74*, *CTLA4*) and 'positive regulation of T cell mediated immunity' (GO:0002711, *IL1R1*, *FOXP3* and *CD1A*). Interestingly, the enrichment analysis also identified the upregulation of genes involved in 'positive regulation of viral entry into host cell' (GO:0046598, *HLA-DRB1* and *CD74*), 'positive regulation of monocyte differentiation' (GO: 0045657, *CD74* and *HLA-DRB1*), and less specific terms such as 'negative regulation of response to external stimulus' (GO:0032102; 8 genes), 'inflammatory response' (GO:0006954; 12 genes), 'response to lipopolysaccharide' (GO:0032496; 6 genes) and 'adaptive immune response' (GO:0002250; 8 genes).

Table 4.1. GO biological process complete analysis of LCH lesion T cell microarray data.

GO biological process complete	No. of genes upregulated	Fold Enrichment
Polysaccharide assembly with MHC II protein complex	2	>100
Positive regulation of nucleotide-binding oligomerisation domain containing 2 signalling pathway	2	>100
Regulation of tolerance induction dependent upon immune response	2	>100
Peptide antigen assembly with MHC II protein complex	2	>100
IL-3-mediated signalling pathway	2	>100
Negative regulation of IL-1-mediated signalling pathway	2	>100
Positive regulation of microtubule nucleation	2	>100
Cellular heat acclimation	2	>100
Negative regulation of cysteine-type endopeptidase activity involved in apoptotic signalling pathway	2	97.42
Negative regulation of Th1 type immune response	2	83.5
Regulation of IL-10 secretion	2	73.06
Positive regulation of TNF-mediated signalling pathway	2	64.94
Positive regulation of monocyte differentiation	2	58.45
Positive regulation of viral entry into host cell	2	58.45
Positive regulation of myeloid leukocyte cytokine production involved in immune response	2	58.45
Tolerance induction	2	53.14
Protein refolding	3	39.85
Chaperone cofactor-dependent protein refolding	4	37.71
Positive regulation of IL-4 production	3	36.53
Negative regulation of IFN-γ production	4	34.38
Positive regulation of neutrophil migration	3	32.47
Regulation of fibroblast migration	3	32.47
Negative regulation of T cell proliferation	6	28.75
Negative regulation of B cell activation	3	27.4

Negative regulation of extrinsic apoptotic signalling pathway in absence of ligand	3	17.89
Embryo implantation	3	21.92
Negative regulation of T cell differentiation	3	21.38
Regulation of macrophage activation	3	19.93
Positive regulation of T cell mediated immunity	3	19.06
Receptor internalisation	4	18.85
Positive regulation of IL-8 production	3	18.65
Antigen processing and presentation of exogenous peptide antigen via MHC II	6	17.89
Negative regulation of TNF production	3	17.54
IL-1-mediated signalling pathway	3	17.19
Positive regulation of chemokine production	3	16.54
Regulation of kidney development	3	16.54
IFN-γ-mediated signalling pathway	4	16.46
Regulation of tissue remodelling	4	15.38
Positive regulation of leukocyte chemotaxis	4	14.8
Positive regulation of proteasomal ubiquitin-dependent protein catabolic process	4	14.43
Negative regulation of intrinsic apoptotic signalling pathway	4	12.05
Leukocyte chemotaxis	5	10.51
Regulation of IL-6 production	4	9.91
Cellular response to unfolded protein	4	9.82
Cellular response to oxidative stress	6	7.83
Negative regulation of response to external stimulus	8	7.77
Inflammatory response	12	7.76
Positive regulation of ERK1 and ERK2 cascade	6	7.37
Negative regulation of secretion	5	6.96
Regulation of animal organ morphogenesis	5	6.93
Neutrophil degranulation	11	6.66
Response to lipopolysaccharide	6	5.81
Positive regulation of secretion by cell	7	5.59
Regulation of inflammatory response	7	5.15
Immune response-activating signal transduction	8	4.82
Immune response-regulating cell surface receptor signalling pathway	7	4.81
Response to wounding	9	4.7
Adaptive immune response	8	4.38
Cellular response to oxygen-containing compound	12	3.69
Tissue development	16	2.75

'Supplemental Data 2 – LCH Lesion CD3 vs. Peripheral LCH CD3 Array Results (L-CD3/P-CD3)' from Allen et al.[65] (available at http://www.jimmunol.org/content/184/8/4557/tab-figures-data).

Reactome pathway analysis (**Table 4.2**) also identified the enrichment of genes (*HLA-DQB1*, *HLA-DRA* and *HLA-DRB1*) involved in 'translocation of ZAP-70 to immunological synapse', 'phosphorylation of CD3 and TCR zeta chains' and 'PD-1 signalling'. Enrichment of genes involved in these pathways suggests that there are increases in the formation of functional immune synapses ('translocation of ZAP-70 to immunological synapse' and 'phosphorylation of CD3 and TCR zeta chains')[173,174] and tolerance (reviewed by Francisco et al.[175]) in T cells from LCH lesions. Other less specific Reactome pathways with enriched genes included 'chemokine receptors bind chemokines' (4 genes), and 'cytokine signalling in immune system' (11 genes).

Table 4.2. GO Reactome pathway analysis of LCH lesion T cell microarray data.

Reactome pathways	Number of genes upregulated	Fold Enrichment
Translocation of ZAP-70 to immunological synapse	3	38.12
Phosphorylation of CD3 and TCR zeta chains	3	33.72
PD-1 signalling	3	32.47
Chemokine receptors bind chemokines	4	20.51
MHC II antigen presentation	6	14.86
Cytokine signalling in immune system	11	5.3

'Supplemental Data 2 – LCH Lesion CD3 vs. Peripheral LCH CD3 Array Results (L-CD3/P-CD3)' from Allen et al.[65] (available at http://www.jimmunol.org/content/184/8/4557/tab-figures-data).

Results by Allen et al.[65] had previously established that T cells within LCH lesions expressed a number of genes consistent with an activated regulatory T cell phenotype, and they also demonstrated upregulation of migration associated genes. The enrichment analyses presented in the current study also suggests activation of, and negative regulation by T cells within LCH lesions, and additionally the results presented here have established that there are potential viral and/or bacterial related processes occurring within the LCH microenvironment, although further investigations are necessary to fully interpret this analysis.

4.2.4. Identification of Tregs in patients with LCH

Foxp3^{+} Tregs were previously reported to be enriched in patients with LCH as defined by their expression of CD3, CD4, CD25 and Foxp3[57,98,100]. Tregs can also be reliably identified using a surrogate definition of CD4^{+}CD25^{+}CD127^{low} T cells, which allows for isolation of live Tregs thus permitting downstream functional assays[102,103]. This method was adopted for the current study and primarily used to identify Tregs in tissues from patients with LCH (**Figure 4.6A**).

4.2.4.1. Circulatory and lesional Treg proportions in LCH

The proportion of Tregs was significantly (*p <0.0001*) increased in the CD4^{+} T cell population in lesions from LCH patients (*median =24.54%*) compared to peripheral blood from healthy donors (*median =2.27%*) (**Figure 4.6B**). The proportion of Tregs in total T cells was also significantly increased in the blood (*p =0.0232, median =1.73%*) and lesions (*p <0.0001, median =12.85%*) from patients with active disease compared to those in the blood from healthy donors (*median =1.03%*) (**Figure 4.6C**). The data presented here using the surrogate staining method are consistent with the existing literature[57,65,98-100]. Additionally, the current study confirmed Foxp3 expression in the lesional T cells that were identified as Tregs (**Figure 4.6D**).

4.2.4.2. *In situ* identification of Foxp3^{+} Tregs and TGF-β

To determine whether Foxp3^{+} Tregs are producing TGF-β *in situ*, immunofluorescent antibody labelling was used to visualise expression of CD3, Foxp3 and TGF-β. Antibody concentrations were optimised using control slides with skin and bowel tissues. In the skin, there was nuclear (DAPI) labelling (**Figure 4.7A. i**) and expression of CD3 (**Figure 4.7A. ii**), Foxp3 (**Figure 4.7A. iii**) and TGF-β (**Figure 4.7A. iv**). The merged image demonstrates co-localisation of DAPI, Foxp3 and TGF-β coated with CD3 (**Figure 4.7B**).

Extracellular TGF-β labelling was deemed specific as confirmed by comparing staining by rabbit anti-TGF-β (**Figure 4.8A**) and cell-specific rabbit anti-cyclin D1 (**Figure 4.8B**) antibodies using the same anti-rabbit secondary antibody in bowel tissue.

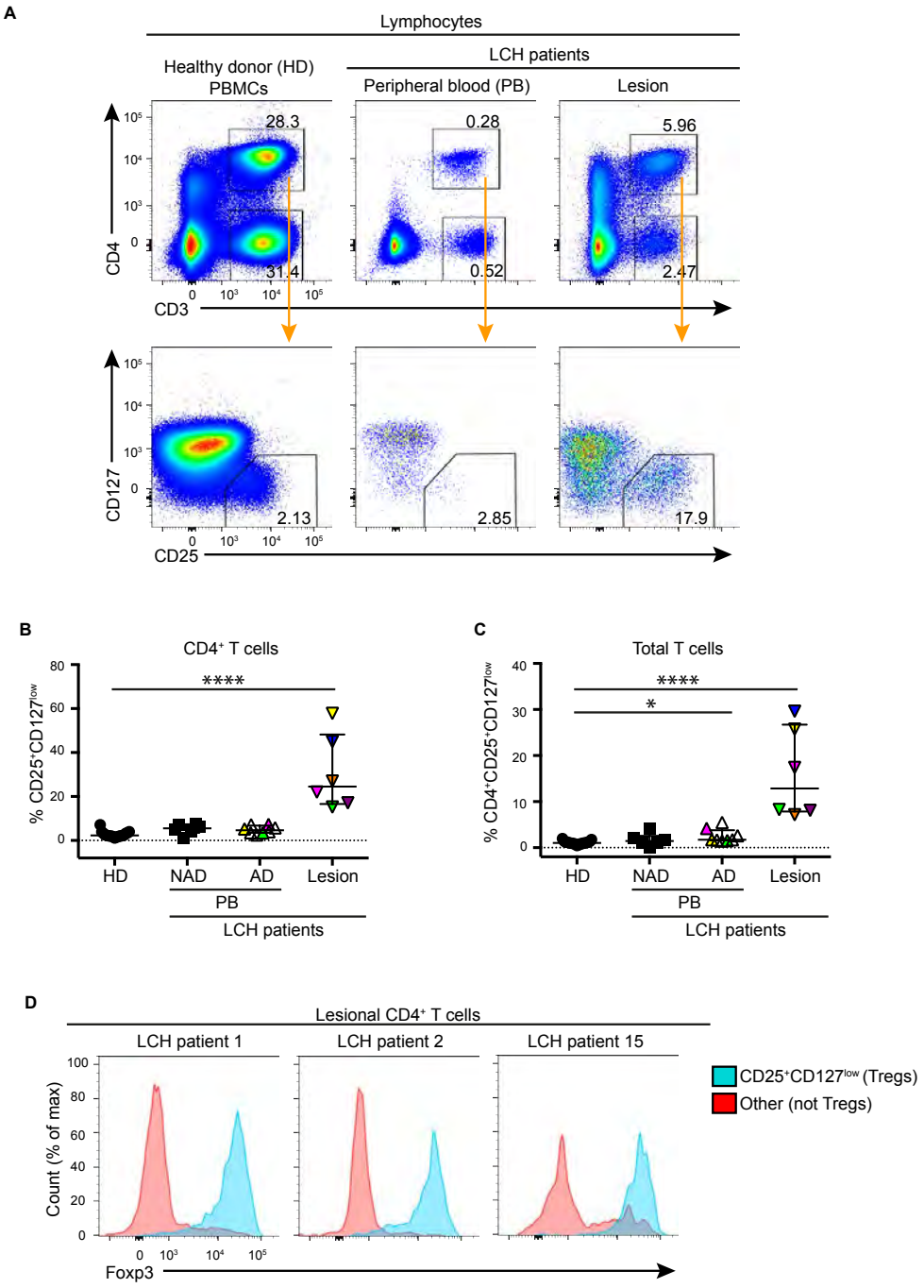


Figure 4.6. Circulatory and lesional Treg proportions in LCH.

*(**A**) Flow cytometry gating strategy for identifying $CD3^+CD4^+CD25^+CD127^{low}$ Tregs in lymphocytes from the peripheral blood from healthy donors (left) and from blood (centre) and lesions (right) from LCH patients. (**B**) Proportions of Tregs in $CD4^+$ T cells from blood from healthy donors and patients with non-active and active LCH, and from lesions from LCH patients. (**C**) Proportions of Tregs in total T cells from the peripheral blood from healthy donors and patients with non-active and active LCH, and from lesions from LCH patients. (**D**) Foxp3 expression by $CD3^+CD4^+CD25^+CD127^{low}$ T cells from lesions from three LCH patients. Kruskal-Wallis tests with Dunn's multiple comparisons were conducted for (**B**) and (**C**). *p <0.05, ****p <0.0001, error bars indicate median + interquartile range. Lesions and matched peripheral blood samples are colour coded on graphs.* ***HD =healthy donor, PB =peripheral blood, NAD =non-active LCH, AD =active LCH.***

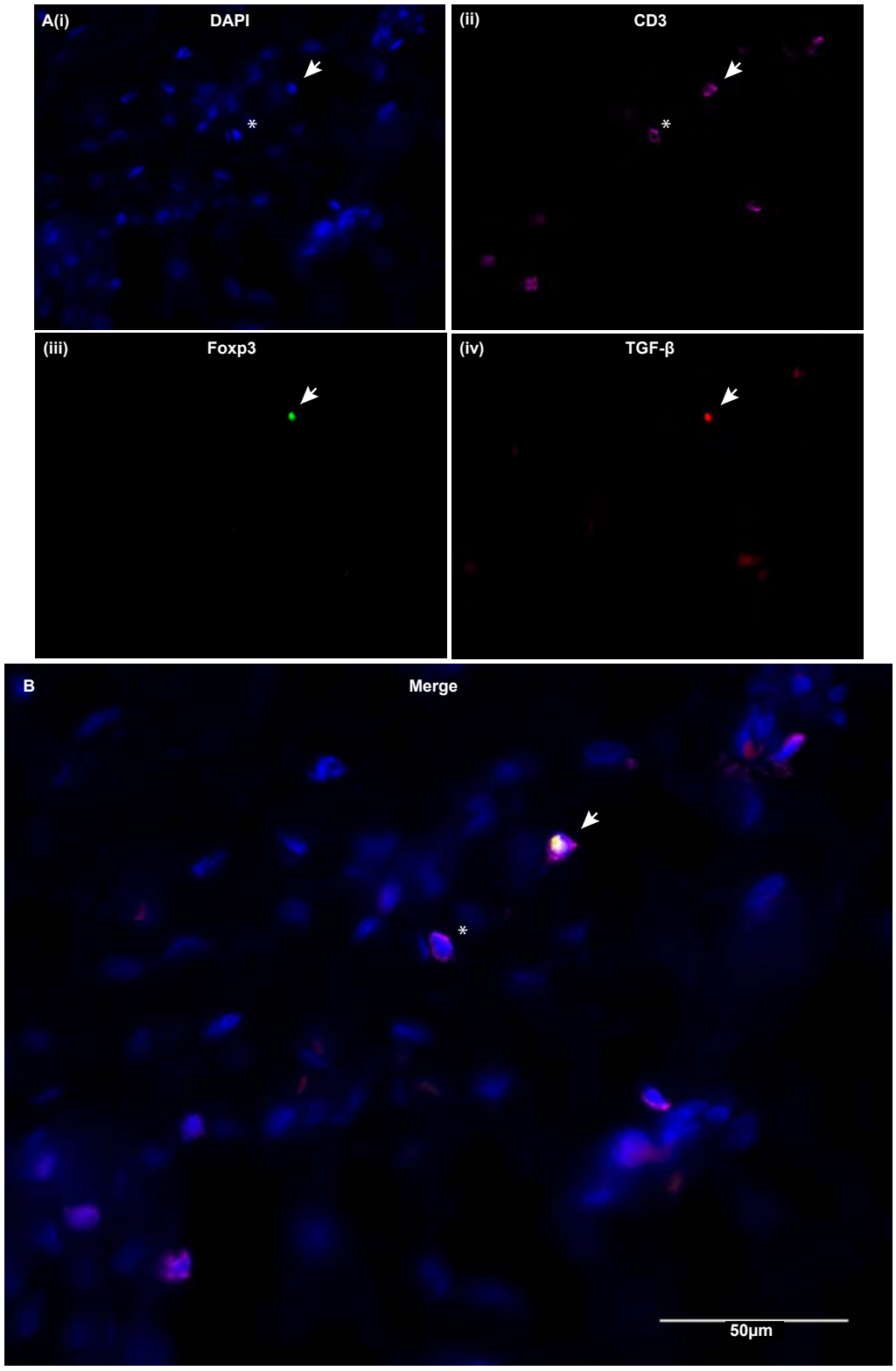


Figure 4.7. CD3 and Foxp3 co-expression and TGF-β production in skin.

*(**A**) Fluorescent microscopy images show a tissue section from skin with nuclear (DAPI) labelling in blue (**i**), CD3 labelling in magenta (**ii**), nuclear Foxp3 expression in green (**iii**) and TGF-β in red (**iv**). (**B**) Merged image shows co-expression of CD3, Foxp3 and TGF-β (arrow) and CD3 alone (asterisk).*

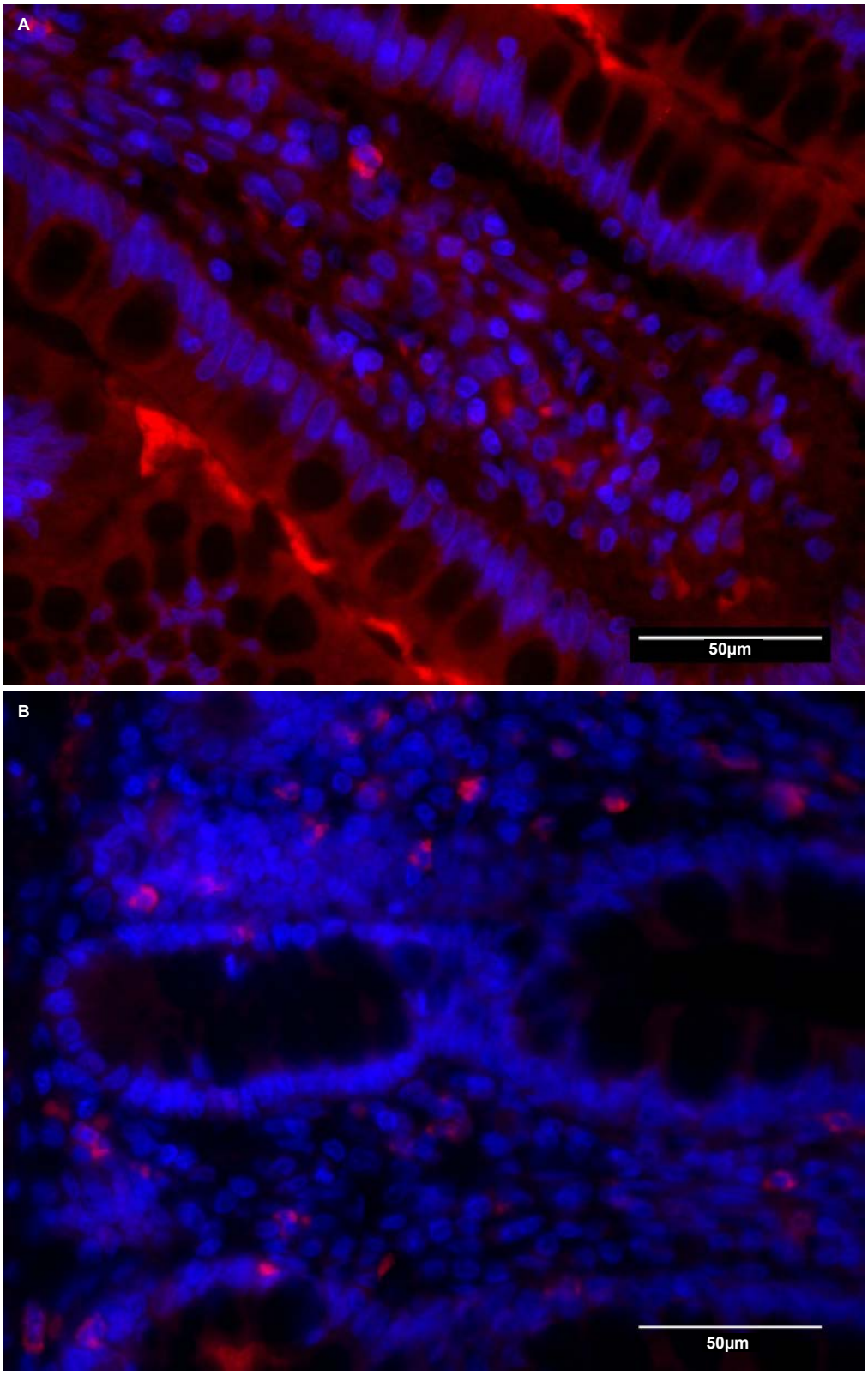


Figure 4.8. Specific secondary antibody labelling in bowel tissue.

*(**A**) Image shows nuclear (DAPI) labelling in blue and TGF β labelling in red (**B**) Image shows nuclear (DAPI) labelling in blue and cyclin D1 (Dako) labelling in red using the same anti-rabbit secondary antibody used in (**A**). Images are tissue sections from bowel.*

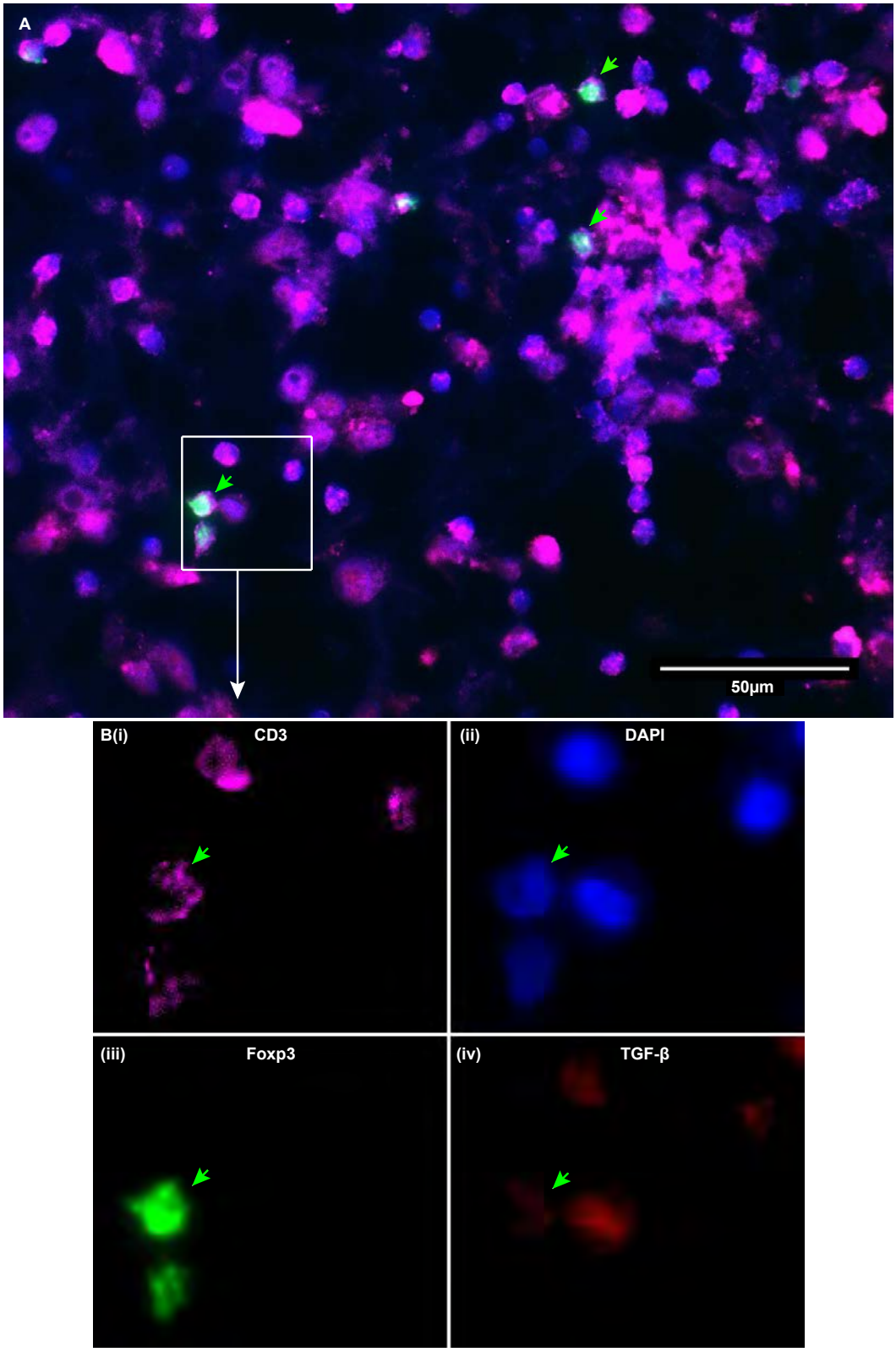
Following optimisation steps, co-expression of CD3 and Foxp3 was confirmed in LCH lesion tissue sections (n =3) (**Figure 4.9A**). In the tissue, there was expression of CD3 (**Figure 4.9B. i**) with nuclear (DAPI) labelling (**Figure 4.9B. ii**), and expression of Foxp3 (**Figure 4.9B. iii**) and TGF-β (**Figure 4.9B. iv**). The images demonstrate specific co-localisation of DAPI and Foxp3 coated with CD3. TGF-β was definitely expressed in LCH lesions (n =3) (**Figure 4.9C. i**), however staining intensity within $Foxp3^{+}CD3^{+}$ cells was not strong as compared to other larger (presumably LCH) cells (**Figure 4.9C. ii**).

4.2.4.3. CD56 expression by Tregs from patients with LCH

Interestingly, Tregs from LCH lesions unexpectedly expressed CD56 on their cell surface, whereas very little CD56 expression was observed on Tregs in peripheral blood samples from healthy donors and LCH patients (**Figure 4.10A**). There was a statistically significant increase in the cell surface expression of CD56 by Tregs in the lesions from LCH patients (*median =36.48%*) compared to expression in the peripheral blood from healthy donors (*p =0.0092, median =2.29%*) and patients with active disease (*p <0.0001, median =0.58%*) (**Figure 4.10B**).

4.2.4.4. CD56 expression by total T cells from patients with LCH

CD56 expression was also examined on total T cells in the peripheral blood from healthy donors and LCH patients, and in LCH lesions (**Figure 4.10C**). CD56 expression on T cells was significantly decreased in the peripheral blood from patients with active disease (*median =1.71%*) when compared to those with non-active disease (*p =0.0490, median =10.95%*) and lesions (*p =0.0136, median =7.04%*) (**Figure 4.10D**). By excluding Tregs it appears that the $CD56^{+}$ T cell population in lesions (*median =2.37%*) is similar to the total $CD56^{+}$ T cell population seen in the blood from patients with active LCH. Additionally, $CD56^{+}$ Tregs are a large constituent (*median =67.08%*) of the total lesional $CD56^{+}$ T cell population (**Figure 4.10E**).


A
50µm
B(i)
CD3
(ii)
DAPI
(iii)
Foxp3
(iv)
TGF-β

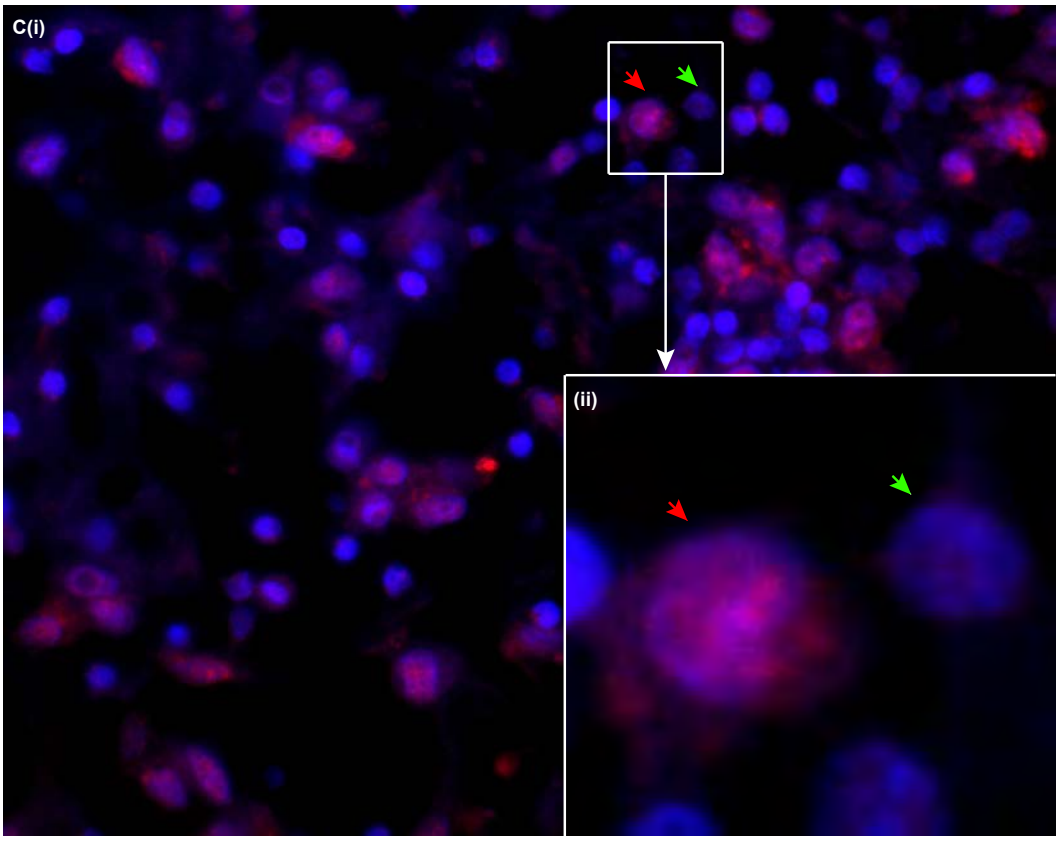


Figure 4.9. In situ Foxp3 expression and TGF-β production in LCH lesions.

*(**A**) Merged image shows tissue section from osseous LCH lesion with DAPI, CD3, Foxp3 and TGF-β labelling. (**B**) Images show a magnified field from (**A**) (white square) with CD3 labelling in magenta (**i**), nuclear (DAPI) labelling in blue (**ii**), nuclear Foxp3 expression in green (**iii**) and TGF-β in red (**iv**). Green arrows indicate $CD3^{+}Foxp3^{+}$ cells. (**C**) (**i**) Image of the field in (**A**) with only nuclear (DAPI; blue) and TGF-β (red) labelling showing previously identified $CD3^{+}Foxp3^{+}$ cell (green arrow) with low intensity nuclear TGF-β compared to larger cell (red arrow) with strong TGF-β expression, field from (**i**) (white square) is magnified in (**ii**). Images are representative of three individual patient lesions.*

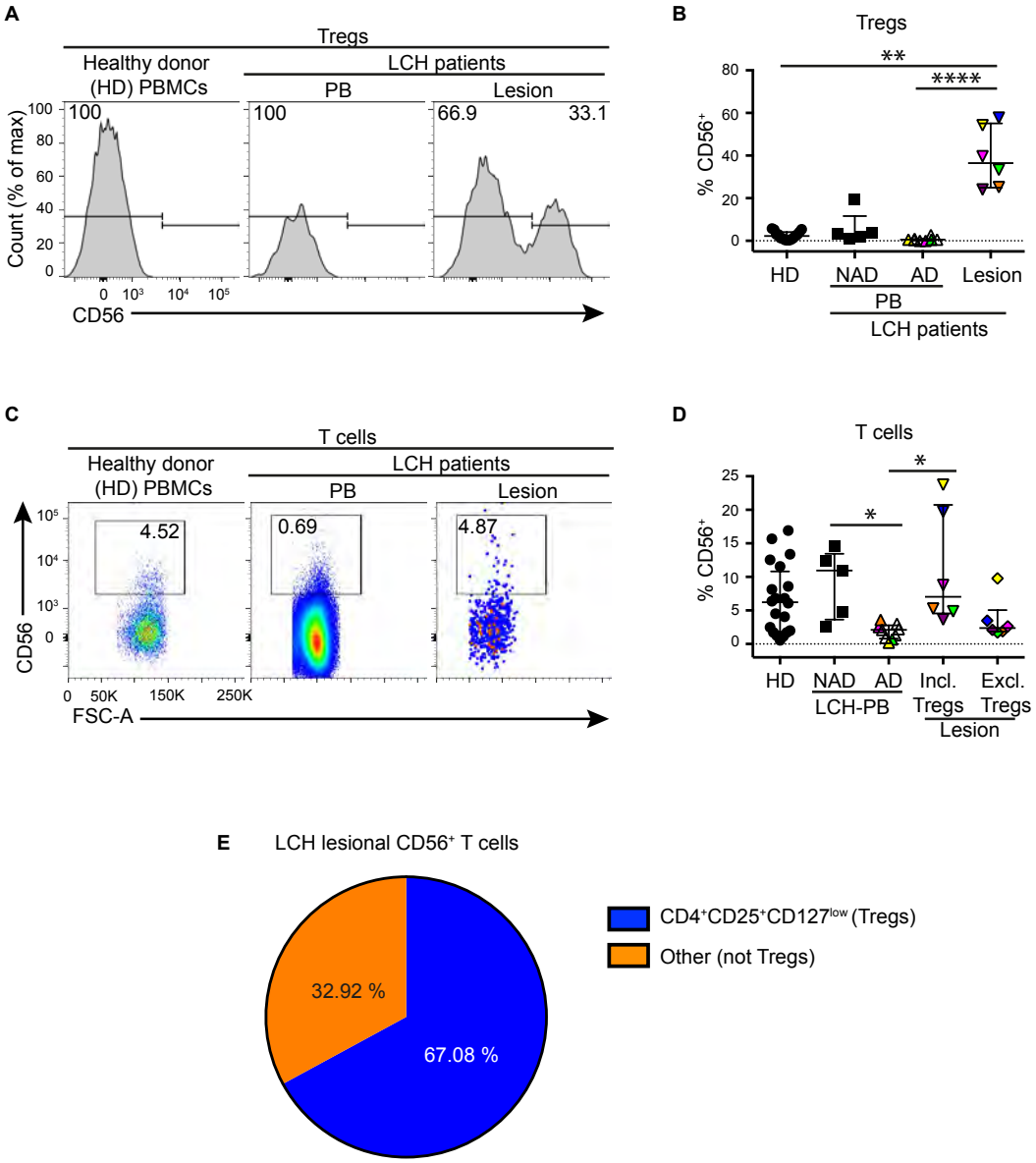


Figure 4.10. CD56 expression on T cells from patients with LCH.

*(**A**) Flow cytometry gating strategy for identifying CD56 expression on Tregs from the peripheral blood from healthy donors (left) and from blood (centre), and lesions (right) from LCH patients. (**B**) Proportions of CD56$^+$ Tregs in total Tregs from blood from healthy donors and patients with non-active and active LCH, and from LCH lesions. (**C**) Gating strategy for identifying CD56 expression on total T cells from the peripheral blood from healthy donors (left) and from blood (centre) and lesions (right) from LCH patients. (**D**) Proportions of CD56$^+$ T cells in total T cells from blood from healthy donors and patients with non-active and active LCH, and from LCH lesions with Tregs included and excluded. (**E**) A representation of the median proportion of Tregs and non-Tregs that make up the total CD56$^+$ T cell population within LCH lesions. Kruskal-Wallis tests with Dunn's multiple comparisons were conducted for (**B**) and (**D**). *p <0.05, **p <0.01, ****p <0.0001, error bars indicate median + interquartile range. Lesions and matched peripheral blood samples are colour coded on graphs.* ***HD =healthy donor, PB =peripheral blood, NAD =non-active LCH, AD =active LCH.***

4.2.5. CD56 expression by $CD3^-$ lymphocytes and T cell subsets in LCH patients

Because of the unexpected expression of CD56 by Tregs in lesions from LCH patients, CD56 expression in the broader lymphocyte population was investigated by assessing CD3 versus CD56 (**Figure 4.11A. i**). Additionally, expression of CD4 and CD8 by $CD56^+$ T cells was characterised (**Figure 4.11A. ii**). The ratio of $CD3^-CD56^+$ lymphocytes, which are most likely NK cells, was not strikingly different in patients with LCH compared to peripheral blood from healthy donors (**Figure 4.11B**). The ratio of $CD3^-CD56^+$ lymphocytes to $CD56^+$ T cells was significantly (*p =0.0048*) decreased within lesions (*median =0.7850*) when compared to blood from patients with active disease (*median =9.660*) (**Figure 4.11C**). The difference in ratios was likely due to the increase in $CD56^+$ T cells in LCH lesions. Investigating $CD56^+$ T cell subsets identified that $CD8^+CD56^+$ T cells were significantly reduced in proportion in the blood (*median =0.32%, p =0.0080*) and lesions (*median =0.16%, p =0.0296*) from patients with active LCH when compared to peripheral blood from healthy donors (*median =3.30%*) (**Figure 4.11D. i**). Additionally, this study tested whether there was a correlation between the proportion of Tregs and the proportion of $CD8^+CD56^+$ T cells in total T cells from LCH patients and identified a significant negative correlation between the two populations (*r =-0.56, p =0.032*) (**Figure 4.11D. ii**). $CD4^-CD8^-CD56^+$ T cells were not considerably different in relative frequency in LCH patients compared to blood from healthy donors (**Figure 4.11E**). Within lesions the proportion of $CD4^+CD56^+$ T cells in total T cells (*median =3.90%*) was significantly higher when compared with blood from healthy donors (*median =0.62%*, *p =0.0218*) and patients with active LCH (*median =0.48%, p =0.0094*) (**Figure 4.11F**). The proportion of Tregs in the $CD4^+CD56^+$ T cell population was significantly (*p =0.003*) higher within lesions (*median =84.00%*) when compared to the blood from healthy donors (*median =1.91%*), and it also trended substantially higher when compared to the blood from patents with active disease (*median =7.51%, p =0.096*) (**Figure 4.11G**).

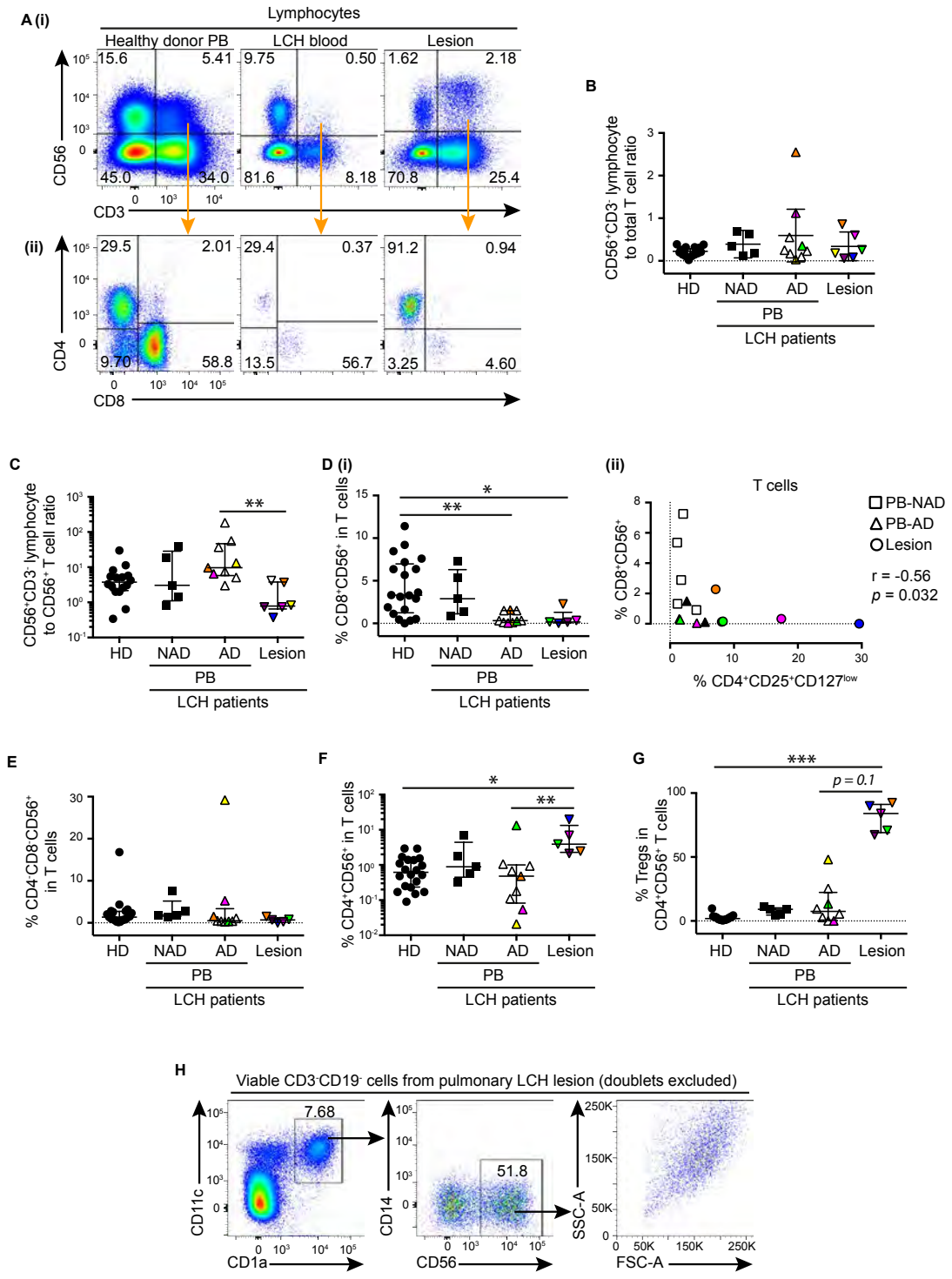


Figure 4.11. Expression of CD56 on CD3$^-$ lymphocytes and T cell subsets in LCH patients.

*(**A**) (**i**) Flow cytometry gating strategy to identify cell surface CD3 and CD56 expression by lymphocytes from the peripheral blood from healthy donors (left) and from blood (centre) and lesions (right) from LCH patients. (**ii**) Gating strategy to identify CD4$^+$, CD8$^+$ and CD4$^-$CD8$^-$ T cells in CD56$^+$ T cells from the peripheral blood from healthy donors (left) and from blood (centre) and lesions (right) from LCH patients. Ratio of CD56$^+$CD3$^-$ (NK) cells to T cells (**B**) and*

*$CD56^+CD3^-$ (NK) cells to $CD56^+$ T cells (**C**) in blood from healthy donors and patients with non-active and active LCH, and in lesions from LCH patients. (**D**) Proportion of $CD8^+CD56^+$ cells in T cells from blood from healthy donors and from blood and lesions from LCH patients (**i**), and correlation between $CD8^+CD56^+$ cells and Tregs in T cells from blood from patients with active (triangles) and non-active (open squares) LCH, and from LCH lesions (circles) (**ii**). Proportion of $CD4^-CD8^-CD56^+$ (**E**) and $CD4^+CD56^+$ (**F**) T cells in total T cells from blood from healthy donors and from blood and lesions from patients with non-active and active LCH, and from LCH lesions. (**G**) Proportion of $CD4^+CD25^+CD127^{low}$ T cells (Tregs) in $CD4^+CD56^+$ T cells from blood from healthy donors and patients with non-active and active LCH, and from LCH lesions. (**H**) Plots demonstrate a single pulmonary LCH lesion where approximately half of the $CD3^-CD19^-CD11c^+CD1a^+$ (LCH cells) express CD56. Kruskal-Wallis tests with Dunn's multiple comparisons were conducted for (**B**-**G**). *p <0.05, **p <0.01, ***p <0.001, error bars indicate median + interquartile range. A Spearman's two-tailed non-parametric correlation test was conducted for (**D. ii**). Lesions and matched peripheral blood samples are colour coded on graphs.* ***HD =healthy donor, PB =peripheral blood, NAD =non-active LCH, AD =active LCH.***

Surprisingly, a pulmonary LCH lesion displayed expression of CD56 on viable $CD3^-CD19^-CD11c^+CD1a^+CD14^-$ cells (**Figure 4.11H**). This was not observed in any other lesions (data not shown) and CD56 expression by $CD19^+$ (B) cells was not detected in two lesional specimens (including the pulmonary lesion) analysed (data not shown).

4.2.6. Relationship between CD56 and Tregs

The relationship between $CD56^+$ Tregs, total Tregs and $CD56^+$ T cells in lesions from LCH patients was investigated. The proportion of total Tregs in lesional T cells was positively correlated to the proportion of $CD56^+$ Tregs (**Figure 4.12A**) and this was statistically significant (*r =1, p =0.028*). There were also positive correlations between the proportions of $CD56^+$ T cells and $CD56^+$ Tregs (**Figure 4.12B**), and total Tregs and $CD56^+$ T cells (**Figure 4.12C**), although these correlations were not statistically significant (*r =0.771, p =0.103 and r =0.771, p =0.103 respectively*).

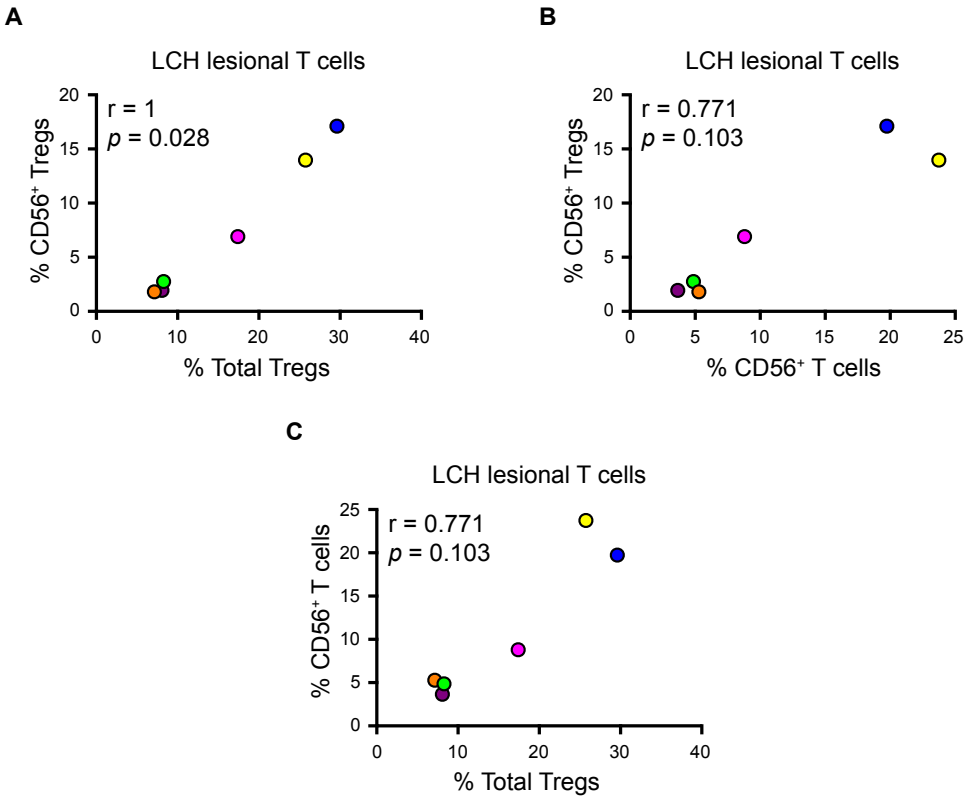


Figure 4.12. Relationship between Tregs, CD56$^+$ Tregs and CD56$^+$ T cells in LCH lesions.

*(**A**) Correlation between the proportion of total Tregs and CD56$^+$Tregs in total T cells from LCH lesions. (**B**) Correlation between the proportion of CD56$^+$ T cells and CD56$^+$ Tregs in total T cells from LCH lesions. (**C**) Correlation between the proportion of total Tregs and CD56$^+$ T cells in total T cells from LCH lesions. Spearman's two-tailed non-parametric correlation tests were conducted for (**A**), (**B**) and (**C**) (95% confidence interval). Lesions and matched peripheral blood samples are colour coded on graphs.*

4.2.7. Summary composition of T cell subsets in LCH

The composition of T cells within LCH patients and healthy donors are summarised (**Figure 4.13**) using the median proportions presented earlier in this chapter. The T cell subsets highlighted in the graphs are CD4$^+$ T cells (excluding Tregs), CD8$^+$ T cells, DN T cells and CD56$^+$ and CD56$^-$ Tregs. This data demonstrates an increase in the proportion of CD4$^+$ T cells and a decrease in the proportion of CD8$^+$ T cells in patients with active LCH compared to the blood from patients with non-active disease and from healthy donors. The data also demonstrate an increase in the proportion of Tregs in the lesions from patients with LCH that appears to be partially due to increased CD56$^+$ Tregs. These changes may be impacting on the lesional environment, therefore the function of Tregs is investigated next.

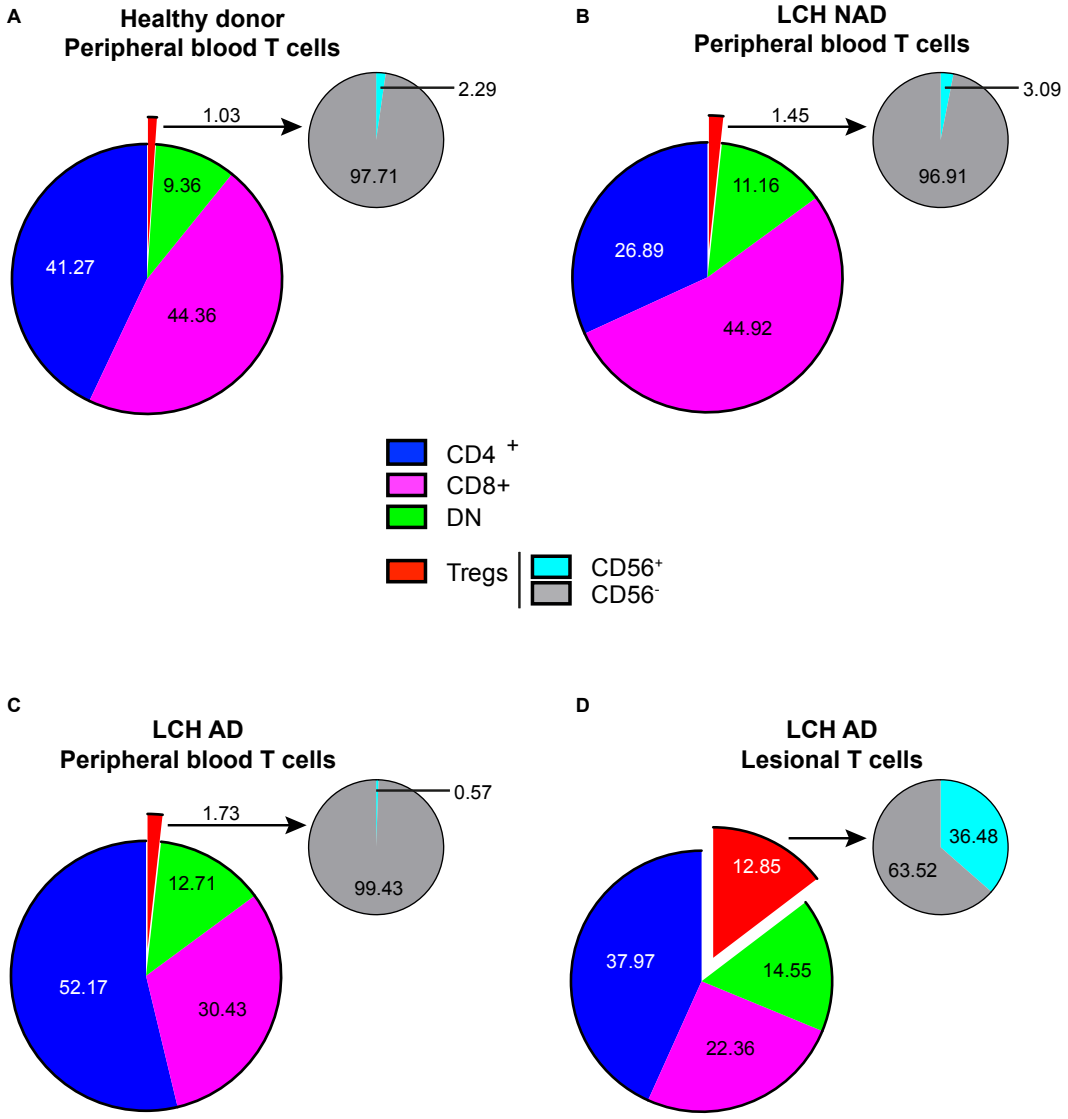


Figure 4.13. Summary composition of T cell subsets from patients with LCH.

*A representation of the proportions of $CD4^+$ T cells, $CD8^+$ T cells, $CD4^-CD8^-$ (DN) T cells, and Tregs from the peripheral blood from (**A**) healthy donors, (**B**) patients with non-active and (**C**) active LCH, and from (**D**) LCH lesions. Tregs are further categorised into $CD56^+$ and $CD56^-$ subsets. Data are a summary of the median proportions reported previously.* ***NAD =non-active LCH, AD =active LCH.***

4.2.8. Measuring suppression of T cells by Tregs

$Foxp3^+$ Tregs are known for their ability to negatively regulate the activity of conventional T cells[105-107], although the function of $Foxp3^+$ Tregs in LCH is not established. Additionally, given Tregs are not commonly known to express CD56 it was especially important to test their function in LCH.

4.2.8.1. Treg suppression assay using small cell numbers

For this study, an *in vitro* Treg suppression assay was developed using CFSE to measure proliferation by T responder cells cultured with Tregs from healthy donor derived PBMCs. Low cell numbers were cultured to reflect what would be isolated from LCH lesions. Tregs and T responder cells were isolated (**Figure 4.14A**) and T responder cells were CFSE labelled, then stimulated and cultured with Tregs (for complete methods see **Section 3.11.4**). While optimising this assay in healthy donors the expected result of reduced proliferation by T responder cells in the presence of Tregs was achieved (**Figure 4.14B**), although this was only intermittently achieved using small cell numbers. Often proliferation in the stimulated T responder cells alone group was lower than desirable, thus preventing accurate detection of suppression. This is likely due to a combination of using small cell numbers and the inhibitory effects that CFSE dye can have on cell proliferation[176]. An attempt to reduce CFSE concentration was also made, however this made the differentiation between labelled, proliferated cells and unlabelled cells difficult.

Consequently, this study measured the expression of Treg associated genes by purified Tregs to establish whether Tregs from LCH patients function similarly to Tregs from healthy donors. These results are described next.

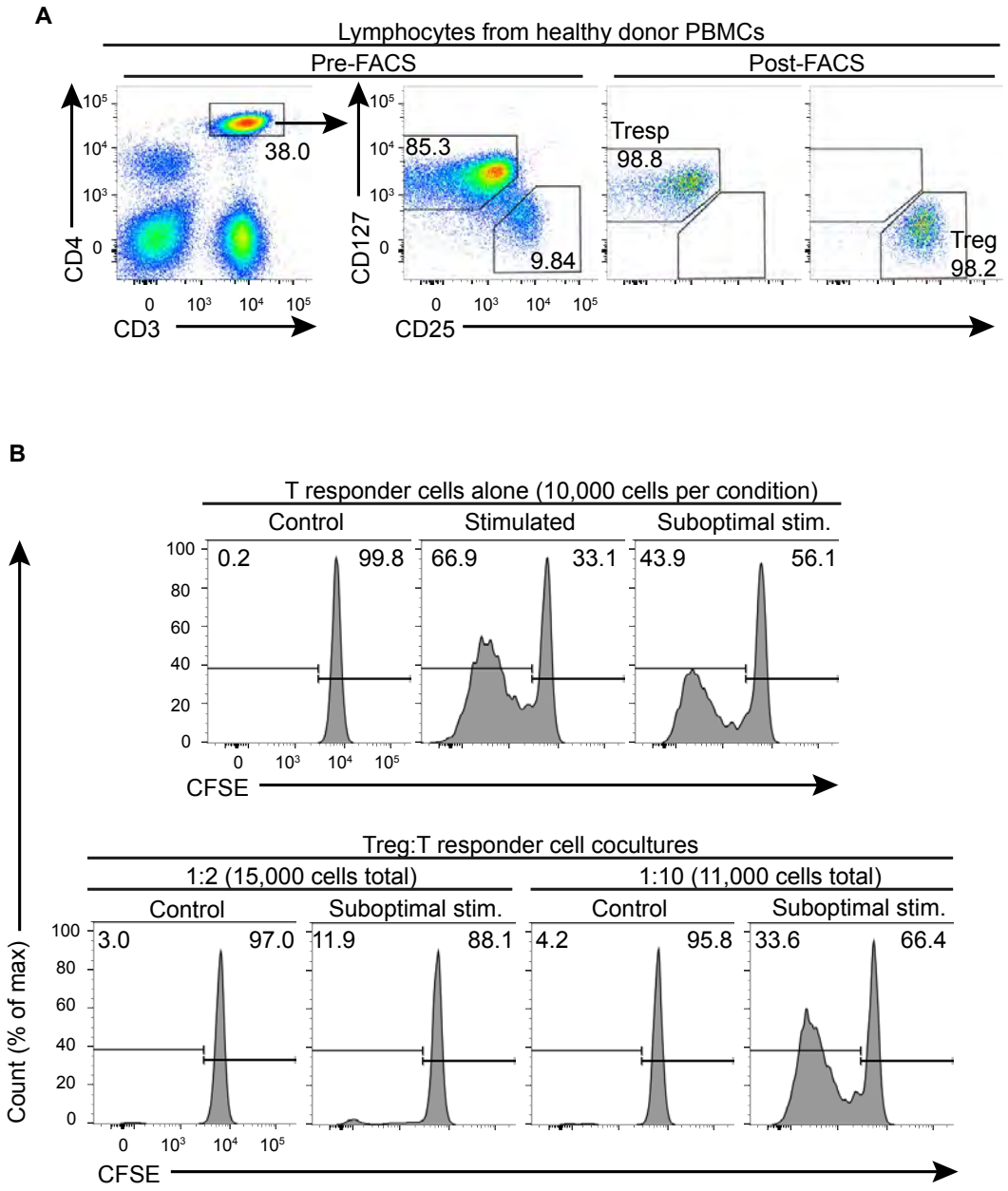


Figure 4.14. Treg suppression assay using small cell numbers.

*(**A**) Flow cytometry gating strategy for identifying and isolating Tregs ($CD4^+CD25^+CD127^{low}$ T cells) and T responder ($CD4^+CD25^{low}CD127^+$) cells (Tresp) and post-FACS analysis of these populations. (**B**) CFSE analysis of Treg and T responder cell co-cultures post-suboptimal anti-CD3 stimulation.*

4.2.8.2. Gene expression analysis of Tregs

Tregs were identified and isolated from the blood from healthy donors and from the blood and lesions from patients with active LCH by FACS as demonstrated previously (**Figure 4.14A**). The post-sort purity was 80% or above for Tregs, although most samples were >90% pure. (**Appendix A, Table A2**). Cell transcriptomes were amplified (for methods see **Section 3.12.1**) to compare the expression levels of a range of genes commonly associated with Foxp3^{+} Tregs (**Figure 4.15A**). This study also investigated the expression of a range of other genes (*NCAM1*, *LIFR*, *CD40LG* and *TNF*) in Tregs isolated from the peripheral blood from healthy donors and from the blood and lesions from patients with active LCH (**Figure 4.15B**). Working with small yields of Tregs from LCH patients resulted in amplification in a lower than desirable number of samples isolated from the blood and lesions from LCH patients. Gene amplification using a range of primers was successful in five of six samples from healthy donors, one of five samples from the peripheral blood from patients with active LCH and in two of five lesional samples, as determined by SYBR based qPCR. Importantly, the expression of *FOXP3* was confirmed in Tregs isolated from the peripheral blood from healthy donors (*n =4*) and patients with active LCH (*n =3*) and from lesions (*n =2*) (**Appendix A, Table A2**).

Since HLA-DR^{+} Tregs were shown to suppress more effectively than HLA-DR^{-} Tregs[177], this study tested for *HLA-DRA* expression to identify potential differences in function between Tregs from LCH patients and from healthy donors, however this gene was undetectable in all but one sample from a healthy donor (**Appendix A, Table A2**).

Expression of the gene encoding for CD56 (*NCAM1*) was confirmed in Tregs isolated from lesions (*n =2*), although this gene was also expressed by Tregs from the peripheral blood from healthy donors (*n =3*) (**Figure 4.15** and **Appendix A, Table A2**).

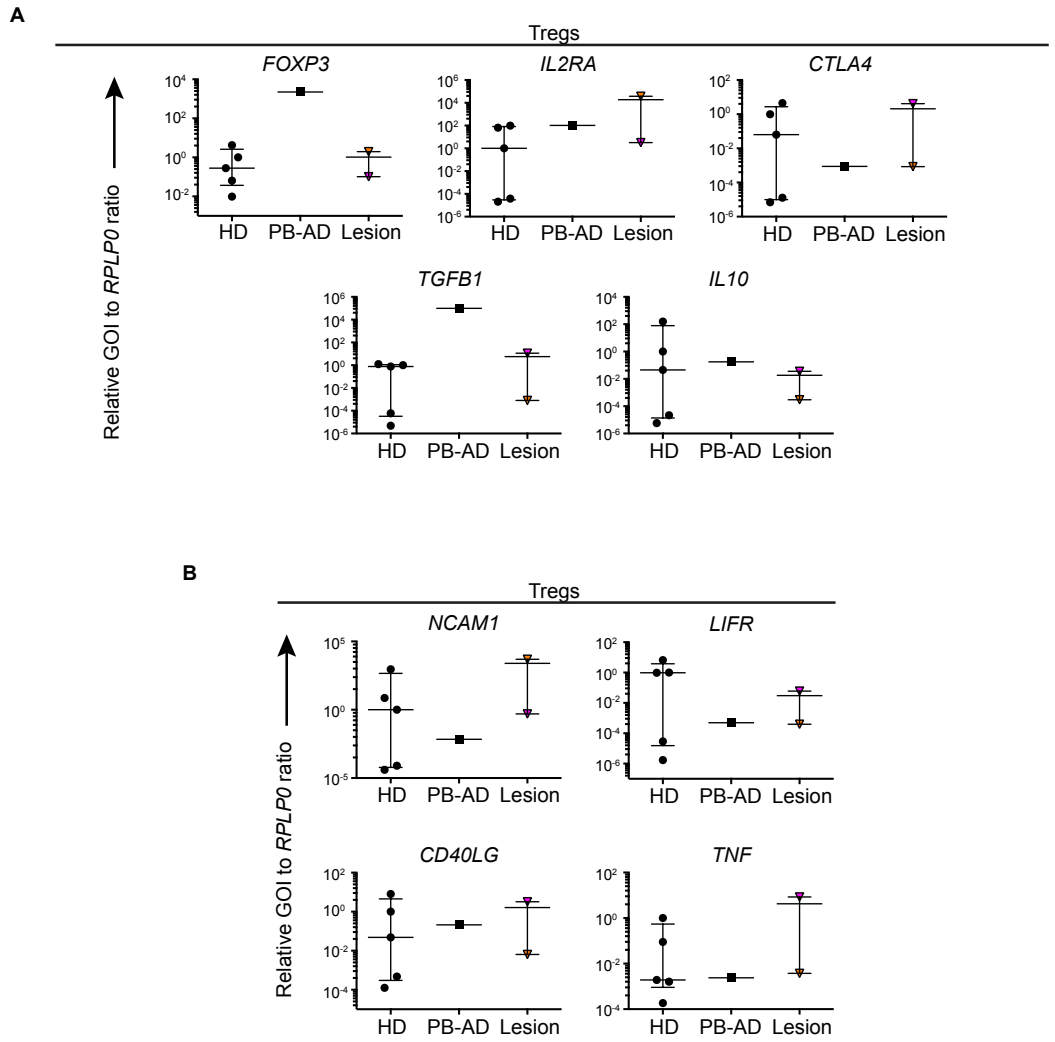


Figure 4.15. Expression of Treg associated genes and other genes of interest in Tregs.

*(**A**) Relative gene of interest to reference gene (RPLP0) copy number ratio for Foxp3^{+} Treg associated genes FOXP3, IL2RA, CTLA4, TGFB1, and IL10 in Tregs from the peripheral blood from healthy donors and from blood and lesions from patients with active LCH. (**B**) Relative gene of interest to reference gene (RPLP0) copy number ratio for NCAM1, LIFR, CD40LG, and TNF in Tregs from the peripheral blood from healthy donors and from blood and lesions from patients with active LCH. Error bars indicate median + interquartile range. Lesions and matched peripheral blood samples are colour coded on graphs.* ***GOI =gene of interest, HD =healthy donor, PB-AD =active LCH peripheral blood.***

4.2.9. Gene expression analysis of Tregs and conventional T cells

This study tested for differences in the expression levels of genes associated with T cell activation in the LCH lesion Treg and conventional T cell populations. Expression of *IL2RA*, *CD40LG* and *TNF* was compared in these groups, although due to the lower than anticipated sample sizes already discussed it is difficult to draw any conclusions (**Figure 4.16**). This study can however confirm the expression of *IL2RA*, *CD40LG* and *TNF* in both the Treg and conventional T cell subsets within LCH lesions.

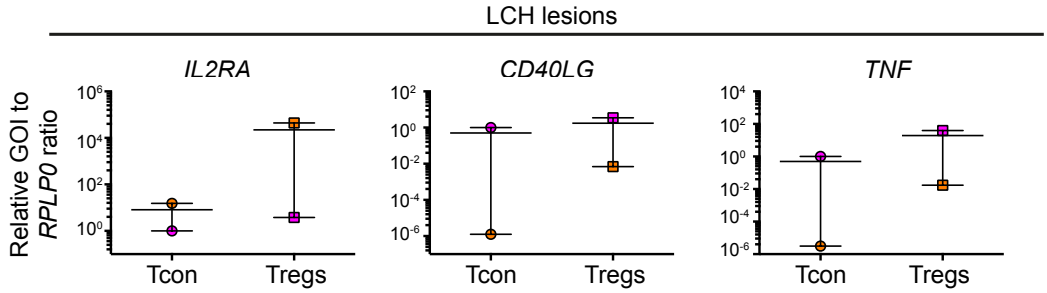


Figure 4.16. T cell activation genes in Tregs and conventional T cells from LCH lesions.

Relative gene of interest (GOI) to reference gene (RPLP0) copy number ratio for IL2RA, CD40LG and TNF in Tregs and conventional T cells (excluding Tregs) from LCH lesions. Error bars indicate median + interquartile range. Lesions and matched peripheral blood samples are colour coded on graphs.

4.2.10. Differentiating between CD56^{+} and CD56^{-} Tregs from LCH lesions

CD56 expression by Foxp3^{+} Tregs is not a common observation, however it is known that activated conventional T cells can downregulate CD127 and upregulate CD25, and this is also true for CD56 expressing NK-like T cells[178]. Furthermore, CD56^{+} T cells have been shown to display MHC-unrestricted cytotoxicity similar to NK cells[179], therefore if the cells identified in LCH lesions are not true Tregs, but activated T cells expressing Foxp3, they could still be a promising target to focus on for developing new immunotherapies to treat LCH patients. Whilst CD56 expression was common to Tregs from LCH lesions, this population did not express CD161, another NK cell associated molecule (data not shown). Interestingly, a recent study identified the expression of NK cell-associated KIR2DL4 in all LCH lesions analysed and hypothesised that this molecule was expressed by LCH cells[180]. Previous LCH studies have also demonstrated

by immunohistochemistry that Foxp3$^+$ Tregs were in close contact with LCH cells in lesions[57], indicating that there may be cell-cell communication between LCH cells and Foxp3$^+$ Tregs.

4.2.10.1. Measuring the fluorescence intensity of CD25 and CD127

The median fluorescence intensities (MFI) for Treg identification markers CD25 and CD127 were compared in the CD56$^+$ and CD56$^-$ fraction of Tregs (**Figure 4.17A**) and there was a significant (*p =0.0313*) increase in CD25 fluorescence in CD56$^+$ Tregs (*MFI =6667*) compared with CD56$^-$ Tregs (*MFI =4904*) but there was no significant difference in CD127 MFI between the groups (**Figure 4.17B**).

4.2.10.2. Foxp3 and HLA-DR expression

In three lesional samples both CD56$^+$ and CD56$^-$ Tregs expressed Foxp3 as determined by transcription factor staining (**Figure 4.17C**). This study also looked for differences in HLA-DR expression in these T cell subsets by flow cytometry, although similar to the results from total lesional T cells, most lesional CD56$^+$ and CD56$^-$ Tregs were negative for HLA-DR, consistent with Tregs from healthy donors (**Figure 4.17D**).

4.2.10.3. Cytokine production by Treg and T responder cells from LCH lesions

The following assay was conducted to establish any differences in cytokine profile between T cell subsets from the lesions from LCH patients and determine whether CD56$^+$ Tregs are functionally inhibitory in nature rather than activated conventional T cells under disguise. CD56$^+$ and CD56$^-$ Tregs were purified along with CD4$^+$ T responder cells and CD8$^+$ T cells (**Figure 4.18A**), and stimulated with PMA and ionomycin for 16 hours with GolgiPlug added after 12 hours (for complete methods see **Section 3.11.5**).

Using intracellular staining it was determined that CD4$^+$ T responder cells (*median =23.32%*) and CD8$^+$ T cells (*median =24.88%*) were able to produce TNF in at least two out of three LCH lesion samples (Figure **4.18B** and **C**), while there was no detectable TNF production in the CD56$^+$ or CD56$^-$ Treg populations (**Figure 4.18B** and **C**). CD25 expression was maintained by the isolated CD56$^+$ (*median =100%*) and CD56$^-$ Treg (*median =99.34%*) populations (**Figure 4.18B** and **D**).

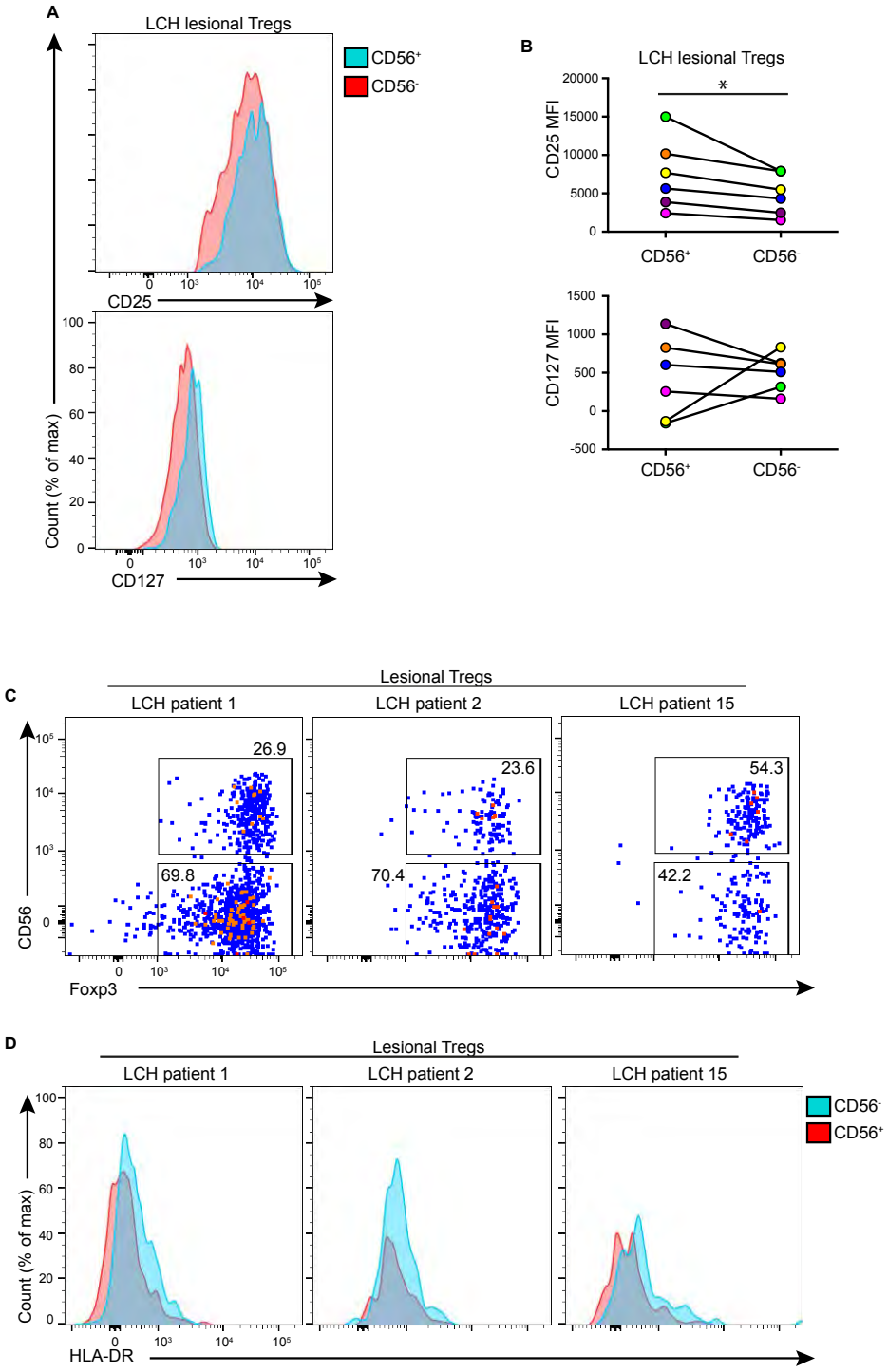


Figure 4.17. Expression of Foxp3 and HLA-DR by Tregs from LCH lesions.

*(**A**) Representative histograms show the expression of CD25 (top) and CD127 (bottom) on CD56$^+$ and CD56$^-$ Tregs from LCH lesions. (**B**) Median fluorescence intensity (MFI) of CD25 (top) and CD127 (bottom) by CD56$^+$ and CD56$^-$ Tregs from LCH lesions. Foxp3 expression (**C**) and HLA-DR expression (**D**) by CD56$^+$ and CD56$^-$ Tregs from lesions from three LCH patients. Two-tailed Wilcoxon matched-pairs signed rank tests were conducted for (**B**). *p <0.05. Lesions and matched peripheral blood samples are colour coded on graphs.*

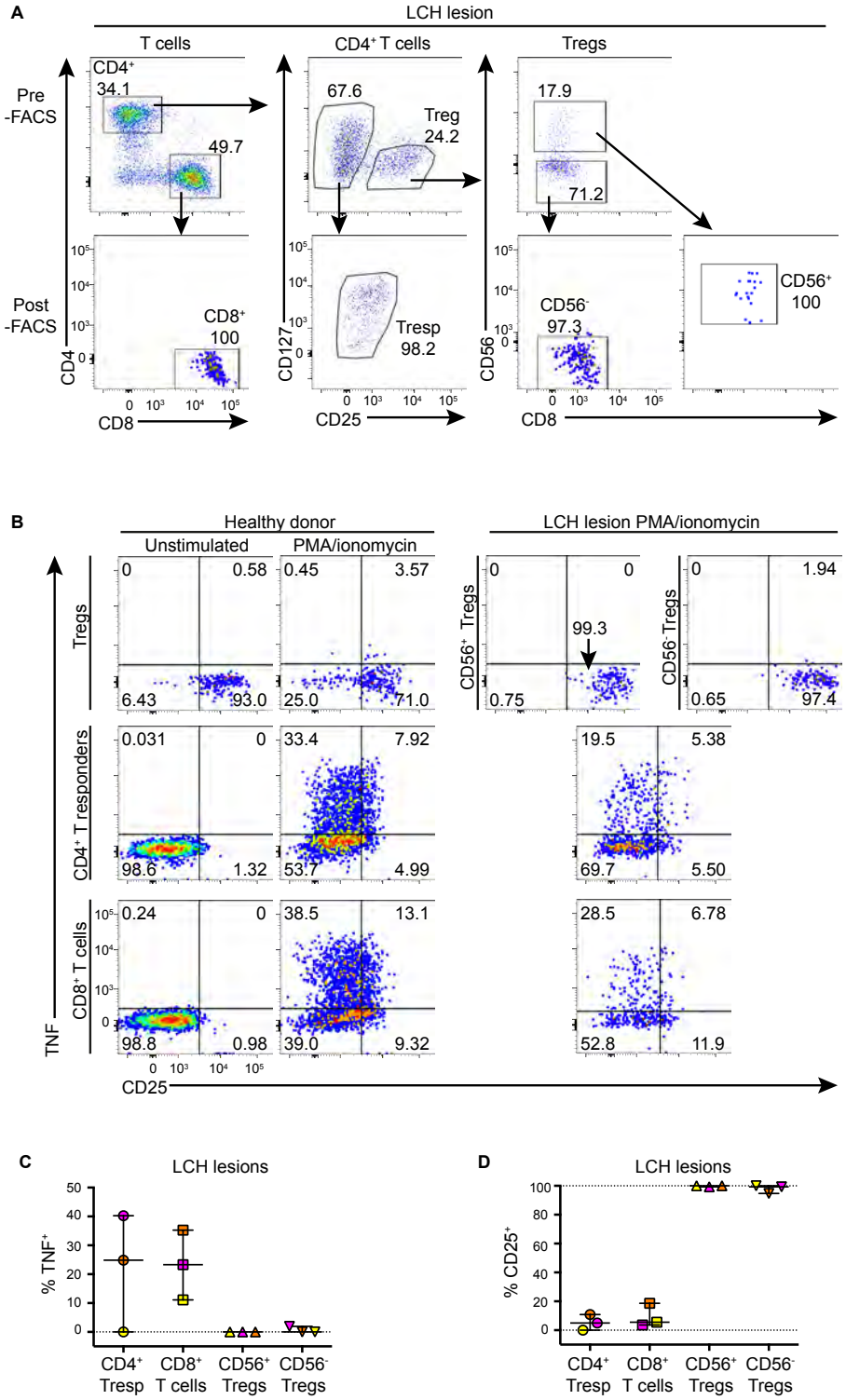


Figure 4.18. Intracellular cytokine production by purified LCH lesion T cell populations.

*(**A**) Gating strategy for identifying and purifying $CD8^+$ T cells, $CD4^+$ T responders (Tresp), and $CD56^+$ and $CD56^-$ Tregs from LCH lesions (top row), and post-FACS analysis of these populations (bottom row). (**B**) Representative plots demonstrating expression levels of intracellular TNF and surface CD25 by purified populations from the peripheral blood from healthy donors and from LCH lesions post-PMA and ionomycin stimulation. Proportions of TNF^+ (**C**) cells and $CD25^+$ cells (**D**) in purified populations from LCH lesions. Error bars indicate median + interquartile range. Lesions and matched peripheral blood samples are colour coded on graphs.*

The concentration of a variety of Th cytokines were next measured using supernatants collected following the cell culture described above (**Table 4.3**). Taking into consideration consistency between cell numbers in $CD4^+$ T responder and $CD8^+$ T cell cultures, and between $CD56^+$ and $CD56^-$ Treg cultures, the highest number of cells possible was cultured for each population purified from LCH lesions (**Table 4.4** and **4.5**). The number of cells from populations purified from healthy donors aimed to match with the higher and lower range of those from LCH patients (**Table 4.4** and **4.5**), although some LCH donor cell numbers did fall below the minimum number of healthy donor T cells cultured, therefore it is conceivable that some cytokines may not be detectable due to the small numbers of cells within LCH lesion cell cultures.

In alignment with the previous results demonstrating TNF production, $CD4^+$ T responder cells and $CD8^+$ T cells purified from LCH patients secreted the Th1 cytokines IFN-γ (*$CD4^+$ T responder median =4.01 pg/mL, $CD8^+$ T cell median =6.36 pg/mL*) and TNF (*$CD4^+$ T responder median =23.98 pg/mL, $CD8^+$ T cell median =4.99 pg/mL*) in the same two out of three samples upon stimulation. In contrast, these cytokines were either not produced within the detectable concentration range (*IFN-γ range =2.11-21,135 pg/mL, TNF range =0.94-17,046 pg/mL*) or above the media alone baseline (*IFN-γ baseline <2.11 pg/mL, TNF baseline =2.31 pg/mL*) in stimulated $CD56^+$ (*IFN-γ median <2.11 pg/mL, TNF median =1.88 pg/mL*) or $CD56^-$ (*IFN-γ median <2.11 pg/mL, TNF median =2.23 pg/mL*) Treg cell cultures (**Figure 4.19A** and **B**). The intracellular cytokine production and extracellular cytokine secretion results presented here suggest that it is not likely that the $CD56^+$ Tregs identified in this study are activated conventional Th1 cells.

While the detection of IL-2, IFN-γ, and TNF was highly sensitive for this assay using small cell numbers, the remaining cytokines measured by the assay were secreted in smaller concentrations as determined using a control population of 50,000 purified $CD4^+$ T responder cells from healthy donors (**Table 4.3**) These cytokines were thus less reliably detectable in the smaller populations from healthy donors (**Table 4.3**) and although it is not likely, we cannot conclusively rule out that the cell populations cultured could display a Th2, Th9, Th22 or Th follicular phenotype.

Table 4.3. Secretion of Th cytokines by purified T cell populations.

	Control concentration (pg/mL)	***Detectable in cell culture supernatant?***						
	50,000 HD Tresp cells	**HD Tresp**	**Lesion Tresp**	**HD $CD8^+$**	**Lesion $CD8^+$**	**HD Treg**	**Lesion $CD56^+$ Treg**	**Lesion $CD56^-$ Treg**
IL-2	>13626.29	Yes 2/2	Yes 2/2	Yes 2/2	Yes 2/2	N/A*	N/A*	N/A*
IFN-γ	6398.69	Yes 2/2	Yes 2/3	Yes 2/2	Yes 2/3	No	No	No
TNF-α	3586.89	Yes 2/2	Yes 2/3	Yes 2/2	Yes 2/3	Yes 1/2	No	No
IL-22	305.10	Yes 1/2	Yes 1/3	No	No	No	No	No
IL-10	236.14	Yes 1/2	Yes 1/3	Yes 1/2	No	No	No	No
IL-6	195.34	Yes 1/2	Yes 1/3	Yes 1/2	Yes 1/3	No	No	No
IL-4	88.57	Yes 1/2	No	Yes 1/2	No	No	No	No
IL-5	85.55	No	No	No	No	No	No	No
IL-17A	36.80	No	No	No	No	No	No	No
IL-13	23.99	Yes 1/2	No	Yes 1/2	No	No	No	No
IL-9	17.36	Yes 1/2	No	Yes 1/2	No	No	No	No
IL-21	9.55	No	No	No	No	Yes 1/2	No	No
IL-17F	1.98	No	Yes 2/3	Yes 1/2	Yes 1/3	No	Yes 2/3	Yes 2/3

HD =healthy donor, Tresp =T responder.

**IL-2 was added to Treg cultures*

Table 4.4. Approximate number of cells cultured in purified T cell populations from lesions from LCH patients.

LCH lesion/patient no.	No. of T responders (100 µl)	No. of $CD8^+$ T cells (100 µl)	No. of $CD56^+$ Tregs (100 µl)	No. of $CD56^-$ Tregs (100 µl)
1	5,000	5,000	1,000	1,000
2	5,000	5,000	350	1,000
15	300	350	200	250

Table 4.5. Approximate number of cells cultured in purified T cell populations from healthy donor PBMCs.

Healthy donor no.	No. of T responders (100 µl)	No. of $CD8^+$ T cells (100 µl)	No. of Tregs (100 µl)
1	500	500	500
2	5,000	5,000	1,000

Interestingly, small concentrations of IL-17F were secreted in stimulated cultures from each LCH lesion within purified populations of $CD4^+$ T responder cells (*range =<1.02-2.82 pg/mL, median =2.12 pg/mL*), $CD8^+$ T cells (*range =1.19-1.90 pg/mL, median =1.85 pg/mL*) and $CD56^+$ (*range =1.04-2.09 pg/mL, median =1.25 pg/mL*) and $CD56^-$ (*range =1.41-2.22 pg/mL, median =1.52 pg/mL*) Treg populations (**Figure 4.19C**). Although concentrations were low, they were within the detectable concentration range (*range =1.02-14,380 pg/mL*) and above the media alone baseline (*baseline <1.02 pg/mL*). The presence of IL-17A in LCH lesions is a controversial topic[65,181,182] and this assay also tested for the secretion of IL-17A, but the cell cultures with small cell numbers were not sensitive enough to rule out IL-17A secretion because it was only detected in a small amount in supernatant from the stimulated control population of 50,000 healthy donor T responder cells (**Table 4.3**).

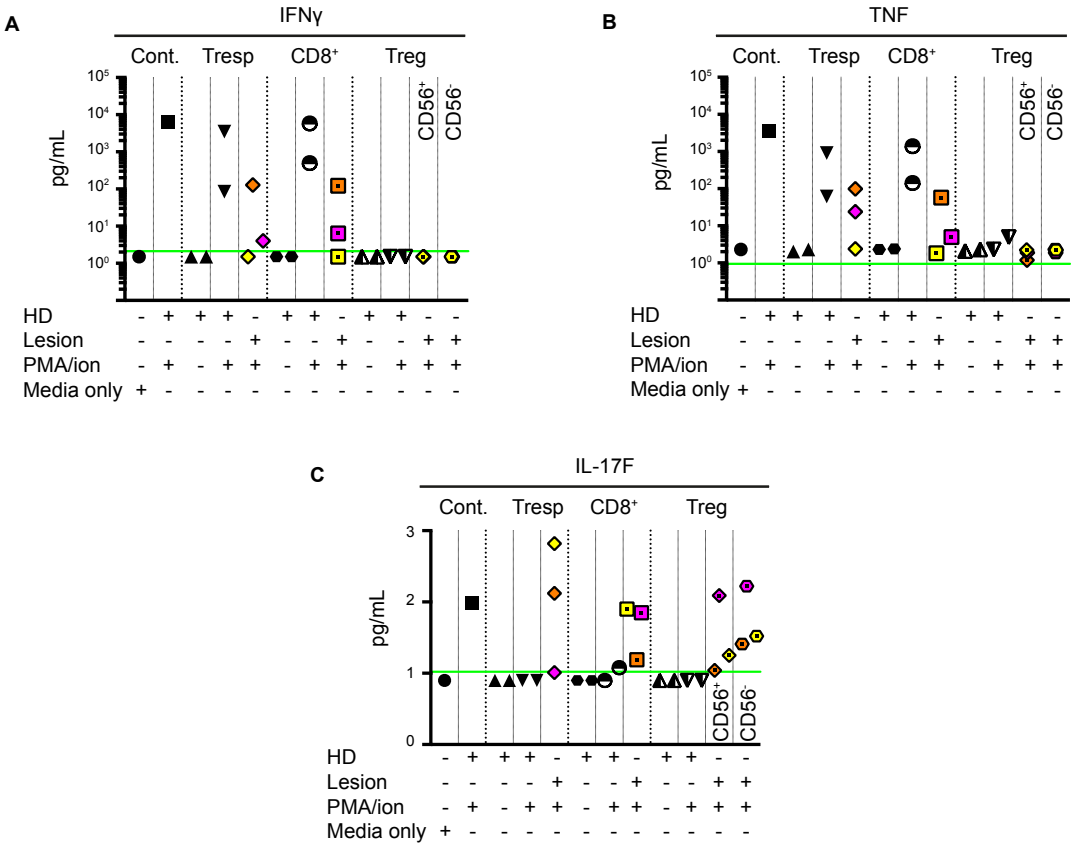


Figure 4.19. Secretion of Th cytokines by purified T cell populations.

*Graphs demonstrate the production of (**A**) IFN-γ, (**B**) TNF and (**C**) IL-17F by purified populations of T responders, $CD8^+$ T cells and Tregs from the peripheral blood from healthy donors and T responder cells, $CD8^+$ T cells and $CD56^+$ and $CD56^-$ Tregs from lesions from LCH patients following stimulation. Green line indicates minimum detectable concentration as determined by standard curve. Black circle indicates media only control (cont.) and black square indicates healthy donor control population of 50,000 purified T responder cells. Lesions and matched peripheral blood samples are colour coded on graphs.* ***HD =healthy donor, Tresp =T responder cells.***

Previous studies on immune-suppressive cytokines within LCH lesions have identified that there are lesions predominantly positive for IL-10 (10/25), lesions predominantly positive for TGF-β (9/25), and mixed lesions positive for both IL-10 and TGF-β (6/25)[100]. One might speculate that CD56 expression by Tregs could align with a specific immune suppressive cytokine, for example $CD56^+$ Tregs may produce IL-10 whereas $CD56^-$ Tregs might produce TGF-β. The assay was not sensitive enough to confirm or rule out IL-10 secretion by Treg populations (**Table 4.3**). In contrast, this study was able to confirm that each population of Tregs isolated from LCH lesions could produce total (**Figure 4.20A**) and active (**Figure 4.20B**) TGF-β, as detected in the supernatant, which

confirms that both $CD56^+$ and $CD56^-$ Treg populations from LCH lesions can produce the inhibitory cytokine TGF-β.

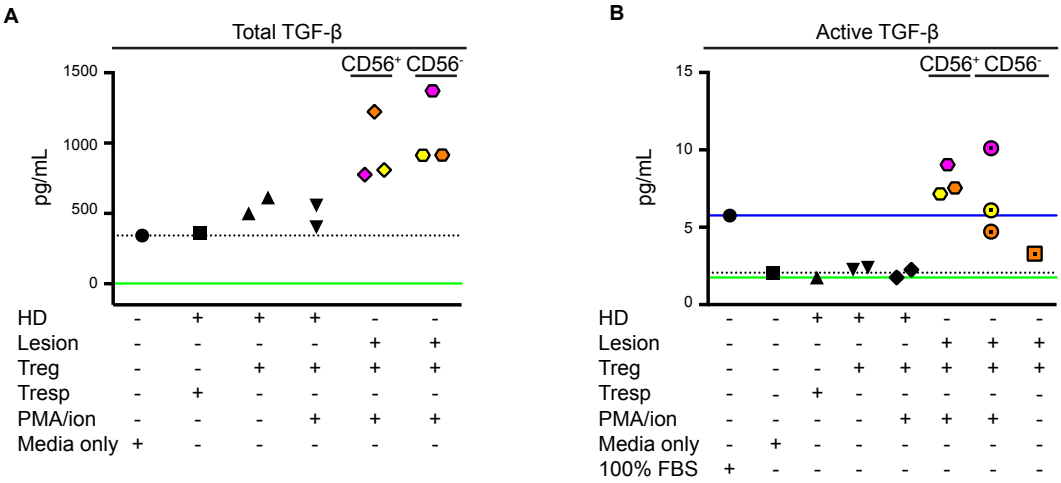


Figure 4.20. TGF-β production by stimulated Tregs from LCH lesions.

*Graphs demonstrate the supernatant levels of total TGF-β (**A**) and active TGF-β (**B**) produced by purified and stimulated populations of Tregs from blood from healthy donors and by $CD56^+$ and $CD56^-$ Tregs from lesions from LCH patients. Green line indicates minimum detectable concentration as determined by standard curve. Dashed line indicates media background control. Blue line indicates detection level of 100% FBS. Lesions and matched peripheral blood samples are colour coded on graphs.* ***HD =healthy donor, Tresp =T responder cells.***

4.2.11. Identification of LCH cells and other myeloid cells in LCH patients

To improve on the poorly defined nature of LCH cells and to understand whether LCH cells interact with T cells in lesions, the LCH cell phenotype was investigated. LCH cells were identified by flow cytometry using anti-CD1a antibody (**Figure 4.21A**). Given the hypothesised myeloid origin of LCH cells, the expression of monocyte and myeloid DC marker anti-CD11c was also used and $CD1a^+$ LCH cells almost ubiquitously expressed CD11c (**Figure 4.21A**). Subsequently, monocytes and myeloid DCs (referred to herein as myeloid cells) were identified in the peripheral blood from healthy donors and LCH patients, and from LCH lesions by gating on $CD3^-CD19^-$ cells (**Figure 4.21B**). In lesions, LCH cells ($CD11c^+CD1a^+$) were distinguished from other myeloid cells ($CD11c^+CD1a^-$) (**Figure 4.21C**).

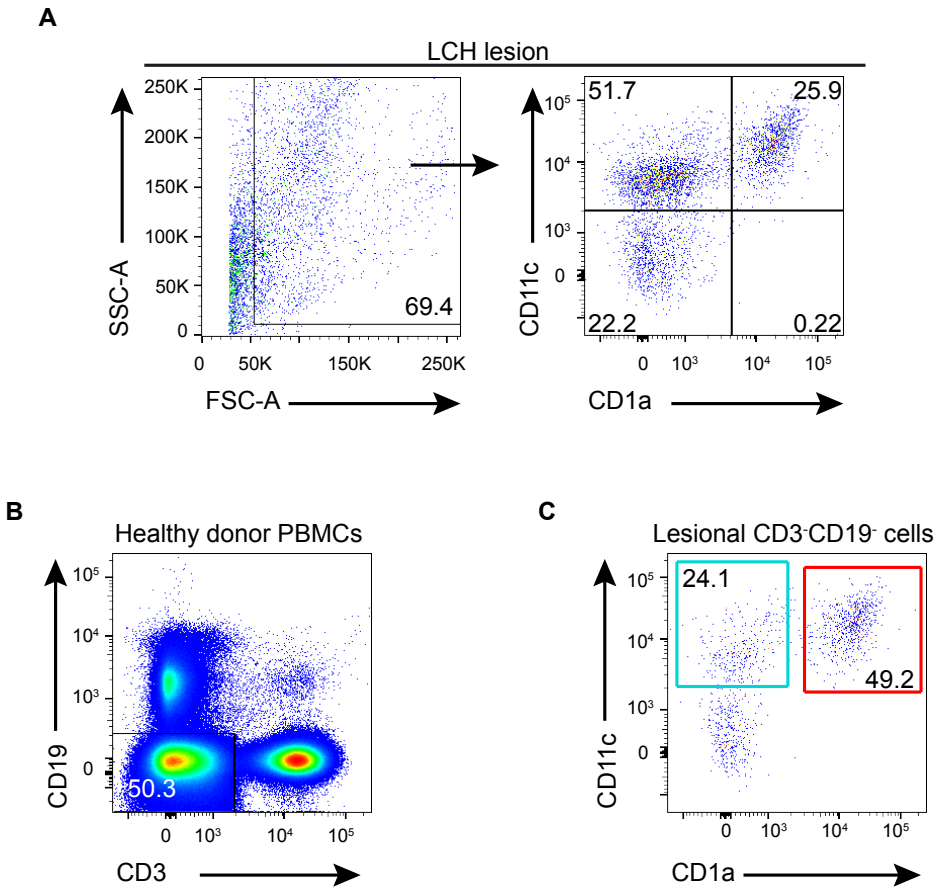


Figure 4.21. Identification of LCH cells and other myeloid cells in patients with LCH.

*(**A**) Representative flow cytometry plot showing expression of CD11c on LCH ($CD1a^+$) cells from lesions. (**B**) Gating strategy for excluding other lineages ($CD3^-CD19^-$) to myeloid cells. (**C**) Gating strategy for identification of LCH cells (red gate) and other myeloid cells (blue gate) within lesions. Viability gates were applied to these analyses (not shown).*

4.2.12. The phenotype of LCH cells

LCH cells were compared to other myeloid cells from lesions and/or to myeloid cells ($CD11c^+$) from the peripheral blood from healthy donors and patients with active disease using cell surface monocyte/macrophage markers. The expression of CD14 and CD4 was examined in these groups and $CD4^{hi}$ cells were identified in the LCH cell ($CD1a^+$) population (**Figure 4.22A**). As expected, a significantly higher proportion of myeloid cells from LCH lesions expressed CD1a (*median =68.64%*) when compared to myeloid cells in the peripheral blood from healthy donors (*p =0.0021, median =0.09%*) and patients with active disease (*p =0.0265, median =0.16%*) (**Figure 4.22B**). Interestingly, there was a significant (*p =0.0219*) decrease in the proportion of LCH cells that expressed CD14 (*median =16.20%*) in comparison to myeloid cells from the

peripheral blood from healthy donors (*median =75.36%*) (**Figure 4.22B**). There was also an increase in $CD4^{hi}$ expression by LCH cells (*median =35.68%*) compared to myeloid cells from the peripheral blood from healthy donors (*p =0.0170, median =0.22%*) and patients with active LCH (*p =0.0003, median =0.07%*) (**Figure 4.22B**). Although it is established that there are circulatory LCH-like cells in some patients with active LCH[148,149], the blood samples from patients in this study showed negligible CD1a expression.

4.2.12.1. Transcriptome analysis of LCH cells

Using FACS, LCH cells were isolated from lesions while myeloid cells were purified from the peripheral blood from healthy donors and patients with active LCH (**Figure 4.23**). Post-sort purity was >90% (**Appendix A, Table A3**). Cell transcriptomes were amplified (for methods see **Section 3.12.1**) and the expression levels of a range of genes associated with antigen presentation and/or with the potential for T cell regulation were examined in these populations (**Figure 4.24**). Working with small yields of isolated cells from LCH patients resulted in amplification in a lower than desirable number of samples. Gene amplification was successful in four of six samples from healthy donors, two of five samples from the peripheral blood from patients with active LCH and three of five lesional samples, as determined by SYBR based qPCR (**Appendix A, Table A3**). In some instances, further samples were excluded from analyses due to the formation of primer dimers during PCR (**Appendix A, Table A3**).

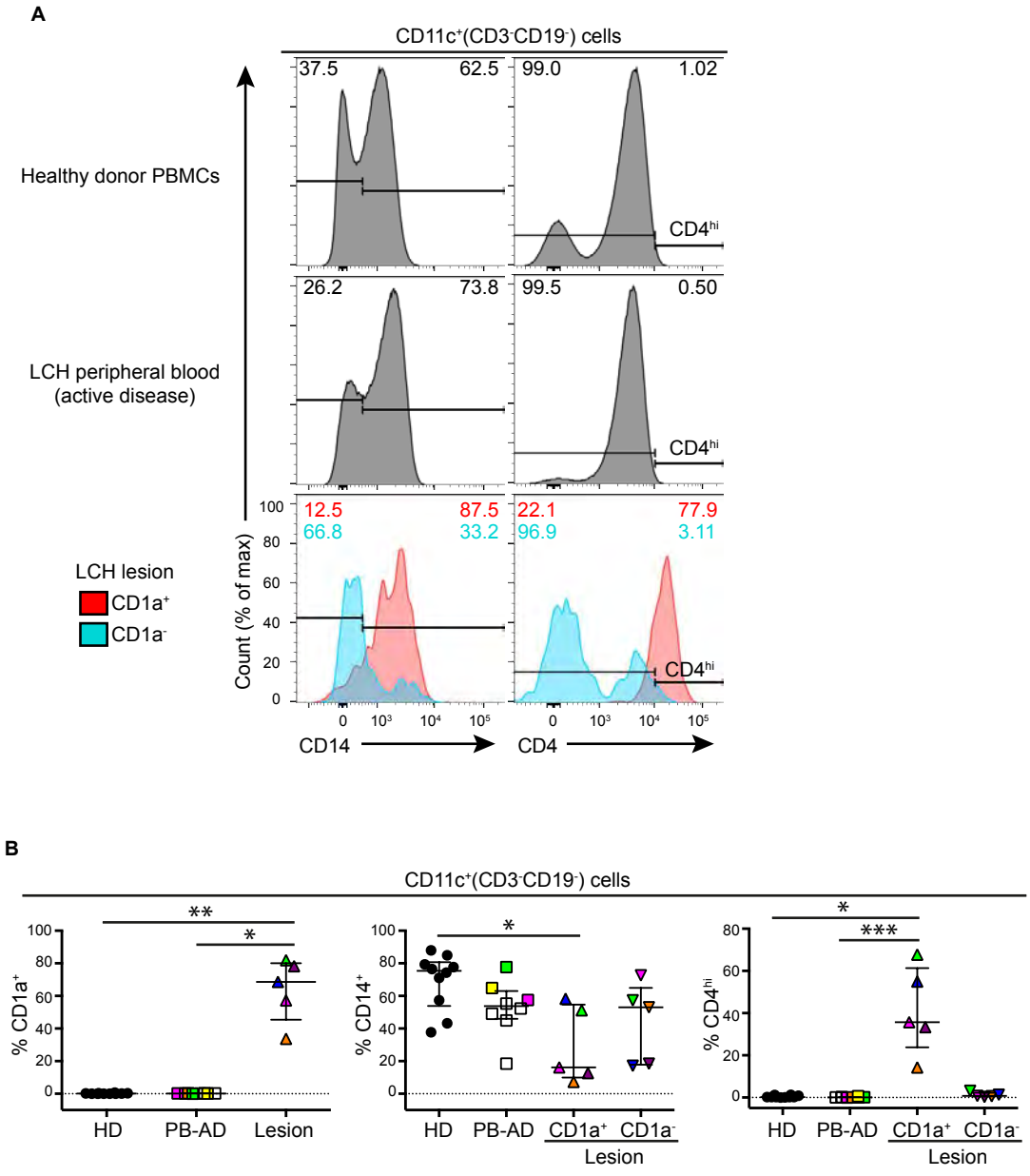


Figure 4.22. The phenotype of LCH cells.

*(**A**) Histograms show representative expression of CD14 (left) and CD4 (right) on myeloid cells from the peripheral blood from healthy donors (top) and from blood (middle) and lesions (bottom) from patients with active LCH. (**B**) Proportions of $CD1a^+$ cells, $CD14^+$ cells and $CD4^+$ cells in myeloid cells from blood from healthy donors and from blood and lesions from patients with active LCH. Kruskal-Wallis tests were conducted for (**B**). *p <0.05, **p <0.01, ***p <0.001, error bars indicate median + interquartile range. Lesions and matched peripheral blood samples are colour coded on graphs.* ***HD =healthy donor, PB-AD =active LCH peripheral blood.***

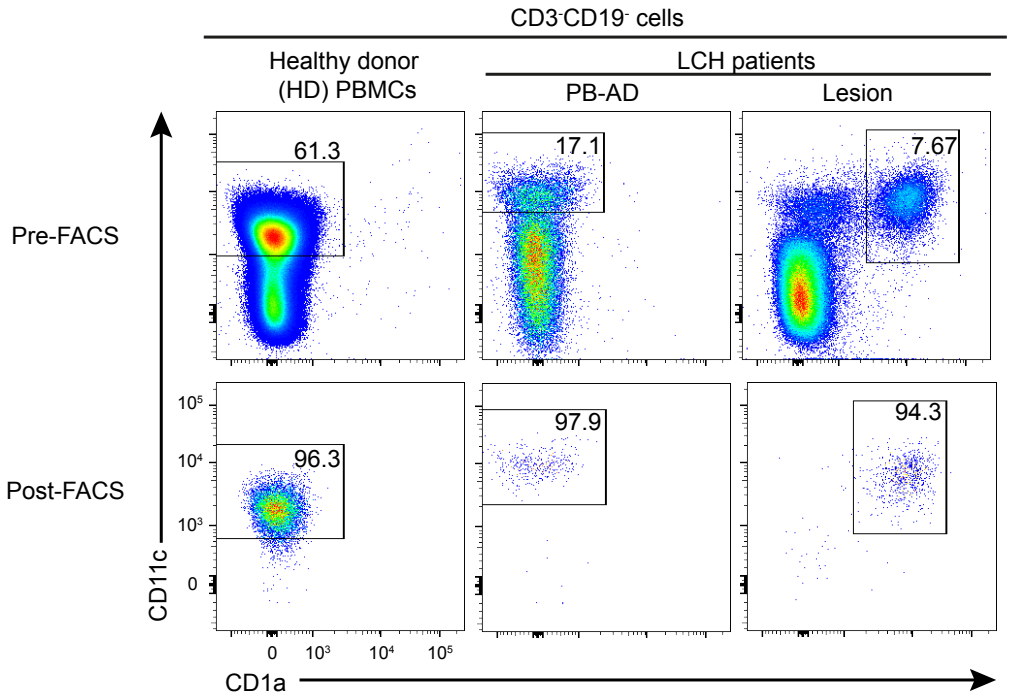


Figure 4.23. Purifying LCH cells from lesions and myeloid cells from peripheral blood.

Flow cytometry gating strategy for identifying and isolating myeloid cells from the peripheral blood from healthy donors (top left) and patients with active LCH (top middle) and LCH cells from lesions (top right), and post-FACS analysis of these populations (bottom row). ***PB-AD =active LCH peripheral blood.***

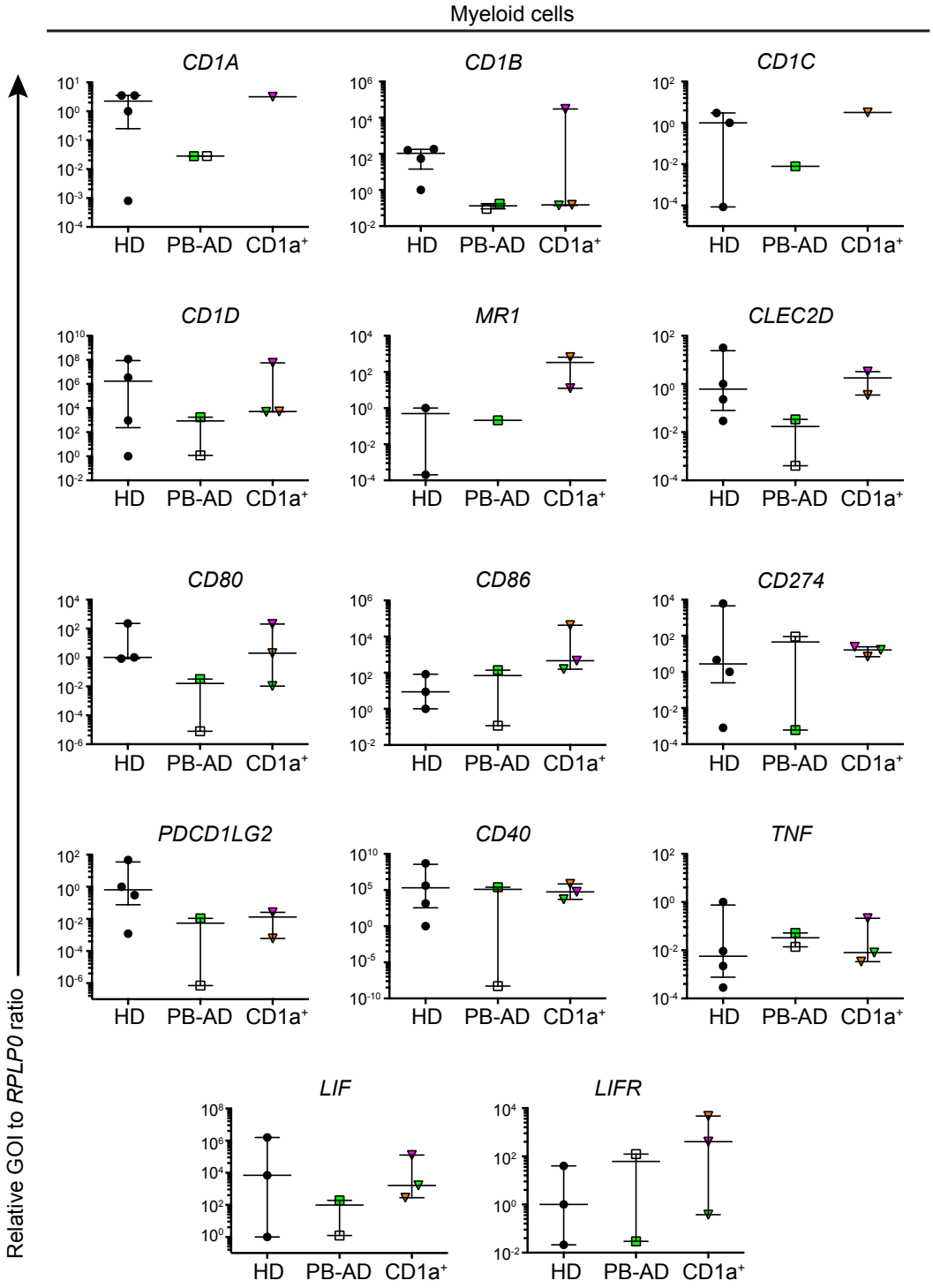


Figure 4.24. Transcriptome analysis of LCH cells.

Graphs demonstrate relative gene of interest to reference gene (RPLP0) copy number ratio for CD1A, CD1B, CD1C, CD1D, MR1, CLEC2D, CD80, CD86, CD274, PDCD1LG2, CD40, TNF, LIF and LIFR in myeloid cells from blood from healthy donors and patients with active LCH and in LCH cells from lesions. Error bars indicate median + interquartile range. Lesions and matched peripheral blood samples are colour coded on graphs. ***GOI =gene of interest, HD =healthy donor, PB-AD =active LCH peripheral blood.***

4.2.12.2. Analysis of publicly available data on LCH cells

To better establish which biological processes and pathways are associated with LCH cells, this study conducted a reanalysis of publicly available microarray data from lesion isolated LCH cells[65] (the data was downloaded from http://www.jimmunol.org/content/184/8/4557/tab-figures-data). Genes considered for the enrichment analysis were increased in expression by a minimum of two-fold in $CD207^+$ LCH cells compared to skin derived $CD207^+$ cells and all enrichment analysis data were selected based on Fisher's exact test with FDR multiple test correction. Raw and FDR adjusted p-values for all processes and pathways listed were <0.05.

Gene Ontology biological process complete analysis (**Table 4.6**) identified the upregulation of genes involved in 'regulation of Th1 cell differentiation' (GO:0045625; *JAK3*, *ANXA1* and *RIPK2*), 'negative regulation of αβ T cell activation' (GO:0046636; *HFE*, *JAK3*, *ANXA1*, *LILRB1* and *LOXL3*), 'positive regulation of T cell proliferation' (GO:0042102; *JAK3*, *EBI3*, *ANXA1*, *VCAM1*, *LILRB2*, *RIPK2*, *NP* and *CD1D*), 'positive regulation of T cell differentiation' (GO:0045582; *ANXA1*, *ADAM8*, *LILRB2*, *TGFB1*, *RIPK2*, *VNN1*, *KIAA0748* and *NP*), 'negative regulation of IL-12 production' (GO:0032695; *JAK3*, *TNFRSF9*, *LILRB1* and *ACP5*), 'negative regulation of adaptive immune response' (GO:0002820; *HFE*, *JAK3*, *SAMSN1*, *LILRB1* and *LOXL3*), 'negative regulation of chemotaxis' (GO:0050922; *PLXNA3, NRP1*, *SEMA3D*, *MIF*, *KLRK1* and *GPR18*) and 'heterotypic cell-cell adhesion' (GO:0034113; *ITGB2*, *ITGA4*, *LILRB2*, *CD2*, *ITGAX* and *CD1D*). Genes involved in 'positive regulation of myeloid cell apoptotic process' (GO:0033034; *CDKN2A*, *ANXA1*, *FCAR* and *MEF2C*), 'mononuclear cell proliferation' (GO:0032943; *EBI3*, *PRKCD*, *BAX*, *TGFB1*, *RIPK2*, *LILRB1* and *MEF2C*), 'negative regulation of glial cell apoptotic process' (GO:0034351; *PRKCH*, *PRKCA* and *PRKCD*), 'negative regulation of intrinsic apoptotic signalling pathway' (GO:2001243; *MDM2*, *IVNS1ABP*, *RRN3*, *ARHGEF2*, *VNN1*, *HTRA2*, *MIF*, *BCL2L1*, *MMP9* and *HIF1A*), 'positive regulation of syncytium formation by plasma membrane fusion' (GO:0060143; *ADAM9*, *EDH1*, *CD53* and *C6orf32*), 'regulation of cell shape' (GO:0008360; *CDC42SE2*, *RHOH*, *WDR1*, *PLXNA3*, *ITGB2*, *WASF3*, *CDC42*, *CDC42EP3*, *ANXA1*, *CENTD3*, *PLXNA1*, *PARVB*, *ICAM1*, *FGR*, *EPS8* and *EDG2*), 'positive regulation of angiogenesis' (GO:0045766; *BTG1*, *ITGB2*, *CYBB*, *PRKCA*, *HADAC9*, *ANXA1*, *GHRL*,

NRP1, *C3AR1*, *HIF1A*), 'regulation of peptidyl-tyrosine phosphorylation' (GO:0050731; *EHD4*, *SOCS3*, *RICTOR*, *PECAM1*, *ARHGEF2*, *GHRL*, *TGFB1*, *RIPK2*, *NRP1*, *ICAM1*, *MIF*, *ANGPT1*, *ADNP*) and 'regulation of leukocyte chemotaxis' (GO:0002688; *C3AR1*, *MIF*, *KLRK1*, *PLA2G7*, *CCR1*, *GPR18*, *RAC2* and *C6orf32*) were also enriched in the analysis of biological processes.

Table 4.6. GO biological process complete analysis of LCH cell microarray data.

GO biological process complete	Number of genes upregulated	Fold Enrichment
Toll-like receptor 2 signalling pathway	3	33.22
Connective tissue replacement involved in inflammatory response wound healing	3	33.22
ISG15-protein conjugation	3	27.69
3'-phosphoadenosine 5'-phosphosulfate biosynthetic process	3	27.69
Negative regulation of cytokine secretion involved in immune response	3	27.69
Positive regulation of myeloid cell apoptotic process	4	24.61
Negative regulation of glial cell apoptotic process	3	20.77
Toll-like receptor 4 signalling pathway	6	19.54
Regulation of Th1 cell differentiation	3	16.61
Positive regulation of cysteine-type endopeptidase activity involved in apoptotic signalling pathway	3	16.61
Cellular response to lipoteichoic acid	3	16.61
Positive regulation of receptor binding	3	16.61
Positive regulation of cellular extravasation	4	15.82
Response to IFN-β	7	14.91
Positive regulation of superoxide anion generation	4	13.84
Negative regulation of IL-12 production	4	13.84
Mast cell activation	5	12.58
Negative regulation of IL-10 production	4	12.31
Response to IFN-α	5	12.04
Positive regulation of bone resorption	4	11.66
Heterotypic cell-cell adhesion	6	11.07
Positive regulation of leukocyte apoptotic process	6	11.07
Regulation of TNF biosynthetic process	4	11.07
Ribonucleoside catabolic process	4	11.07
Regulation of myoblast fusion	4	10.55
Regulation of leukocyte degranulation	5	6.44
Lipopolysaccharide-mediated signalling pathway	6	10.38
Positive regulation of myeloid leukocyte mediated immunity	4	10.07
Cellular response to IL-6	6	10.07

Negative regulation of extrinsic apoptotic signalling pathway via death domain receptors	6	9.23
Cell adhesion mediated by integrin	4	9.23
Positive regulation of syncytium formation by plasma membrane fusion	4	9.23
Regulation of IL-8 secretion	4	9.23
Negative regulation of biomineral tissue development	4	9.23
Cellular extravasation	5	8.65
Type I IFN signalling pathway	10	8.39
Negative regulation of viral genome replication	8	8.2
Integrin-mediated signalling pathway	13	7.82
IFN-γ-mediated signalling pathway	10	7.8
Positive regulation of response to biotic stimulus	6	7.73
Negative regulation of αβ T cell activation	5	7.69
Positive regulation of response to cytokine stimulus	7	7.45
Positive regulation of TNF production	9	7.44
Negative regulation of toll-like receptor signalling pathway	5	7.29
Positive regulation of blood vessel endothelial cell migration	7	7.18
Protein trimerisation	7	7.05
Regulation of mast cell activation	5	6.92
Endodermal cell differentiation	5	6.92
Negative regulation of adaptive immune response	5	6.75
Leukocyte cell-cell adhesion	6	6.51
Regulation of bone mineralization	8	6.51
Negative regulation of chemotaxis	6	6.15
Regulation of cell shape	16	5.75
Negative regulation of intrinsic apoptotic signalling pathway	10	5.71
Rho protein signal transduction	8	5.61
Actin filament bundle assembly	6	5.45
Positive regulation of T cell differentiation	8	5.4
Regulation of smooth muscle cell migration	6	5.36
Actin cytoskeleton reorganisation	6	5.27
Positive regulation of type I IFN production	6	5.19
Mononuclear cell proliferation	7	5.1
Regulation of reactive oxygen species biosynthetic process	7	5.1
Positive regulation of NF-kappaB transcription factor activity	13	4.96
Cell-matrix adhesion	11	4.91
Regulation of Rho protein signal transduction	12	4.82
Positive regulation of IL-6 production	7	4.79
Positive regulation of T cell proliferation	8	4.52
Regulation of IFN-γ production	8	4.47
Defence response to virus	15	4.47
Positive regulation of muscle cell differentiation	7	4.46

Regulation of leukocyte chemotaxis	8	4.34
Platelet degranulation	10	4.33
Regulation of cytokine-mediated signalling pathway	12	4.31
Actomyosin structure organisation	8	4.26
B cell activation	11	4.23
Positive regulation of cytokine secretion	9	4.19
Neutrophil degranulation	36	4.13
Regulation of peptidyl-serine phosphorylation	10	4.07
Positive regulation of peptidyl-tyrosine phosphorylation	13	3.25
T cell differentiation	9	3.83
Positive regulation of intracellular protein transport	11	3.83
Cytokine production	10	3.82
Positive regulation of response to external stimulus	18	3.61
Regulation of I-kappaB kinase/NF-kappaB signalling	14	3.49
Positive regulation of angiogenesis	10	3.4
Cell chemotaxis	12	3.36
Extracellular matrix organization	19	3.32
Regulation of plasma membrane bounded cell projection assembly	10	3.28
Negative regulation of cell migration	14	3.16
Cellular response to TNF	11	3.06
Regulation of endocytosis	13	3.01
Apoptotic signalling pathway	15	3.01
Regulation of neuron death	15	2.85
Regulation of protein stability	13	2.72
Regulation of ERK1 and ERK2 cascade	15	2.68
Regulation of actin cytoskeleton organisation	16	2.66
Response to steroid hormone	16	2.65
Regulation of cellular component size	18	2.63
Regulation of MAP kinase activity	16	2.6
Regulation of supramolecular fibre organization	16	2.56
Response to peptide hormone	17	2.5
Regulation of neuron projection development	21	2.44
Positive regulation of cell projection organisation	16	2.43
Negative regulation of hydrolase activity	18	2.3
Response to inorganic substance	22	2.3
Viral process	24	2.28
Positive regulation of intracellular signal transduction	42	2.22
Cellular response to organonitrogen compound	20	2.21
Regulation of signalling receptor activity	22	2.19
Negative regulation of cellular component organisation	27	2.18
Adaptive immune response	21	2.18
Regulation of growth	26	2.09

Negative regulation of cellular protein metabolic process	37	2.07
Regulation of ion transport	24	2.03
Regulation of catabolic process	30	1.94
Response to drug	33	1.93
Positive regulation of transcription by RNA polymerase II	38	1.8
Homeostatic process	46	1.64

'Supplemental Data 1 – LCH CD207 vs. Control Skin CD207 Expression Array Results (LCH CD207/Skin CD207)' from Allen et al.[65] (available at http://www.jimmunol.org/content/184/8/4557/tab-figures-data).

Interestingly, similarly to the T cell enrichment analysis, genes involved in the following bacterial and viral biological processes were enriched in LCH cells: 'toll-like receptor 2 signalling pathway (GO:0034134; *TLR2*, *RIPK2* and *PIK3AP1*), 'toll-like receptor 4 signalling pathway' (GO:0034142; *ITGB2*, *ITGAM*, *TLR4*, *RIPK2*, *PIK3AP1* and *CD14*), 'negative regulation of toll-like receptor signalling pathway' (GO:0034122; *TLR4*, *LILRA2*, *CD14*, *CD300LF* and *OTUD4*), 'cellular response to lipoteichoic acid' (GO:0071223; *TLR2*, *RIPK2* and *CD14*), 'lipopolysaccharide mediated signalling pathway' (GO:0031663; *TLR2*, *TLR4*, *IRAK2*, *TGFB1*, *RIPK2* and *CD14*), 'negative regulation of viral genome replication' (GO:0045071; *OASL*, *IFITM3*, *IFITM1*, *BST2*, *APOBEC3G*, *IFITM2*, *TRIM6* and *ISG15*), and 'positive regulation of response to biotic stimulus' (GO:0002833; *PUM1*, *SIGLECP16*, *PRKCA*, *RIPK2*, *MIF* and *KLRK1*). Less specific terms such as 'response to drug' (GO:0042493; 33 genes), 'response to inorganic substance' (GO:0010035; 22 genes), 'response to peptide hormone' (GO:0043434, 17 genes), 'response to steroid hormone' (GO:0048545; 16 genes), 'regulation of ERK1 and ERK2 cascade' (GO:0070372; 15 genes), 'regulation of MAP kinase activity' (GO:0043405; 16 genes), 'viral process' (GO:0016032; 24 genes), 'defence response to virus' (GO:0051607; 15 genes), and 'positive regulation of response to external stimulus' (GO:0032103; 18 genes) were also enriched.

Reactome pathway analysis (**Table 4.7**) also identified the enrichment of genes involved in the following pathways: 'immunoregulatory interactions between a lymphoid and a non-lymphoid cell' (19 genes), 'IFN-γ signalling' (11 genes), and toll-like receptor associated pathways 'MyD88:Mal cascade initiated on plasma membrane' (9 genes), and 'TRIF-mediated TLR3/TLR4 signalling' (8 genes).

Table 4.7. GO Reactome pathway analysis of LCH cell microarray data.

Reactome pathways	Number of genes upregulated	Fold Enrichment
Transport and synthesis of PAPS	3	27.69
TRAIL signalling	3	23.73
HuR (ELAVL1) binds and stabilises mRNA	3	20.77
Ligand-dependent caspase activation	4	15.82
Interferon α/β signalling	11	9.23
Integrin cell surface interactions	12	7.82
Cell surface interactions at the vascular wall	14	7.68
Immunoregulatory interactions between a lymphoid and a non-lymphoid cell	19	6.74
IFN-γ signalling	11	6.69
Semaphorin interactions	7	5.79
MyD88:Mal cascade initiated on plasma membrane	9	5.48
TRIF-mediated TLR3/TLR4 signalling	8	4.61
Rho GTPase cycle	10	4.47
Platelet degranulation	9	3.99
DAP12 interactions	17	2.74
Signalling by NGF	21	2.65

'Supplemental Data 1 – LCH CD207 vs. Control Skin CD207 Expression Array Results (LCH CD207/Skin CD207)' from Allen et al.[65] (available at http://www.jimmunol.org/content/184/8/4557/tab-figures-data).

Previous analysis by Allen et al.[65] focussed on similarities in gene expression between LCH cells and immature myeloid DCs. Collectively, the pathway enrichment analyses from the results presented here contribute that LCH cells possess an enrichment of genes associated with exposure to endogenous antigens, and regulating the recruitment, activation, and proliferation of T cells. According to this enrichment analysis, LCH cells could also be responsible for the formation of multinucleated giant cells as implied by the enrichment of genes involved in syncytium formation by plasma membrane fusion. Further investigations are necessary to better understand these processes and pathways.

4.3. Discussion

In this study, LCH cells, conventional T cells and Foxp3$^+$ Tregs from LCH patients were characterised. The proportion of CD4$^+$ T cells in total T cells was significantly increased

in the peripheral blood and lesions from patients with active disease compared to the peripheral blood from patients with non-active disease, however it is unestablished whether this increase was due to the presence of an increase in Tregs or has occurred in parallel.

In contrast this study identified a significant decrease in the proportion of $CD8^+$ T cells in the blood from patients with active LCH compared to healthy donors. $CD8^+$ T cells can directly kill pathogenic cells and a relative reduction in these cells in the peripheral blood from LCH patients could be due to an increase in another subset, such as $CD4^+$ T cells, or the recruitment of peripheral $CD8^+$ T cells to an inflamed tissue environment. Given the proportion of $CD8^+$ T cells in the peripheral blood from patients with non-active disease was similar to those from healthy donors, it is not likely that $CD8^+$ T cells from LCH patients have a defective proliferative ability. It is important to emphasise that the decrease in the proportion of $CD8^+$ T cells observed is not due to the increase in Tregs and could be negatively affecting immune regulation in LCH patients, thus contributing to disease progression.

For this project, lesions analysed were intentionally derived from a variety of tissues and their immune profiles were compared to those from peripheral blood. It would be of interest to examine a number of LCH lesions derived from independent donors but originating from a single tissue type such as osseous tissue and compare them to matched control tissues to determine whether the $CD8^+$ T cell proportion is higher than usual at lesional sites, which might suggest that these cells are migrating to LCH lesions.

4.3.1. Conventional T cell function

This study investigated Th1 cytokine production by T cells to determine whether there were any functional defects in this lineage in patients with LCH. A novel finding from this chapter is the ability of T cells from LCH patients to produce Th1 cytokines, although it is of interest that one patient with LCH demonstrated poor intracellular cytokine production, and another showed poor Th1 cytokine production and secretion

upon stimulation. This suggests that additional studies in a larger cohort of LCH patients should be conducted to determine whether the poor ability to produce cytokines is evident in more patients. These studies could also exclude Tregs and compare the ability of conventional T cells against more physiological stimuli such as anti-CD3 and anti-CD28 to mimic the *in vivo* environment more closely. Abnormalities might be identified through measurements such as the rate of proliferation, the ability to produce perforin and/or granzymes and expression of exhaustion markers.

4.3.2. A potential role for unconventional T cells in LCH

The decrease in $CD8^+$ T cells identified in this study could also be due to a reduction or relocation in a specific subpopulation of $CD8^+$ T cells. For example, it is known that the $CD8^+$ T cell population comprises approximately 10% MAIT cells[108]. It is also of interest that DN T cells from patients with LCH were more variable in proportion than those from healthy donors. DN T cells comprise a range of T cell subsets including unconventional T cells, such as NKT cells, MAIT cells and most γδ T cells. DN T cells or a subset thereof could be poorly regulated in LCH patients, which warrants investigation into unconventional T cells in LCH. This study investigated the expression of genes encoding for non-MHC antigen presenting molecules by LCH cells. Albeit in a limited number of samples, the expression of *CD1A*, *CD1B*, *CD1C*, *CD1D* and *MR1* in these cells was confirmed, which indicates that additional to CD1a, other CD1 proteins and MR1 could be expressed by LCH cells. Thus, LCH cells may be able to interact directly with a range of unconventional T cell lineages and activate them via antigen presentation. Unconventional T cells are examined and more broadly discussed in **Chapter 5**.

4.3.3. CD56 expression and $Foxp3^+$ Treg function in LCH lesions

Prior to this study functional analyses of $Foxp3^+$ Tregs from LCH lesions had not been completed. The current study established that the surrogate identification method substituting Foxp3 for CD127 is sufficient for detecting Tregs in the context of LCH lesions. Furthermore, the results using this identification method are consistent with and more definitive than the previous studies that report an enrichment of Tregs in

LCH patients[57,65,98-100]. In the six lesions analysed, expression of the IL-2 receptor and activation marker CD25 was mainly confined to cells with a Treg phenotype and similarly, little HLA-DR expression was observed on total T cells.

Expression of HLA-DR by Tregs is indicative of a highly effective suppressor cell[177]. *HLA-DRA* gene expression was not able to be measured in this study using qPCR, however it was confirmed by flow cytometry that cell surface HLA-DR expression by LCH lesion Tregs was low and similar to the expression levels on Tregs from healthy donors. The absence of *HLA-DRA* mRNA is possibly due to low copy numbers in samples, or alternatively because the cells may have been unexpectedly in a resting rather than active state of function across all groups tested. A caveat to the 'cells to cDNA' approach is the inability to measure RNA integrity. RNA can be affected by variables such as sample viability, therefore low sample viabilities coupled with lower FACS yields from several LCH patient samples is likely why overall detectable amplification was more attainable in samples from healthy donors. It is not likely that the absence of *HLA-DRA* mRNA was due to poor RNA integrity because it was barely detectable in samples derived from healthy donors, and in contrast a range of other genes were detectable in these same samples.

This study was the first to identify CD56 expression by a considerable proportion of Tregs within LCH lesions. CD56 is also known as neural cell adhesion molecule, although it is not typically foreign to immune cells. CD56 is one of the defining markers used to identify NK cells, and it is also expressed on the surface of unconventional T cells such as NKT cells and MAIT cells[117,183,184]. Investigating other $CD56^+$ cell populations demonstrated that infiltrating $CD3^-CD56^+$ lymphocytes, likely to be NK cells, were at consistent levels relative to T cells in healthy donors and LCH patients. This suggests that NK cells are not likely enriched in LCH lesions in which the $CD56^+$ Tregs are. An interesting and unforeseen finding was the expression of CD56 by approximately 50% of lesional LCH cells in a single patient with pulmonary LCH. CD56 expression has previously been reported on myeloid cells in chronic myeloid leukaemia[185] and is associated with a poor prognosis in this setting[186]. Furthermore, aberrant CD56 expression by myeloid lineage monocytes and granulocytes has been

described in essential thrombocythaemia, primary myelofibrosis, and polycythaemia vera[187].

Regarding CD56 expression by Tregs, the literature is sparse, with only two reports to the best of my knowledge. The first study to report CD56 expression by Foxp3$^+$ Tregs found this population in hepatocellular carcinoma[188], while a more recent study demonstrated that CD56$^+$Foxp3$^+$ Tregs were enriched at the site of *Leishmania donovani* infection[189]. Both studies indicated that these cells were suppressive in nature, with the first demonstrating that CD56$^+$Foxp3$^+$ Tregs were able to inhibit the proliferation of CD4$^+$CD25$^-$ T cells following anti-CD3 stimulation[188], while the latter confirmed that CD56$^+$Foxp3$^+$ Tregs produced TGF-β[189]. Likewise, our study demonstrated TGF-β production by CD56$^+$Foxp3$^+$ Tregs from LCH lesions.

While this study did not detect any major differences between CD56$^+$ and CD56$^-$ Tregs, it does confirm that neither CD56$^+$ nor CD56$^-$ Tregs derived from LCH lesions produced the inflammatory cytokines TNF or IFN-γ when challenged, and confirms that LCH lesion Tregs can produce the immune suppressive cytokine TGF-β. Given their cytokine potential, the function of Foxp3$^+$ Tregs in LCH lesions is biased towards suppression. This means that Foxp3$^+$ Tregs could potentially contribute to the well documented expression of TGF-β within lesions that was previously attributed entirely to LCH cells[30,100,169].

Overall, the proportion of CD56$^+$ T cells was reduced in the blood from patients with active LCH when compared to lesions, and also when compared to the proportion in blood from patients with non-active disease. Conversely, the CD4$^+$CD56$^+$ T cell population was increased in LCH lesions compared to the blood from patients with active disease and healthy donor controls. Interestingly the enriched LCH lesional CD4$^+$CD56$^+$ and the total CD56$^+$ T cell populations comprised mostly Tregs. These results suggest that during active LCH, CD56$^+$ T cells from the blood could be relocating to lesional tissue sites. CD56$^+$ T cells have previously been shown to assume Foxp3 and CD25 expression in the presence of TGF-β[188] and furthermore the correlation identified between CD56$^+$ Tregs and total Tregs in LCH lesions suggests a direct

relationship between these two cell populations. One possible explanation is that $CD4^+$ T cells, including $CD4^+CD56^+$ T cells are recruited to the LCH lesional microenvironment and the production of TGF-β by LCH cells and $Foxp3^+$ Tregs induces Foxp3 and CD25 expression along with TGF-β production by other $CD4^+$ T cells.

The recruitment of immune cells to the lesional environment is likely initiated by LCH cells. For instance, $Foxp3^+$ Tregs may be recruited as a consequence of the immature LCH cell phenotype (reviewed by Janikashvili et al.[190]), although other factors such as the CXCR3-CXCL11 axis may be important. To elaborate, we know that CXCL11 is a chemoattractant for CXCR3[191] and CXCR3, which is commonly found on T cells with an activated memory phenotype was observed on T cells within LCH lesions, while CXCL11 was suggested to be expressed by LCH cells (unpublished data reported in study by Annels et al.[88]). Results presented in the current study established a reduction in the proportion of $CD8^+CD56^+$ T cells in both the blood and lesions from patients with active LCH compared to healthy donors, therefore similar to Kumari et al.[189] one might suggest that the induction of Tregs in LCH patients is responsible for apoptosis or inhibited proliferation of the $CD8^+CD56^+$ T cell population. The negative correlation between Tregs and $CD8^+CD56^+$ T cells detected in the current study supports this hypothesis. The findings presented here have potential implications for cancer immunology, because $CD8^+CD56^+$ T cells have potent cytolytic abilities[192] and patients with advanced cancers have responded to injections of $CD8^+CD56^+$ T cells into tumours[193].

4.3.3.1. A potential role for CD1a in $Foxp3^+$ Treg recruitment

An alternative mechanism to consider is that $CD4^+$ T cells may be recruited and expanded within lesions due to a unique antigen specificity. LCH cells are reported to have mostly internalised MHC II[85,86], which rules out the likelihood of peptide antigen presentation by LCH cells to $CD4^+$ T cells within lesions. In contrast to their expression of MHC II, LCH cells almost ubiquitously express CD1a on their cell surface, and interestingly, CD1a is known to house a range of endogenous and exogenous lipids, some of which can prime T cells for specific immune responses[145-147,150,194-197]. A recent study also showed that $Foxp3^+$ Tregs proliferated in response to LCH-like cells derived

from notch-activated monocytes[198]. Thus, there is potential for CD1a-dependent stimulation of T cells in LCH lesions and this is discussed further in **Chapter 6**.

4.3.4. IL-17F

An interesting finding from this chapter was the ability of T cells from various subsets from LCH patients to secrete small concentrations of IL-17F in contrast to control cultures with approximately similar cell numbers. IL-17F production can be regulated by TGF-β[199] and can promote the production of GM-CSF[200], monocyte chemoattractant protein, CCL2[199] and also mediate downstream MAPK activation[200]. IL-17F, is therefore a plausible cytokine to be implicated in the pathogenesis of LCH and should be further focussed upon.

4.3.5. LCH cells

This study also set out to better define the immunophenotype of LCH cells and found that LCH cells almost ubiquitously expressed CD11c on their surface, which aligns with the hypothesis that LCH cells are myeloid derived cells[49,65,149]. Additionally, there was a reduction in the expression of CD14 by LCH cells compared to myeloid cells derived from the peripheral blood from healthy donors. Monocyte/macrophage immunophenotypic marker and antigen receptor CD14 is a pattern recognition receptor for lipopolysaccharide, an endotoxin found in the cell wall of Gram-negative bacteria (reviewed by Jersmann[164]). CD14 was reported to be expressed by LCH cells by a number of groups[48,65,85] but expression by LCH cells was not ubiquitous[85]. Interestingly, *BRAF V600E* mutated LCH cells were shown to co-express CD14[48]. In addition, circulating monocytes[171] and $CD14^+$ cells isolated from human skin were shown to differentiate into $CD207^+$ cells in the presence of TGF-β *in vitro*[201]. It is therefore conceivable that LCH cells are derived from a $CD14^+$ lineage and the recent study by Schwentner et al.[198] comparing notch-activated monocytes to LCH cells has confirmed this soundly. Given this together with the understanding that CD14 is downregulated upon monocyte differentiation[85], CD14 expression could be an indicator of LCH cell immaturity and could be an important prognostic indicator for patients.

$CD4^{hi}$ expression was also increased on LCH cells compared to myeloid cells in the peripheral blood from healthy donors and from patients with active LCH. CD4 is a T cell co-receptor required for MHC II-presented antigen recognition. CD4 is also expressed by LCs[202] and other myeloid progenitor derived cells. CD4 is known to trigger macrophage differentiation upon ligation by MHC II[203] and could conceivably play a role in LCH cell/DC maturity. An alternate explanation for the high expression of CD4 on LCH cells could be the induction of surface CD4 expression by myeloid cells under inflammatory conditions. There is greater anti-CD4 antibody staining intensity on LCs in inflammatory states compared to clinically normal skin[202] therefore it is possible that CD4 is upregulated broadly by myeloid cells in the presence of inflammatory signals.

Recent proposals about the origin of LCH cells include JAG2-mediated notch induced monocyte-derived DCs[172,198] that are transcriptionally distinct from epidermal LCs, plasmacytoid DCs and myeloid DCs[172]. Other groups have suggested that circulating $CD1c^{+}$ myeloid DCs align phenotypically with LCH cells in the presence of known LCH microenvironment cytokines, GM-CSF and TGF-β[204,205]. The most recent report demonstrated that LCH cells are most similar transcriptionally and phenotypically to JAG2-mediated notch-activated monocytes[198]. The results presented in this study suggest that LCH cells express CD1a and CD4 similarly to LCs, but they can also express CD14, albeit in a reduced proportion. CD14 is poorly expressed by LCs[206] but was reported to be co-expressed by *BRAF V600E* mutated LCH cells[48], thus CD14 could be a marker of undifferentiated LCH cells, similar to monocytes. Whilst our definition and unequivocal origin of LCH cells is not completely established, it is likely that the cytokine milieu is impacting on the expression of these molecules by LCH cells and this has consequences for the downstream activation of T cells. The gene enrichment analysis conducted on LCH cells also suggests that LCH cells have great influence over the recruitment, activation, and proliferation of T cells in the environment.

4.3.6. Future studies

As our understanding surrounding the basic biology of Foxp3$^+$ Tregs increases along with new technologies, we should in the future be able to assess whether Tregs directly supress T responder cells through assays such as those that measure proliferation, however in agreeance with Quispel et al.[100] this is beyond the scope of current LCH studies. Furthermore, through co-culture studies we may be able to determine whether LCH cells specifically have the ability to stimulate Foxp3$^+$ Tregs to produce suppressive cytokines such as TGF-β, although again this is currently technically challenging because attempts to establish LCH cell lines have resulted in phenotype alterations[207,208]. Utilising fresh tissue biopsies would likely have improved the outcome of transcriptome analyses, but since the incidence of LCH is low, fresh tissue biopsies in sufficient numbers to achieve statistical power are difficult to obtain. New LCH studies should further investigate the function of CD56$^+$ and CD56$^-$ Tregs and confirm the presence of IL-17F in LCH lesions.

4.3.7. Conclusion

In summary, this study identified a decrease in the proportion of CD8$^+$ T cells in LCH patients, which could be negatively impacting on immune regulation and contributing to disease progression. High variability in the proportion of the DN T cell population warrants investigation into unconventional T cells in patients with LCH. This study has identified phenotypical differences in LCH cells compared to myeloid cells from the peripheral blood from patients with active LCH, which has contributed to our overall understanding of LCH cells. The results presented here are the first to identify the secretion of IL-17F by T cells from LCH lesions, which could be implicated in the pathogenesis of LCH, however further research into this topic is required. This study has established that the alternative identification method allowing for downstream functional studies on Tregs is appropriate in the context of assaying Tregs from LCH lesions. It also highlights that CD56$^+$ T cells collectively could have an important role in the pathogenesis of LCH. Importantly, Foxp3$^+$ Tregs are likely essential to LCH pathogenesis because additional to our current understanding that Foxp3$^+$ Tregs and TGF-β are present within LCH lesions, results from the present study established that

Tregs from LCH lesions are biased towards TGF-β production *in vitro*. This study takes us closer to elucidating the pathogenesis of LCH and encourages a path towards prospective immunotherapy treatments for LCH patients.

5.0. ALTERED POPULATIONS OF UNCONVENTIONAL T CELL LINEAGES IN PATIENTS WITH LCH

5.1. Introduction

The composition and inflammatory characteristics of LCH lesions suggest a localised dysregulation of immune cells, however in LCH a link between innate and adaptive immunity at the site of inflammation remains to be elucidated. The characteristic presence of LCH cells implies their involvement in the pathogenesis of this disease and this could occur through T cell signalling that leads to cytokine release and even dysregulation of T cells within the microenvironment.

There are also signs of immune abnormalities outside of LCH lesions. For example, in the circulation of patients with active LCH there are reports of $CD1a^+$ LCH-like cells with myeloid characteristics[32,148,149] and increased $Foxp3^+$ Tregs[36,57]. These abnormalities suggest problems with immune regulation in LCH, but nevertheless several lineages of immune cells with regulatory functions have not been reported on in LCH patients. Prime candidates are unconventional T cells such as MAIT cells, γδ T cells and NKT cells, all of which are capable of rapid cytokine responses and orchestrating innate and adaptive immunity. They are proposed to play fundamental roles in regulating aspects of tumour immunity, infection and autoimmunity[29,36,45,109,111-113,209], piquing our curiosity in their capabilities. While their importance in LCH is not established, one study found γδ T cells at a high frequency in LCH lesions[101], and there is overexpression of *CD1D* in LCH cells compared with skin-resident LCs[65]. NKT cells respond to lipids and are restricted by CD1d rather than conventional MHC/HLA molecules, hence higher expression of *CD1D* in lesions suggests a role for NKT cells in LCH. MAIT cells are also potent cytokine producers and were initially associated with anti-microbial responses, but more recently they were shown to have an altered function in colorectal tumours[112,113] and type two diabetes[210], and they are associated with several autoimmune diseases[137,138]. Like NKT cells, MAIT cells have not been studied in LCH, but CD161 is a defining characteristic

of MAIT cells and it is noteworthy that the gene encoding for its C-type lectin receptor (*CLEC2D*) is overexpressed by LCH cells compared with skin-resident LCs[65].

A unique characteristic of unconventional T cells is their ability to produce large amounts of cytokines that can bias immune responses depending on the setting[122,124,126]. Consequently, unconventional T cells are a logical target of investigation in an inflammatory setting like LCH. Therefore, the frequency of several unconventional T cell lineages was investigated in blood as well as tissue lesions from LCH patients to determine if they were present, participating in, or altered by the local inflammation when compared with healthy individuals. The hypothesis is that LCH progression is caused by immune dysregulation and given the regulatory nature of unconventional T cells and their potential to interact with LCH cells, they are contributing to LCH progression.

5.2. Results

5.2.1. Identification of unconventional T cells in patients with LCH

Unconventional T cells were stringently identified according to the following criteria: MAIT cells were classified as $CD3^+V\alpha7.2\ TCR^+CD161^+$ lymphocytes, γδ T cells as $CD3^+$γδ TCR^+ and NKT cells as $CD3^+$PBS44-loaded CD1d $tetramer^+$ lymphocytes. Specimens analysed were from the peripheral blood from healthy donors and from blood and lesions from LCH patients (**Figure 5.1A**).

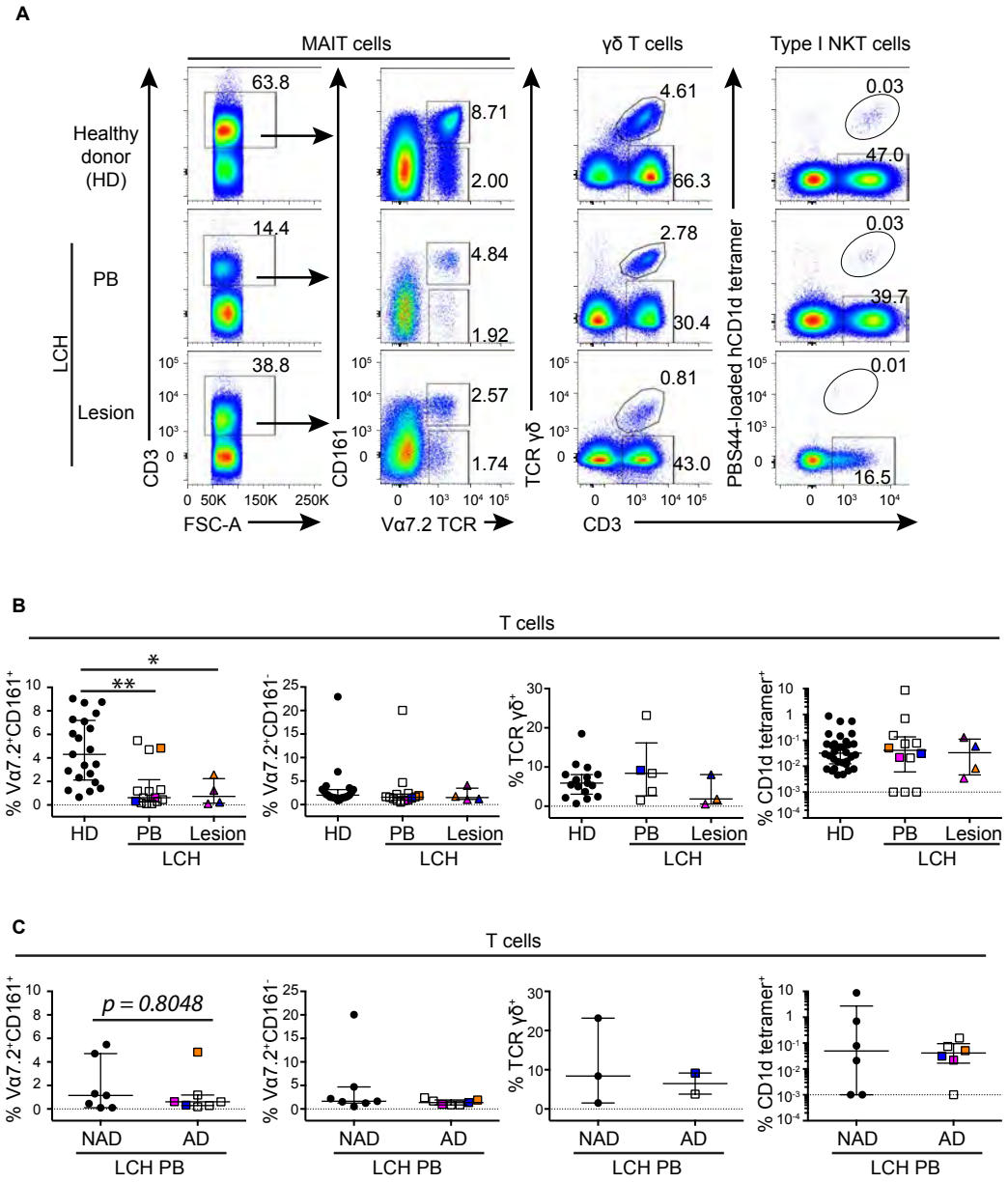


Figure 5.1. Circulatory and lesional unconventional T cell proportions in LCH.

*(**A**) Flow cytometry gating strategy for identifying MAIT cells (first and second columns), γδ T cells (third column) and type I NKT cells (fourth column) in circulating lymphocytes from healthy donors (first row) and LCH patients (second row) and in lymphocytes from LCH lesions (third row). (**B**) Proportions of MAIT cells and Vα7.2 TCR^{+}CD161^{-} T cells, γδ T cells and type I NKT cells in total T cells. (**C**) Proportions of MAIT cells, Vα7.2 TCR^{+}CD161^{-} T cells, γδ T cells and type I NKT cells in circulating T cells from LCH patients stratified by active and non-active disease. Kruskal-Wallis tests with Dunn's multiple comparisons were conducted for (**B**) and two-tailed Mann Whitney tests for (**C**). *p <0.05, **p <0.01, error bars indicate median + interquartile range. 10^{-3} on logarithmic scale indicates 'undetectable'. Lesions and matched peripheral blood samples are colour coded on graphs.* ***HD =healthy donor, PB =peripheral blood, NAD =non-active LCH, AD =active LCH.***

The frequency of MAIT cells expressed as a proportion of total T cells was significantly lower in blood (*p =0.0010, median =0.61%*) and lesions (*p =0.0353, median =0.73%*) from LCH patients compared to blood from healthy donors (*median =4.30%*) (**Figure 5.1B**). The measure of MAIT cell frequency can sometimes be confounded by downregulation of CD161, however Vα7.2 TCR^{+}CD161^{-} T cells did not increase in blood or lesions from LCH patients (**Figure 5.1B**). No significant difference was seen in the proportions of γδ T cells or NKT cells across the groups (**Figure 5.1B**). Interestingly, there was greater variability in the proportion of circulatory NKT cells in LCH patients (*range =0.000-8.68%*) compared to controls (*range =0.005-0.871%*) (**Figure 5.1B**).

Peripheral blood samples from LCH patients were further stratified into active and non-active disease, however there were no significant differences in the proportions of circulatory MAIT cells (*active LCH =0.06%, non-active LCH =1.15%*), γδ T cells (*active LCH =6.47%, non-active LCH =8.38%)* or NKT cells (*active LCH =0.041%, non-active LCH =0.050*) when investigating these groups (**Figure 5.1C**). Thus, while no discernible differences were observed in unconventional T cells between samples from patients with active and non-active LCH, MAIT cells were significantly reduced in LCH patients compared to controls derived from healthy donor blood.

5.2.2. MR1 tetramer labelling of MAIT cells

Vitamin B metabolite 5-OP-RU is the MAIT cell cognate antigen and 5-OP-RU-loaded MR1 tetramer is the gold standard reagent for MAIT cell identification[211]. This was not accessible at the commencement of this study, therefore it was confirmed in healthy donor and LCH peripheral blood samples that Vα7.2 TCR^{+}CD161^{+} MAIT cells corresponded with 5-OP-RU-loaded MR1 tetramer^{+} T cells and vice versa (**Figure 5.2A**). Importantly, proportions of Vα7.2 TCR^{+}CD161^{+} MAIT cells and 5-OP-RU-loaded MR1 tetramer^{+} MAIT cells were not different in four LCH peripheral blood samples (*p =0.3750*) (**Figure 5.2B**).

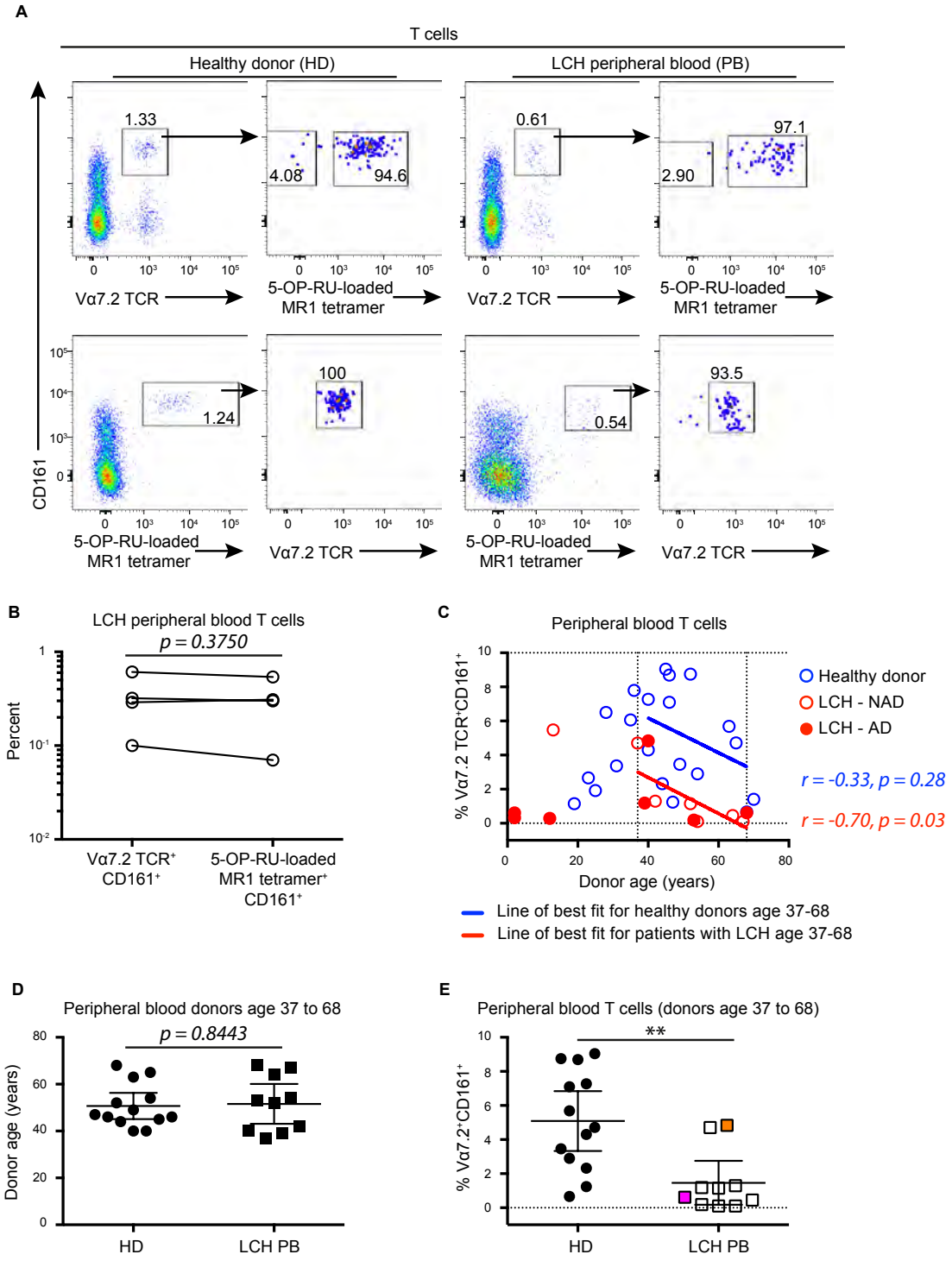


Figure 5.2. MAIT cell identification methods and age stratification.

*(**A**) Flow cytometry gating strategy for identifying 5-OP-RU-loaded MR1 tetramer$^+$ cells within the Vα7.2 TCR$^+$CD161$^+$ T cell population (top row) and Vα7.2 TCR$^+$ cells within the 5-OP-RU-loaded MR1 tetramer$^+$CD161$^+$ T cell population in (bottom row) from peripheral blood from healthy donors (left) and LCH patients (right). Data are representative of at least three individuals per group. (**B**) Matched proportions of Vα7.2 TCR$^+$CD161$^+$ T cells and 5-OP-RU-loaded MR1 tetramer$^+$CD161$^+$ T cells in total T cells from blood from LCH patients. (**C**)*

*Proportions of MAIT cells in peripheral blood T cells versus age for healthy donors (blue circles) and patients with active (closed red circles) and non-active LCH (open red circles) with a line of best fit for a subset of healthy donors (solid blue line) and LCH patients (solid red line) aged 37-68). (**D**) Plot shows the age distribution of a subset of healthy donors and LCH patients aged 37-68. (**E**) Proportions of MAIT cells in total T cells from blood from healthy donors and LCH patients aged 37-68. A two-tailed Wilcoxon matched-pairs signed rank test was conducted for (**B**). Pearson's two-tailed correlation tests were conducted for (**C**). Two-tailed unpaired t tests with Welch's correction were conducted for (**D**) and (**E**). **p <0.01, error bars indicate mean + 95% confidence interval. Lesions and matched peripheral blood samples are colour coded on graph (**E**).* ***HD =healthy donor, PB =peripheral blood, NAD =non-active LCH, AD =active LCH.***

5.2.3. MAIT cell proportion and age

While age is reported as a factor in MAIT cell frequency[184,212] it is important to emphasise that the median proportion of MAIT cells in total T cells was 0.61% and 0.73% in the peripheral blood and lesions from LCH patients respectively. These values were comparable to the lowest relative frequency observed in the healthy donor cohort (*minimum =0.66%*). The median age for healthy donors was 45 years (*range =19-70 years*), while for LCH peripheral blood samples it was 41 years (*range =2-68 years*) and for lesions it was 24.5 years (*range =2-68 years*). Peripheral blood MAIT cell proportions were plotted against age and a subset of 13 healthy donors and 10 LCH patients in the age range of 37-68 was identified, for which MAIT cell proportion was measured. The data outside these age brackets was excluded and a Pearson's two-tailed correlation test (95% confidence interval) was performed and a line of best fit was determined for age versus MAIT cell proportion for each group (**Figure 5.2C**). For healthy donors age 37-68 the MAIT cell proportion was not significantly correlated to age (*r =-0.33, p =0.28*), while in LCH patients, there was a significant negative correlation to age (*r =-0.70, p =0.03*). The mean age for healthy donors (*50.69*) and LCH patients (*51.60*) between 37 and 68 were well matched and there was no significant difference in age between the groups (*p =0.8443*) (**Figure 5.2D**). Importantly, when comparing the proportion of MAIT cells in total T cells there remained a significant (*p =0.0015*) decrease in the peripheral blood from LCH patients age 37-68 (*mean =1.46%*) compared to healthy donors age 37-68 (*mean =5.09%*) (**Figure 5.2E**). While age was reported as a factor associated with MAIT cell declines, data from the current study suggests that it is not the only factor responsible for the lower proportions of MAIT cells in the peripheral blood from LCH patients. It is

important to emphasise that both age and a range of diseases are associated with peripheral blood MAIT cell reductions (reviewed by Kumar and Ahmad[213]), and often diseases are age related, therefore age related measurements of MAIT cells could also be confounded by increased incidence of disease.

5.2.4. CD161$^+$ T cells in patients with LCH

Given that all MAIT cells express CD161 and the majority also express CD8[214], the overall changes to CD8 and CD161 T cell subsets were investigated (**Figure 5.3A**). Patients with active LCH had significantly (*p =0.0344*) lower proportions of CD8$^+$CD161$^+$ cells in T cells from the peripheral blood (*median =0.66%*) compared to healthy donors (*median =2.65%*) (**Figure 5.3B**). These patients also displayed a significant (*p =0.0303*) reduction in CD8$^+$CD161$^-$ cells in T cells (*median =28.03%*) compared to patients with non-active LCH (*median =44.95%*), but significantly (*p =0.0286*) higher proportions of CD8$^-$CD161$^-$ cells (*active LCH median =63.79%, non-active LCH median =47.20%*) (**Figure 5.3B**). Given that Vα7.2 TCR$^+$CD161$^-$ proportions were stable across the groups (**Figure 5.1B**) it is not likely that the reduction in MAIT cells is due to an overall decrease in CD161.

5.2.5. Activation status of unconventional T cells in LCH lesions

LCH lesions are thought to harbor activated T cells[30,56,100], therefore CD25 expression was assessed. There was no considerable CD25 expression by either MAIT cells or NKT cells in LCH lesions (**Figure 5.4**) and given that most CD25$^+$ T cells within LCH lesions are Foxp3$^+$ Tregs, it is not likely that γδ T cells express CD25 either.

5.2.6. Ability of LCH cells to activate unconventional T cells

While γδ T cells can be stimulated by antigen in a variety of MHC-dependent and -independent methods[215], CD1d and MR1 are necessary for antigenic stimulation of NKT cells and MAIT cells respectively. It is already known that *CD1D* is expressed by LCH cells[65] and to understand whether LCH cells are able to present antigen to MAIT cells, this study included testing for the expression of *MR1* by purified LCH (CD1a$^+$) cells in the previously described transcriptome analysis (**Section 4.2.12.1**.) *MR1* was

expressed by LCH cells (**Figure 4.24**) and this suggests that MR1 presentation by LCH cells is not defective at the mRNA level. It is also important to note, however, that MAIT cells can be stimulated independently of antigen by cytokines such as IL-12 and IL-18, and IL-18 has incidentally been detected at elevated levels in the blood from LCH patients[170].

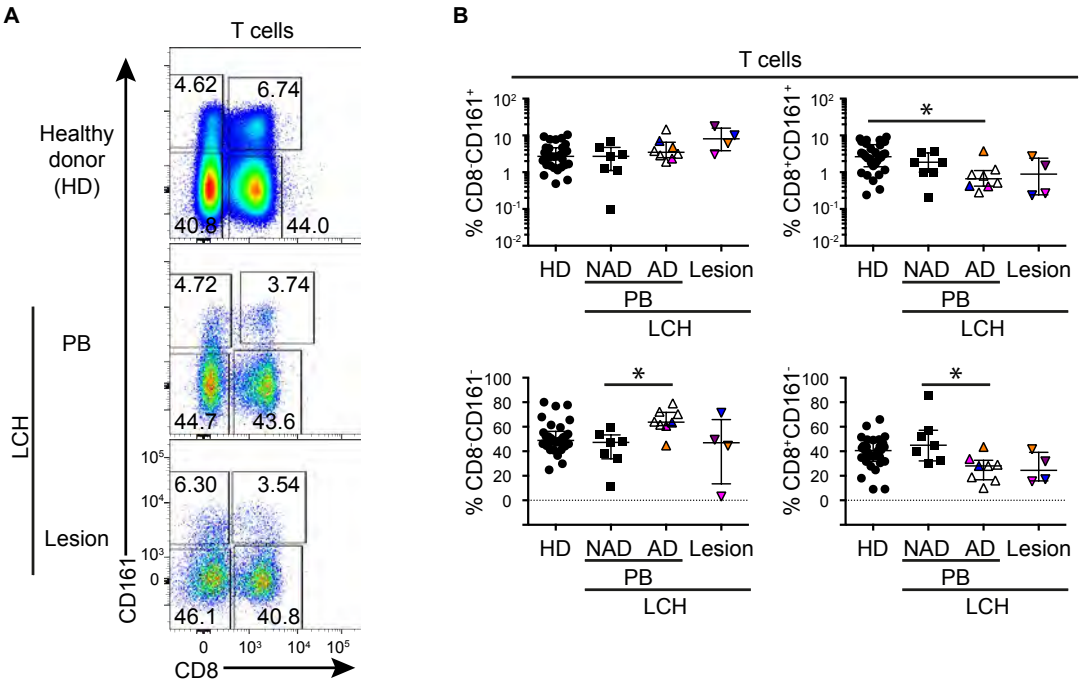


Figure 5.3. CD161⁺ T cells in LCH.

*(**A**) Flow cytometry gating strategy for identifying CD8⁺CD161⁻, CD8⁺CD161⁺, CD8⁻CD161⁻ and CD8⁺CD161⁻ cells in T cells from the peripheral blood from healthy donors (top) and from blood (middle) and lesions (bottom) from LCH patients. (**B**) Proportions CD8⁺CD161⁻ T cells (top left), CD8⁺CD161⁺ T cells (top right), CD8⁻CD161⁻ T cells (bottom left) and CD8⁺CD161⁻ T cells (bottom right) in T cells from blood from healthy donors and patients with non-active and active LCH, and from lesions from LCH patients. Kruskal-Wallis tests with Dunn's multiple comparisons were conducted for (**B**). *p <0.05, error bars indicate median + interquartile range. Lesions and matched peripheral blood samples are colour coded on graphs.* ***HD =healthy donor, PB =peripheral blood, NAD =non-active LCH, AD =active LCH.***

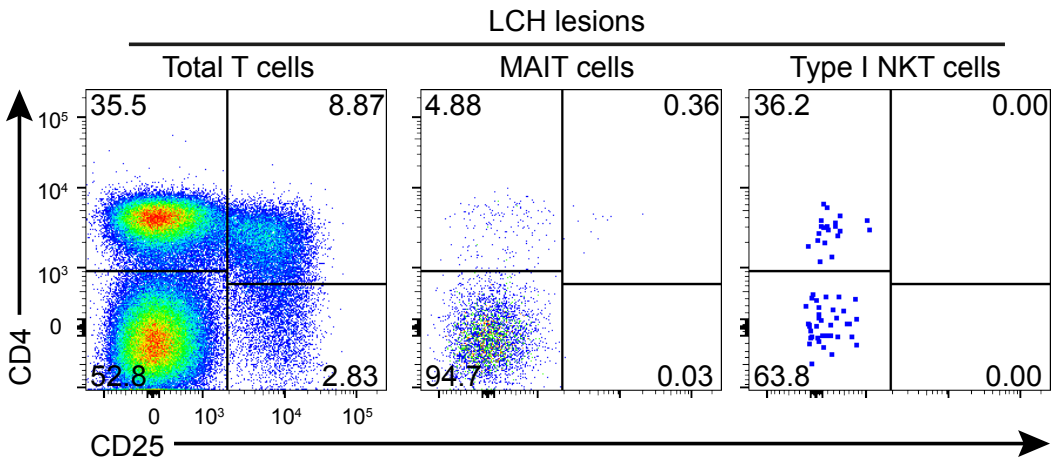


Figure 5.4. Activation status of unconventional T cells in LCH.

*(**A**) Representative flow cytometry plots demonstrate that most CD25$^+$ T cells in LCH lesions are CD4$^+$ (left) and there is little to no CD25 expression by MAIT cells (centre) or type I NKT cells (right). Data are representative of at least four individuals.*

5.2.7. Summary composition of regulatory T cell subsets in LCH patients

The composition of T cells from LCH patients was summarised in comparison to healthy donors using the median proportions described earlier (including in **Chapter 4**), and the T cell subsets highlighted in the graphs are regulatory T cell subsets (**Figure 5.5**). The data presented here demonstrates that regulatory T cell subsets collectively (γδ T cells, MAIT cells, NKT cells and Tregs) make up approximately 2% less of the total T cell population in the blood from patients with active LCH compared to healthy donors and patients with non-active disease, for which MAIT cells may be responsible. Within lesions, despite a decrease in the proportion of MAIT cells, and a lower median proportion of γδ T cells (though this was not significant), the striking increase in Tregs brings the collective population to over 15% of the total T cell population. Given Tregs are enriched within LCH lesions while other populations are decreased, they could either be inhibiting the proliferation of other T cell lineages or be proliferating to outnumber them, either of which is likely an important factor in the progression of LCH.

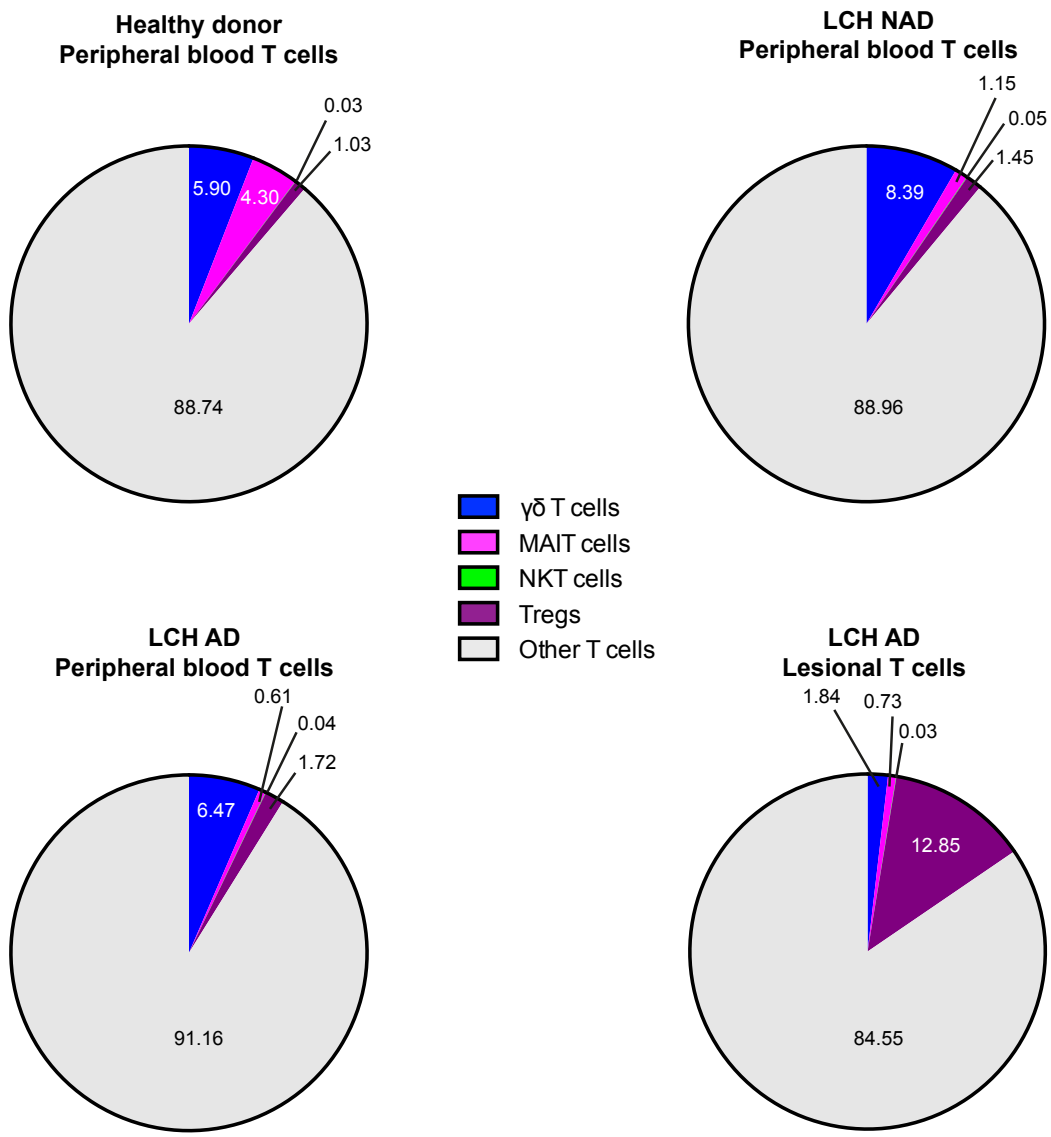


Figure 5.5. Summary composition of regulatory T cell subsets in patients with LCH.

A representation of the proportions of γδ T cells, MAIT cells, type I NKT cells, Tregs, and all remaining (other) T cells from the peripheral blood from healthy donors (top left) and patients with non-active (top right) and active (bottom left) LCH, and from LCH lesions (bottom right). Data are a summary of the median proportions reported previously. ***NAD =non-active LCH, AD =active LCH.***

5.2.8. Changes to unconventional T cell subpopulations in LCH patients

Since there are functionally distinct subsets of unconventional T cells, this study investigated the relative frequency of known MAIT cell, NKT cell and γδ T cell subpopulations.

5.2.8.1. MAIT cell subsets in patients with LCH

For MAIT cells, CD8 and CD4 subsets were examined (**Figure 5.6A**) and LCH patients had significantly (*p =0.0162*) increased blood $CD4^+$ cells in $V\alpha7.2\ TCR^+CD161^+$ T cells (*median =3.28%*) when compared to healthy donors (*median =1.05%*). A similar trend was also observed when comparing lesions (*median =6.02%*) to healthy donors, although this was not significant (*p =0.1142*) (**Figure 5.6B**). No differences (*p =0.9452*) were observed upon further stratification of LCH blood samples into active disease and non-active disease (*active LCH median =3.55%, non-active LCH median =3.08%*) (**Figure 5.6B**).

Given recent developments regarding the $CD4^+$ MAIT cell subset using the surrogate staining method ($V\alpha7.2\ TCR^+CD161^+$ T cells)[184] a tetramer based approach should be used in the future to determine the significance of this finding. The LCH blood specimens that were co-stained with anti-$V\alpha7.2$ TCR and 5-OP-RU-loaded MR1 tetramer were comparable, however the cell numbers were too low to determine whether specimens with the higher proportions of CD4 were 5-OP-RU antigen specific (**Figure 5.6C**). It is important to note that although $CD4^+V\alpha7.2\ TCR^+CD161^+$ T cells may not be as reliably defined as MAIT cells compared with the $CD8^+$ and DN subsets, the expression of Eomes, PLZF and CCR4 are differentially expressed in this population when compared to $CD8^+$ and DN MAIT cells[214].

There were no differences observed in the proportions of $CD8^+$ or DN MAIT cell populations across the groups (**Figure 5.6B**), indicating that the MAIT cell reduction in LCH patients is not due to the overall reduction of $CD8^+$ T cells observed in **Chapter 4**. In retrospect, the study next tested whether the reduced MAIT cell population was responsible for the reduction in the proportion of $CD8^+$ cells in total T cells identified

in **Chapter 4**. MAIT cells (and Tregs) were excluded from the previous data and statistical analysis demonstrated a trend towards a decrease in the proportion of $CD8^+$ cells in total T cells from patients with active LCH, although this was not significant (**Figure 5.6D**). It is important to point out that the statistical power of this test was reduced compared to that from the previous chapter because MAIT cells and Tregs were not detected in concert in all of the specimens analysed. It appears that there are independent reductions in both MAIT cells (significant) and $CD8^+$ T cells (trend) in LCH although this analysis requires expanding for conclusiveness.

5.2.8.2. γδ T cell subsets in patients with LCH

Typically most γδ T cells are $CD8^-CD4^-$ and there are known transcriptional differences between $CD161^+$ and $CD161^-$ γδ T cells[216]. Therefore, DN, $CD8^+$ and $CD4^+$ γδ T cells and $CD8^-CD161^+$, $CD8^+CD161^+$, $CD8^+CD161^-$ and $CD8^-CD161^-$ γδ T cell subsets were investigated (**Figure 5.7A**). There was a significant (*p =0.0465*) increase in the proportion of $CD4^+$ cells in γδ T cells from LCH lesions (*median =3.36%*) when compared to peripheral blood from healthy donors (*median =0.34%*) (**Figure 5.7B**) but no significant differences were detected in the CD161 subsets between the healthy donor and LCH patient groups (**Figure 5.7C**).

5.2.8.3. NKT cell subsets in patients with LCH

For NKT cells CD8 and CD4 subsets, CD8 and CD161 subsets and expression of CD56 were investigated (**Figure 5.8A**). There were no differences in expression of CD56 by NKT cells from patients with LCH compared to healthy donors (**Figure 5.8B**) and there were similar proportions of $CD8^+$ cells in NKT cells across the groups (**Figure 5.8C**). There was a trend towards a decrease in DN cells and an increase in $CD4^+$ cells in NKT cells in blood from patients with active LCH (*DN median =33.14%, $CD4^+$ median =62.62%*) when compared to non-active disease (*DN median =89.44%, $CD4^+$ median =7.04%*) (**Figure 5.8C**). This study also found a significant (*p =0.0404*) decrease in circulatory $CD8^+CD161^-$ cells in NKT cells in patients with active LCH (*median =0.001%*) compared to heathy donors (*median =0.974%*) (**Figure 5.8D**). As previously mentioned, there was little sign of activation (CD25) in the overall NKT cell population in patients with LCH.

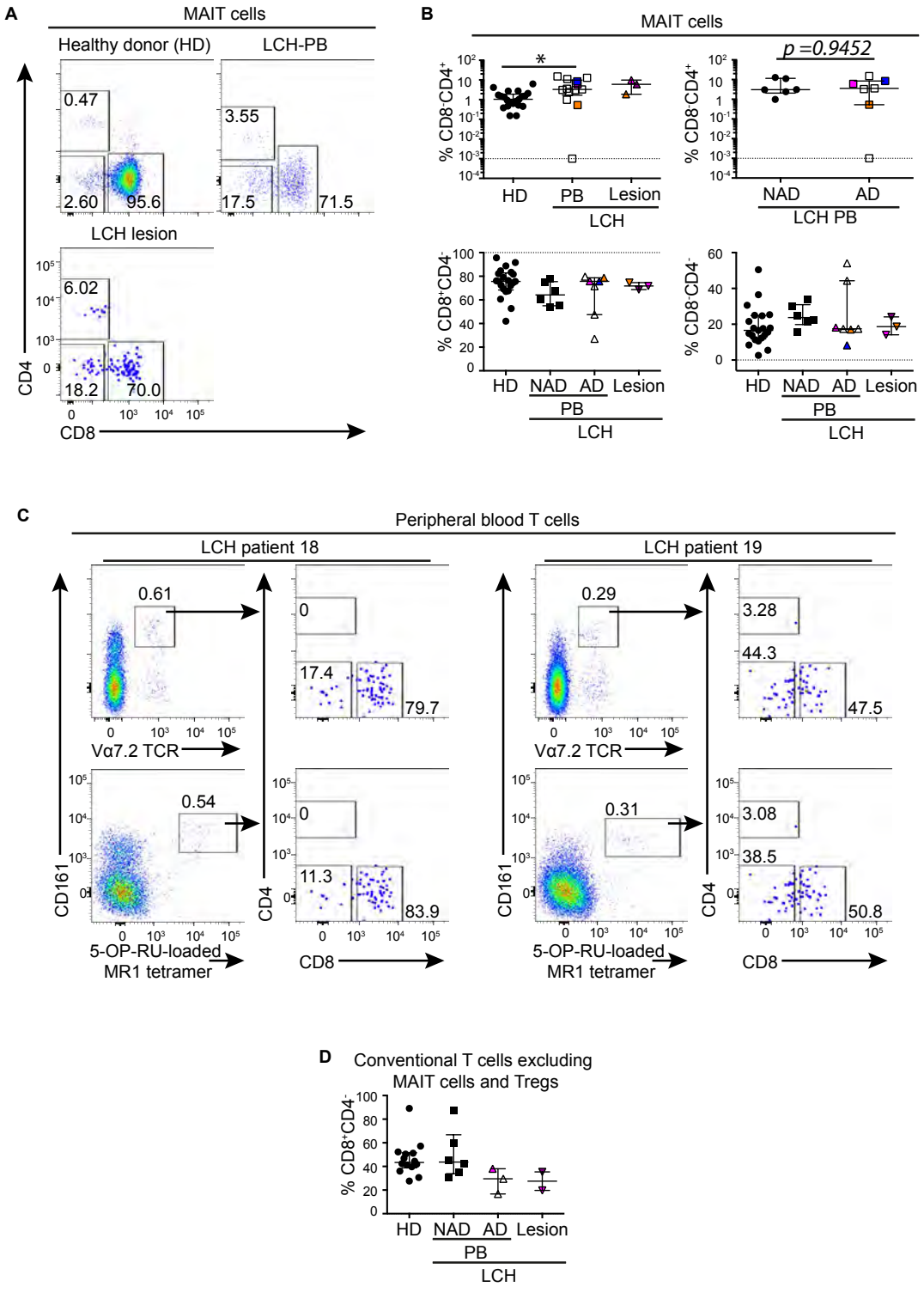


Figure 5.6. MAIT cell subsets in patients with LCH.

*(**A**) Flow cytometry gating strategy for identifying CD8 and CD4 cells in MAIT cells from the peripheral blood from healthy donors (left) and from blood (centre) and lesions (right) from LCH patients. (**B**) Proportions of $CD4^+$, $CD8^+$, and $CD8^-CD4^-$ cells in MAIT cells from blood from healthy donors and patients with non-active and active LCH, and from LCH lesions. (**C**)*

*Comparison of CD4 expression in MAIT cells identified as Vα7.2 TCR$^+$CD161$^+$ T cells (top row) or 5-OP-RU-loaded MR1 tetramer$^+$CD161$^+$ T cells (bottom row) from the peripheral blood from LCH patients. (**D**) Proportions of CD8$^+$ T cells in conventional T cells (specifically excluding MAIT cells and Tregs) from blood from healthy donors and patients with non-active and active LCH, and from LCH lesions. Kruskal-Wallis tests with Dunn's multiple comparisons were conducted for (**B**) and (**D**) except where only two groups were compared (two-tailed Mann Whitney test). *p <0.05, error bars indicate median + interquartile range. 10^{-3} on logarithmic scale indicates 'undetectable'. Lesions and matched peripheral blood samples are colour coded on graphs.* ***HD =healthy donor, PB =peripheral blood, NAD =non-active LCH, AD =active LCH.***

5.2.9. *In vitro* stimulation of unconventional T cells from LCH patients

Next, the functional capacity of unconventional T cells from LCH patients was investigated. Dysfunction in cytokine production among these lineages is strongly associated with increased incidence and progression of disease, so it was important to establish if these cells could mount a normal cytokine response to stimulation. Purified T cells were stimulated with PMA and ionomycin (for methods see **Section 3.11.3**) and TNF and IFN-γ production by MAIT cells (**Figure 5.9A**), γδ T cells (**Figure 5.9B**) and NKT cells (**Figure 5.9C**) was examined. Proportions of cytokine producing MAIT cells, γδ T cells and NKT cells in the peripheral blood from patients with active LCH were not significantly different to those from patients with non-active disease and healthy donors (**Figure 5.9D**). This data suggests that although decreased in proportion in LCH patients, MAIT cells are still responsive, although whether this is true *in vivo* is not established.

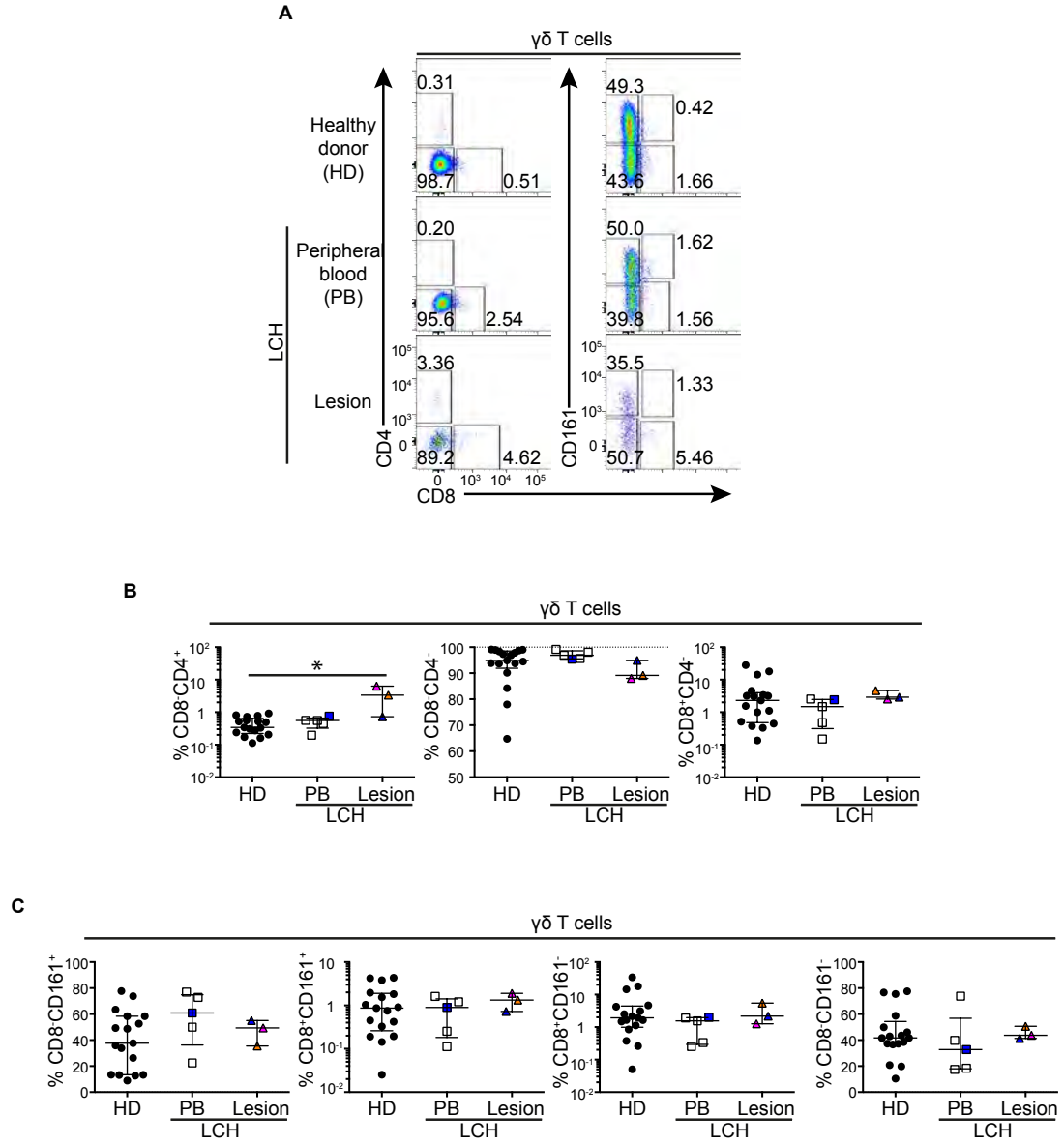


Figure 5.7. γδ T cell subsets in patients with LCH.

*(**A**) Flow cytometry gating strategy for identifying γδ T cell subsets in peripheral blood from healthy donors and in blood and lesions from LCH patients. (**B**) Proportions of $CD4^+CD8^-$, $CD8^-CD4^-$ and $CD8^+CD4^-$ cells in γδ T cells from blood from healthy donors and from blood and lesions from LCH patients. (**C**) Proportions of $CD8^-CD161^+$, $CD8^+CD161^+$, $CD8^+CD161^-$ and $CD8^-CD161^-$ cells in γδ T cells from blood from healthy donors and from blood and lesions from LCH patients. Kruskal-Wallis tests with Dunn's multiple comparisons were conducted for (**B**) and (**C**). *p <0.05, error bars indicate median + interquartile range. Lesions and matched peripheral blood samples are colour coded on graphs.* ***HD =healthy donor, PB =peripheral blood.***

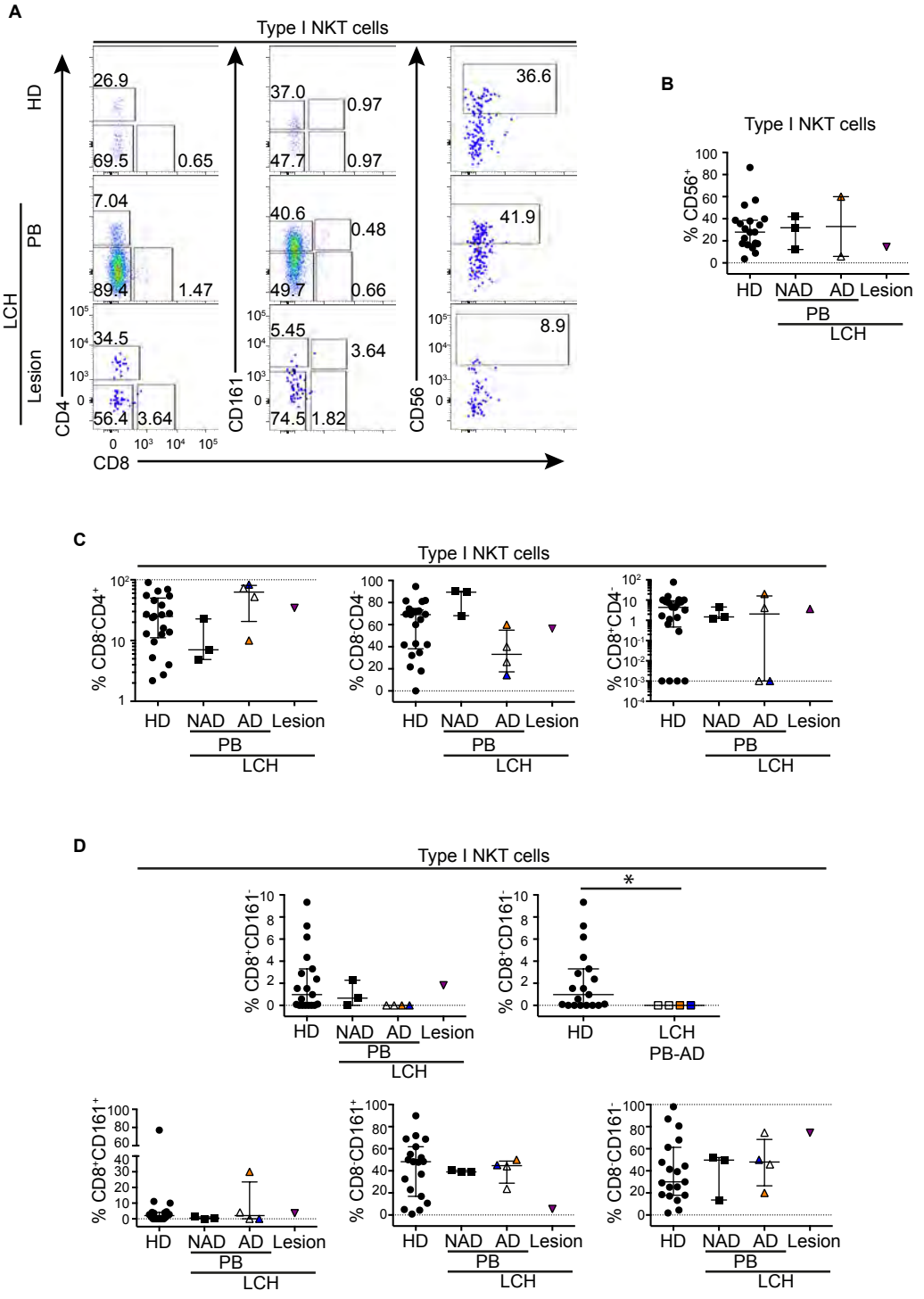


Figure 5.8. NKT cell subsets in patients with LCH.

*(**A**) Flow cytometry gating strategy for identifying type I NKT cell subsets in peripheral blood from healthy donors and in blood and lesions from LCH patients. Proportions of $CD56^+$ cells (**B**), $CD4^+CD8^-$, $CD8^-CD4^-$ and $CD8^+CD4^-$ cells (**C**), and $CD8^+CD161^-$, $CD8^+CD161^+$, $CD8^-CD161^+$ and $CD8^-CD161^-$ (**D**) in type I NKT cells from healthy donors and LCH patients. LCH lesion group is excluded from tests. Kruskal-Wallis tests with Dunn's multiple comparisons were conducted for (**B**), (**C**) and (**D**) except where only two groups were compared (two-tailed Mann Whitney test). *p <0.05, error bars indicate median + interquartile range. 10^{-3} on logarithmic scale indicates 'undetectable'. Lesions and matched peripheral blood samples are colour coded on graphs.* ***HD =healthy donor, PB =peripheral blood, NAD =non-active LCH, AD =active LCH.***

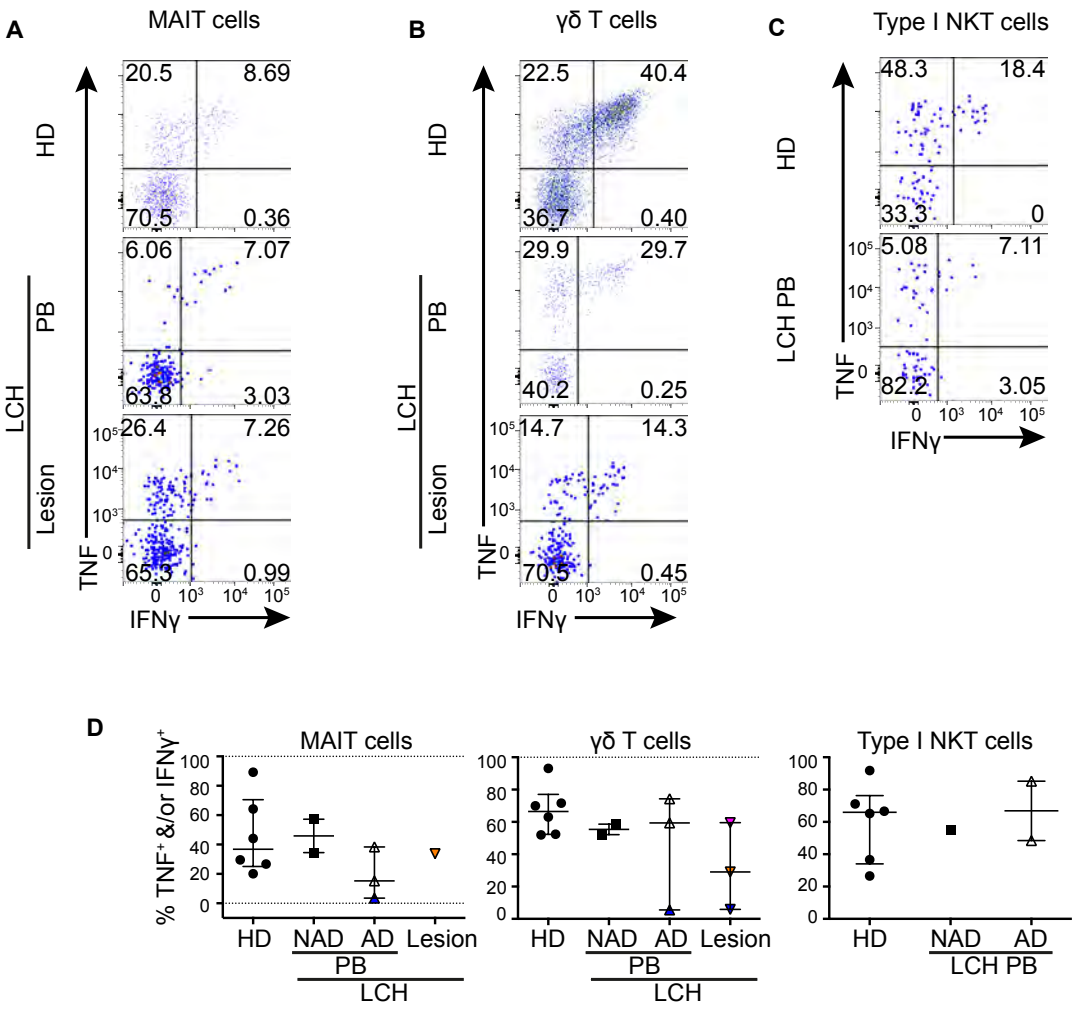


Figure 5.9. Th1 cytokine production by unconventional T cells.

*Flow cytometry gating strategy for post-stimulation identification of TNF and IFN-γ producing MAIT cells (**A**), γδ T cells (**B**) and type I NKT cells (**C**) from the peripheral blood from healthy donors and from blood and lesions from LCH patients. (**D**) Proportions of TNF^+ and/or $IFN\text{-}\gamma^+$ T cells in MAIT cells, γδ T cells and type I NKT cells from healthy donors and LCH patients. Groups with only one value are excluded from tests. Kruskal-Wallis tests with Dunn's multiple comparisons were conducted for (**D**), except where only two groups were compared (two-tailed Mann Whitney test). Error bars indicate median + interquartile range. Lesions and matched peripheral blood samples are colour coded on graphs.* ***HD =healthy donor, PB =peripheral blood, NAD =non-active LCH, AD =active LCH.***

5.2.10. The effect of TGF-β on MAIT cell stimulation

TGF-β is suggested as one of the drivers of the LCH cell phenotype[148,171,172] and it is also known to induce Foxp3 and CD25 expression by T cells[188]. Given these influences, the effect of TGF-β on the activation of MAIT cells was examined to understand whether the LCH microenvironment may be affecting the ability of the MAIT cells to elicit an appropriate immune response. PBMCs from four healthy donors were cultured overnight in the presence of TGF-β (0.1 ng/mL, 1 ng/mL and 10 ng/mL) and cell cultures were stimulated with the MAIT cell cognate antigen 5-OP-RU for 6 hours (for complete methods see **Section 3.11.6**). Following the stimulation, CD69 expression and TNF production by MAIT cells and by total T cells was measured (**Figure 5.10A**). There was no significant difference in the proportion of MAIT cells in total T cells across the different conditions (*p =0.6937*), however the median MAIT cell proportion was most consistent in the unstimulated cell cultures (*median =1.270-1.385%*) when compared to the 5-OP-RU stimulated cell cultures (*median =0.805-2.32%*) (**Figure 5.10B**).

While MAIT cells were stimulated in an antigen-specific manner with 5-OP-RU, the effects of TGF-β on cultures would not likely be limited to this population. This assay therefore investigated the effects of activation on MAIT cells and on total T cells. There was a trend towards decreased CD69 expression by unstimulated MAIT cells cultured with higher concentrations of TGF-β (1-10 ng/mL) when compared to the lower concentrations (0-0.1 ng/mL) (**Figure 5.10C**). This trend was also apparent in the total T cell population. Additionally, CD69 expression was significantly reduced in T cells cultured in 10 ng/mL TGF-β compared to cells cultured in 0.1 ng/mL (*p =0.0370*) or no TGF-β (*p =0.0370*) (**Figure 5.10C**). In the 5-OP-RU stimulated cultures there was also a trend towards decreased CD69 expression by T cells cultured in 0.1 ng/mL TGF-β when compared to cells cultured in 10 ng/mL TGF-β (*p =0.0557*), but CD69 expression in 5-OP-RU stimulated MAIT cells specifically did not appear significantly different across the different TGF-β concentrations (**Figure 5.10C**). It is important to note that GolgiPlug was added to cultures one hour post adding 5-OP-RU and GolgiPlug inhibits the trafficking of CD69 to the cell surface[217], therefore any changes to CD69

upregulation occurring in the latter 5 hours of culture might be confounded by this effect.

By investigating the ability of 5-OP-RU stimulated MAIT cells and total T cells to produce TNF, this study found a trend towards decreased TNF production by total T cells in cultures with 10 ng/mL TGF-β (*median =34.25%*) when compared to cultures with 0.1 ng/mL (*median =47.35%*), 1 ng/mL (*median =46.95%*) or no TGF-β (*median =43.40%*), and a similar response was observed in total T cells (**Figure 5.10D**). These results suggest that there might be an optimal titre of TGF-β to enhance inflammatory responses, whereas large concentrations of the cytokine likely inhibit these responses. This study is preliminary and increased sample sizes are now required along with additional assays to test whether TGF-β regulates other responses such as antigen independent activation, proliferation and cytotoxicity in MAIT cells.

5.2.11. Longitudinal analysis of circulatory MAIT cells in LCH patients

Since MAIT cells have an established immunoregulatory ability and are reduced in the circulation of LCH patients, this study retrospectively tracked MAIT cell proportions in LCH patients at different stages of disease and treatment. The relative frequency of circulatory MAIT cells was analysed following lesion excision and during treatment in 2 patients for a period of 20 or more months (**Figure 5.11**). For patient one the MAIT cell proportion and total lymphocyte count fluctuated only slightly whilst the patient had active disease and was receiving chemotherapy (vinblastine or methotrexate) and prednisolone for the duration of the period. For patient two, the total lymphocyte count also fluctuated slightly while the MAIT cell proportion increased during treatment with vinblastine and prednisolone, and decreased at cessation of treatment and during the period of non-active disease. This patient's MAIT cell proportion increased again at approximately the time of disease reactivation and treatment recommencement. Whether these changes in MAIT cell proportion are disease specific or treatment specific is uncertain, nevertheless this is an indication that MAIT cell frequency could potentially be associated with LCH disease activity.

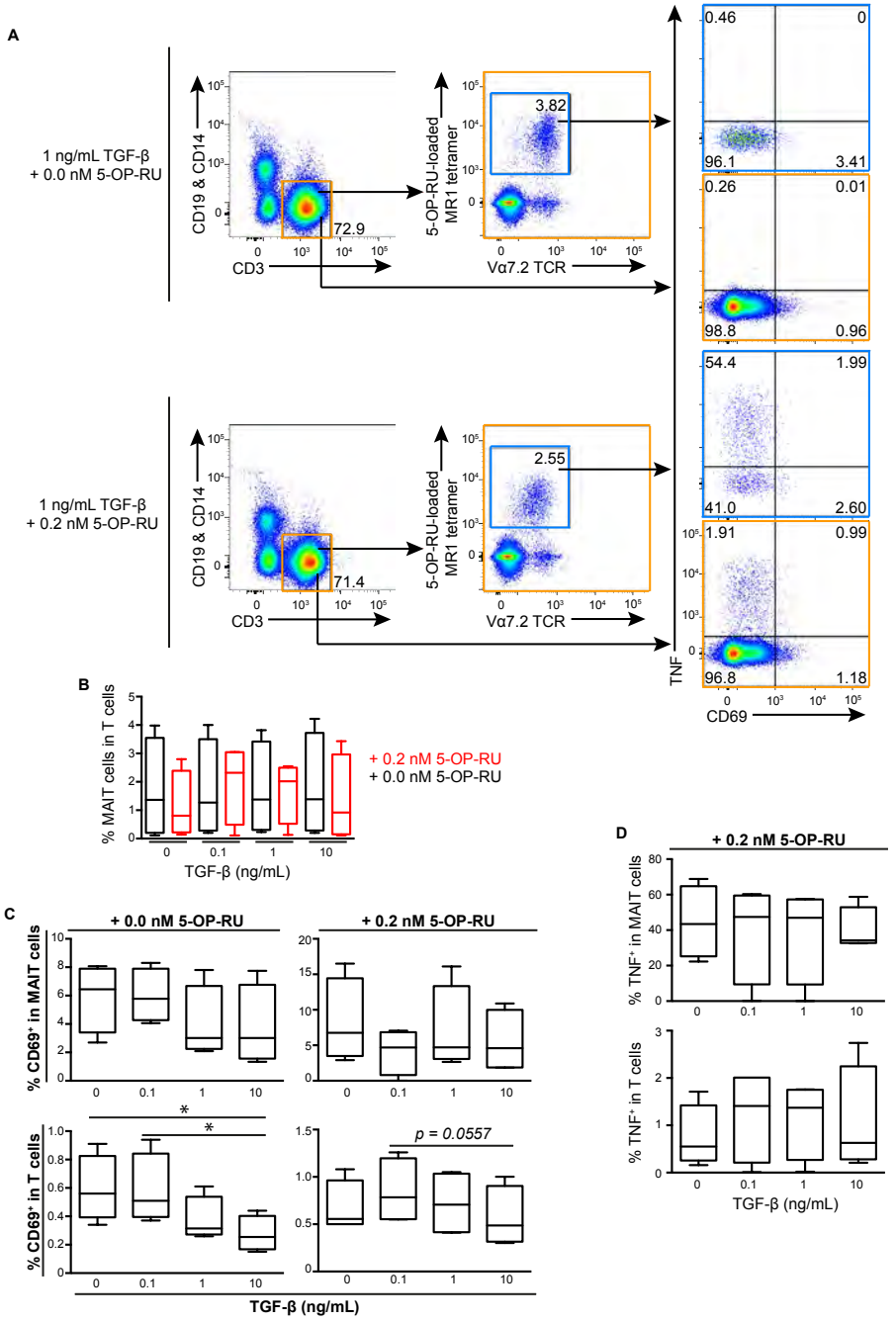


Figure 5.10. The effect of TGF-β on MAIT cell stimulation.

*(**A**) Flow cytometry plots demonstrate gating strategy for identifying TNF and CD69 expression in total T cells (orange) and MAIT cells (blue) from healthy donor derived PBMCs following 5-OP-RU stimulation in the presence of TGF-β. Data are representative of four individual donors. (**B**) Graph shows the proportion of MAIT cells in total T cells from PBMCs in unstimulated control (black) and 5-OP-RU stimulated (red) cultures in the presence of varying concentrations of TGF-β (n =4) (**C**) Graphs show proportions of CD69^{+} T cells in MAIT cells (top) and in total T cells (bottom) from PBMCs in unstimulated control (left) and 5-OP-RU stimulated (right) cultures in the presence of varying concentrations of TGF-β (n =4). (**D**) Graphs show proportions of TNF^{+} T cells in MAIT cells (top) and in total T cells (bottom) from PBMCs in 5-OP-RU stimulated cultures in the presence of varying concentrations of TGF-β (n =4). Friedman tests with Dunn's multiple comparisons were conducted for (**B**), (**C**) and (**D**). Box and whiskers plots indicate interquartile range and range with median, *p <0.05.*

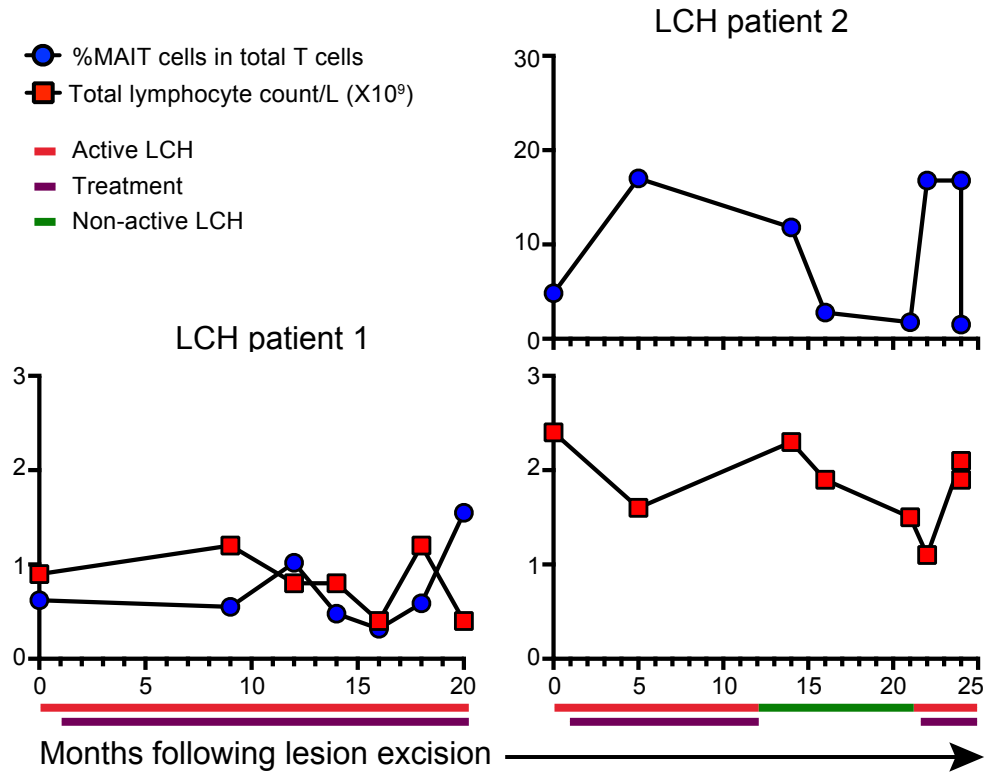


Figure 5.11. Relative frequency of circulatory MAIT cells over time.

Graphs show the proportion of MAIT cells in total T cells and the corresponding total lymphocyte counts over a period of months following lesion excision and during chemotherapy. Patient one received chemotherapy (vinblastine or methotrexate) and prednisolone for the duration of the period. Patient two received vinblastine and prednisolone where indicated.

5.3. Discussion

In this study, the frequency, phenotype, and functional potential of key unconventional T cell lineages derived from LCH patients were investigated. MAIT cells were reduced in proportion in the blood from patients with LCH and the lesional proportions were consistent with that seen in the blood from patients. Interestingly, the proportion of circulating MAIT cells was similar in patients with active and non-active disease, which suggests that a MAIT cell deficiency may precede the onset of LCH and could be a potential predisposing factor. Of note, longitudinal analysis of one patient revealed a substantial rise in the relative MAIT cell frequency after treatment and a subsequent fall when treatment ceased and the disease became inactive. Fluctuations in MAIT cell proportion, which were remarkably at 17% of total T cells at 3 separate time points, will be interesting to pursue in a cohort of patients. Albeit in a

single patient, the changes are consistent with the hypothesis that increasing MAIT cell frequency could be beneficial in the resolution of LCH. It is vital to emphasise that this result is a preliminary observation. When investigating γδ T cells, this study did not observe an increase such as that reported by Alaibac and Chu[101], although an important difference in the current study was that lesions from various organs were assayed, whereas the earlier study was limited to cutaneous lesions.

Unconventional T cells have functionally distinct subsets and it is noteworthy that there were changes in the subset proportions in γδ T cells and NKT cells as well as in the relative frequency of MAIT cells. Most γδ T cells do not express CD4 and CD8, but unexpectedly there was a significant increase in the proportion of lesional $CD4^+$ cells in γδ T cells. The functional significance of this is still under investigation, but an increase in the proportion of $CD4^+V\alpha7.2\ TCR^+CD161^+$ T cells was also detected in LCH patients. As with γδ T cells, the $CD4^+\ V\alpha7.2\ TCR^+CD161^+$ T cells are usually a minor subset, but their expansion in LCH parallels a similar observation reported for patients with colorectal cancer[113]. It is important to note that this increase in $CD4^+V\alpha7.2\ TCR^+CD161^+$ T cells may be due to the overall reduction in MAIT cells, therefore further tetramer based studies are required to determine the implication of this finding.

The frequency of circulatory NKT cells in healthy donors usually ranges from 0.01-0.1% of total T cells, but is rarely >1%[218]. Surprisingly, NKT cell frequency in patients with LCH was more variable and even extended to a patient where NKT cells comprised almost 9% of circulatory T cells. A previous case study of an individual with 5% NKT cells was considered exceptional[218]. The findings presented in the current study could potentially be a statistical anomaly, although it is noteworthy that the patient in this study had inactive disease at the time of blood collection following a spontaneous resolution without treatment. Because NKT cells are immunoregulatory in nature, it is conceivable that the high systemic NKT cell frequency was a factor in this remission, although further studies are required to elucidate this. In striking contrast were patients with LCH that had undetectable NKT cells. This too is unusual in healthy individuals and could have a large impact on immune regulation and potentially the progression of LCH.

This study investigated whether the frequency abnormalities of unconventional T cell subsets extended to the functional capacity of these cells, as this has previously been reported for many patient groups with cancer[113,117]. Unconventional T cells from patients with LCH retained the ability to produce Th1 cytokines when challenged. The rarity of NKT cells meant it was technically challenging to analyse subsets, especially those from LCH lesions. This study therefore focussed on assaying the overall cytokine responses of NKT cells because this is the primary mechanism they use to regulate other immune cells and dysfunctions in NKT cell cytokine production has previously been associated with several forms of cancer and autoimmune disease[117]. The present study established that NKT cells from the blood from LCH patients were functionally competent, however the subset distributions of circulating NKT cells from patients with active LCH were abnormal and distinct from controls.

High levels of IL-17A have been reported in the blood from patients with LCH[219] and more controversially[65,181] in lesions[182]. **Chapter 4** demonstrated that LCH patient derived T cells produced negligible IL-17A, indicating that T cells in LCH do not respond with rapid IL-17A release, although we cannot rule out that an *in vivo* stimulus could cause IL-17A production. From these results we can deduce that the MAIT cells in LCH patients have not likely switched to a Th17 response as seen in colorectal cancer[113], although we cannot rule a Th17 response out altogether because of the detection of IL-17F across a variety of T cell subsets (**Chapter 4**).

The pilot study on the potential effects of TGF-β on MAIT cell activation is a promising avenue of investigation. TGF-β, which is found within the blood and lesions from LCH patients could be responsible for reducing the expression of CD69 by MAIT cells and also by T cells collectively without 5-OP-RU stimulation. Since the addition of GolgiPlug is known to inhibit CD69 from trafficking to the cell surface[217], further investigations that include intracellular labelling for CD69 are needed to assess whether CD69 expression is altered in 5-OP-RU stimulated cultures. This study identified trends that suggest differing concentrations of TGF-β may have contrasting effects on TNF production by MAIT cells. These studies should be continued in a larger cohort to determine whether the trends observed in this study become significant.

It will be important to determine the source of the immune abnormalities identified in this study and in particular, whether they precede LCH, or are caused by it. An important question is to determine the outcome(s) of interactions between LCH cells and unconventional T cells. For example, activated MAIT cells can express CD40 ligand[136], which is known to mature LCH cells *in vitro* and increase their allostimulatory capacity[85]. While there is no CD40 ligand defect in LCH[89] it is conceivable that MAIT cells might be more efficient at CD40 binding than the T cells present in lesions, which could in turn lead to maturation of LCH cells and a subsequent reduction in $Foxp3^+$ Tregs in lesions.

The results presented here show for the first time that the frequency of unconventional T cells, especially MAIT cells, is abnormal in patients with LCH. MAIT cell frequencies were similar in the periphery of patients with active and non-active disease, although a rise in proportion was observed upon treatment in one patient that suggests MAIT cells may be associated with LCH onset/progression, however further studies are required to confirm this. Changes in unconventional T cell subset distributions and frequency in LCH patients are consistent with the established role of unconventional T cells in immune dysregulation. It will be especially informative to determine the direct roles of these cells in LCH and whether the abnormalities identified in this study are a cause or consequence of the disease. The analyses conducted in this study strengthen earlier reports of immune dysfunction in LCH by showing the abnormalities extend beyond $Foxp3^+$ Tregs to unconventional T cell lineages that could be equally important factors in LCH. Their distinctive functions and the development of specific agonists make unconventional T cells promising candidates as biomarkers or targets of immune based therapies, but a more detailed analysis is now required for translation to clinical applications.

6.0. THE IMPACT OF CD1A EXPRESSION IN LCH

6.1. Introduction

CD1 molecules differ to classical MHC molecules by displaying a large hydrophobic groove[220] that can house and present lipids and glycolipids to T cells[221] rather than the typical MHC presentation of peptides to T cells (**Figure 6.1**). The CD1 groove consists of two large pockets, namely the A' pocket and the F' pocket, which can accommodate lipids with either short fatty acid tails combined with spacer lipids, or lipids with longer fatty acid tails[222-224] (**Figure 6.1**). CD1 complexes do have some structural similarities to MHC I, for example both CD1 and MHC I molecules are associated with β2 microglobulin (**Figure 6.1**). CD1 molecules may also share functional features with MHC II, as the invariant chain chaperone (CD74) that is involved in assembly and trafficking of MHC II is potentially associated with CD1 molecules and their trafficking[225].

CD1a is a hallmark of LCH, is expressed on the surface of LCH cells and is one of the diagnostic indicators for this disease[29]. The role of CD1a in LCH is unknown, but given its ubiquity, it could be an important factor in LCH pathogenesis. CD1a can be expressed by DCs under normal conditions and it is commonly expressed on the membrane of skin-resident LCs. CD1a is also expressed by thymocytes, however this is only at immature stages of T cell development. Interestingly, along with CD1a expression by LCH cells in lesions, CD1a expression on the surface of mature T cells in lesions was reported[31]. Expression of CD1a by T cells outside of the thymus is rare, although cases have been reported where CD1a was expressed by immature T cells in cancers[95,96] and by extrathymic T cells in normal tonsil tissue[97]. Similarly, human skin-derived group 2 innate lymphoid cells were also reported to express CD1a[94]. The functional significance of CD1a expression on the surface of T cells outside of developmental stages is not established, but it may have consequences for the induction or progression of LCH. Another CD1 molecule, CD1d, is known to be upregulated by activated T cells[151] and it is possible that CD1a could similarly be expressed by T cells upon activation.

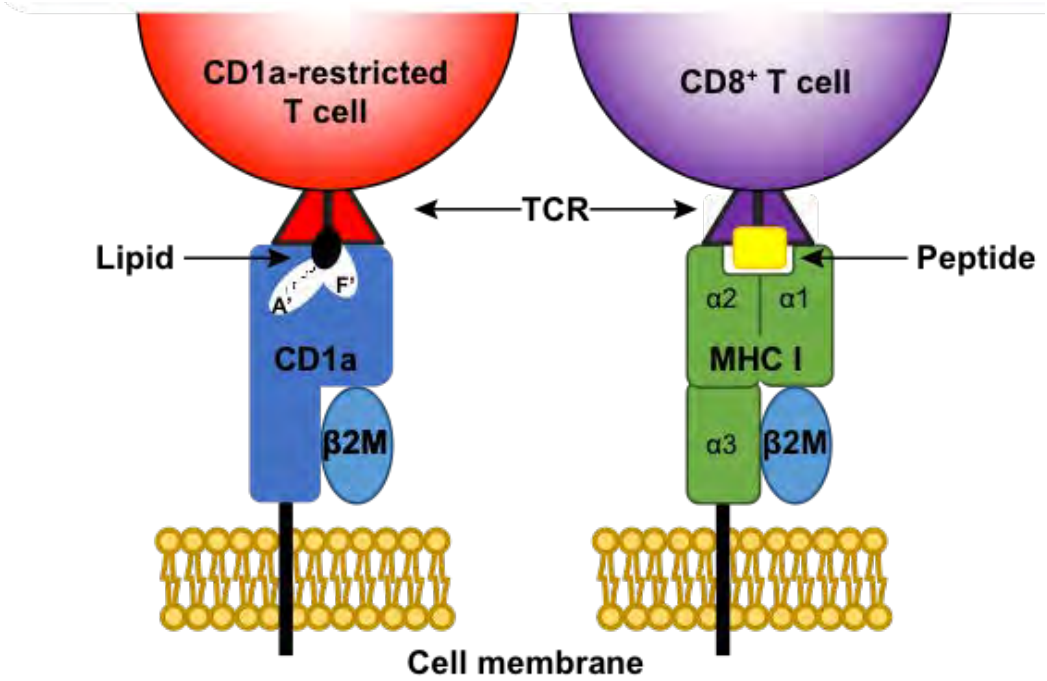


Figure 6.1. Antigen presentation by CD1a and MHC I.

Both CD1a and MHC I are membrane bound complexes that are able to present antigens to T cells via the TCR. Both complexes associate with β2 microglobulin (β2M). MHC I presents peptide antigens to CD8⁺ T cells, whereas CD1a complexes can house lipids in both the A' pocket and F' pocket of their large hydrophobic grove and present these lipids to CD1a-restricted T cells.

Indeed, there are many studies that suggest varied conditions affect CD1a expression in APCs[226-228], though information regarding the regulation of CD1a expression by T cells is lacking. CD1a can be upregulated by monocytes in the presence of stimuli such as phospholipids (phosphatidylcholine) or lectins (PHA)[226]. Furthermore, polycyclic aromatic hydrocarbons, which are environmental pollutants, were shown to inhibit CD1a expression in monocytes in the presence of the DC differentiation cytokines, GM-CSF and IL-4, but did not alter CD1a expression on already mature DCs[227]. Polycyclic aromatic hydrocarbons also impaired monocyte-derived DC maturation and consequently these cells poorly stimulated T cells in mixed leukocyte reactions[227]. This impairment is similar to that observed in LCH cells, although in the study by Geissmann et al.[85] this was corrected by the addition of CD40 ligand. It was not specified what effect this had on CD1a expression levels[85]. Culturing monocytes in the presence of human serum rather than FBS, along with DC differentiating cytokines, also inhibits the expression of CD1a along with other group 1 CD1 molecules, CD1b and CD1c, but

not CD1d[228]. Interestingly, CD1a is also known to be upregulated in lower than optimal temperature conditions (34° C) by stably transfected HeLa cells[229]. Given the abundance of information on factors that can impact the regulation of CD1a, the current investigation aimed to explore the dynamics of CD1 expression by T cells following activation under a range of cell culture conditions.

CD1a expression by both T cells and LCH cells in lesions is likely important because this complex can bind a variety of lipids, some of which can prime T cells for specific immune responses[150]. CD1a can bind the mycobacterial lipopeptide antigen, dideoxymycobactin (DDM)[147], and it can also bind with several naturally occurring endogenous lipid ligands such as squalene, wax esters and triacylglycerides commonly found in skin oil[150]. Additionally, CD1a is known to bind sphingomyelin, which is found in cell membranes and myelin sheath[197]. A study by de Jong et al.[150] identified CD1a-autoreactive T cells within the skin, suggesting a role for CD1a-restricted T cells in skin immunity. These cells were also detected in the blood, and their circulatory presence suggests that CD1a-restricted T cells may be present in other host organ tissues too[150]. Kasmar et al.[147] found CD1a-DDM tetramer$^+$ T cells in peripheral blood from tuberculosis patients and showed that DDM is capable of binding to a recombinant TCR following incubation with CD1a[147]. Additionally, the self-lipid galactosylceramide 3-sulfate (sulfatide) is able to stimulate multiple categories of CD1-restricted T cells including CD1a-restricted T cells[230]. Phosphatidylcholine can activate CD1a-restricted T cells[231] and T cells were also reported to recognise CD1a-bound lysophosphatidylcholine (LPC)[197], both of which can be microbial- or self-derived. Other studies demonstrated that bee-venom derived phospholipase can generate small neo-antigens, for example free fatty acids and lysophospholipids from common phosphodiacylglycerides, which can in turn activate T cells via CD1a[194]. Additionally, plant-derived urushiol was shown to trigger CD1a-dependent skin inflammation by CD4$^+$ Th cells[195]. There are perhaps still many unknown endogenous lipid ligands and exogenous lipid antigens that can bind with CD1a, of which some may be activating or inhibitory towards T cells.

Upon recognition of ligands and antigens presented by CD1a molecules, CD1a-restricted T cells have been shown to produce TNF, IFN-γ and IL-22, indicating that they might bias the immune response towards a Th1 or Th22 response[145-147]. In addition to this, studies have shown that TCRs can directly bind CD1a when permissive lipids such as phosphatidylcholine and other endogenous ligands form a complex with CD1a but do not protrude from the groove[197], which suggests that TCRs are able to directly recognise CD1a molecules. This implies that in addition to the type of lipid within the environment, the expression levels of cell surface CD1a in a particular environment may be an important factor in immunogenicity irrespective of the specific lipids being housed. It is possible that CD1a-restricted T cells are capable of additional immune responses but our knowledge of their functions is currently limited. Group 1 CD1-restricted T cells collectively, which includes CD1a-restricted T cells, have been suggested as prime candidates of immunotherapeutic potential by experts in the field[232,233] due to primary studies demonstrating their recognition of tumour derived lipids and cytotoxicity towards tumour cells[234].

It is estimated that there are as many CD1a-restricted T cells in humans as there are MR1-restricted MAIT cells[108], although they are not currently as technically identifiable as MAIT cells because they have a more diverse TCR repertoire. It is unknown whether CD1a-restricted T cells are present in LCH lesions, but given the high levels of CD1a expressed by LCH cells it is possible that there could be direct interactions between LCH cells and CD1a-restricted T cells, and indeed there are already established interactions between LCH cells and T cells within lesions (**Figure 6.2**). If CD1a-restricted T cells are activated by LCH cells they might proliferate within lesions and/or display an activated phenotype. We could even expect an alteration to the frequency found in the peripheral blood from LCH patients. If CD1a-restricted T cells are present within LCH lesions, this may be a result of CD1a expression by LCH cells, therefore investigating CD1a-restricted T cells could be essential to our understanding of LCH. If CD1a-restricted T cells observed in lesions from LCH patients are increased in frequency or display signs of activation, this could suggest antigenicity within lesions.

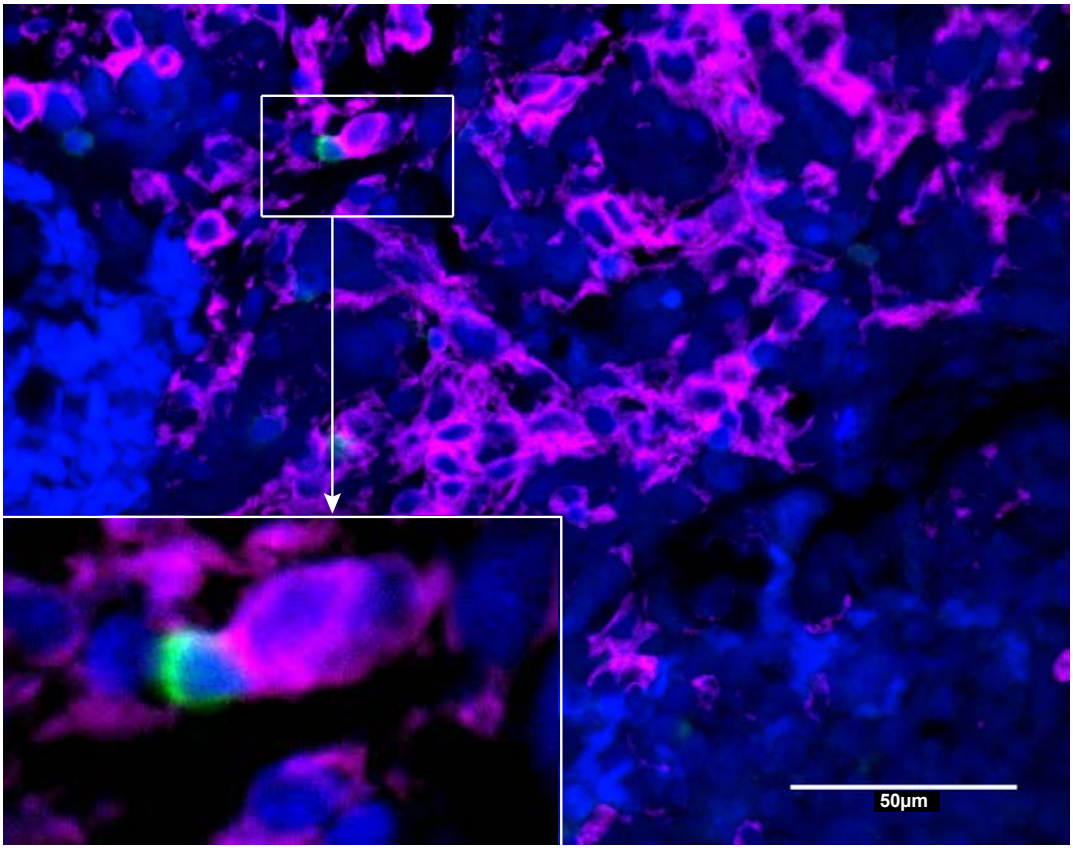


Figure 6.2. LCH cells and T cells are in close contact in LCH lesions.

An immunofluorescent stained formalin-fixed paraffin-embedded tissue section of an LCH lesion demonstrating contact between a CD1a^{+} LCH cell (magenta), and a CD3^{+} T cell (green).

6.2. Results

6.2.1. CD1a expression by T cells from LCH lesions

CD1a expression by T cells from LCH lesions was observed in a previous study by West et al.[31], therefore the current study initially set out to confirm these results. CD1a^{+} T cells were identified in LCH lesions (**Figure 6.3A**) and the median proportion of T cells that expressed CD1a was significantly (*p =0.0003*) increased in T cells from LCH lesions (*median =0.16%*) compared to those from the peripheral blood from healthy donors (*median =0.007%*) (**Figure 6.3B**).

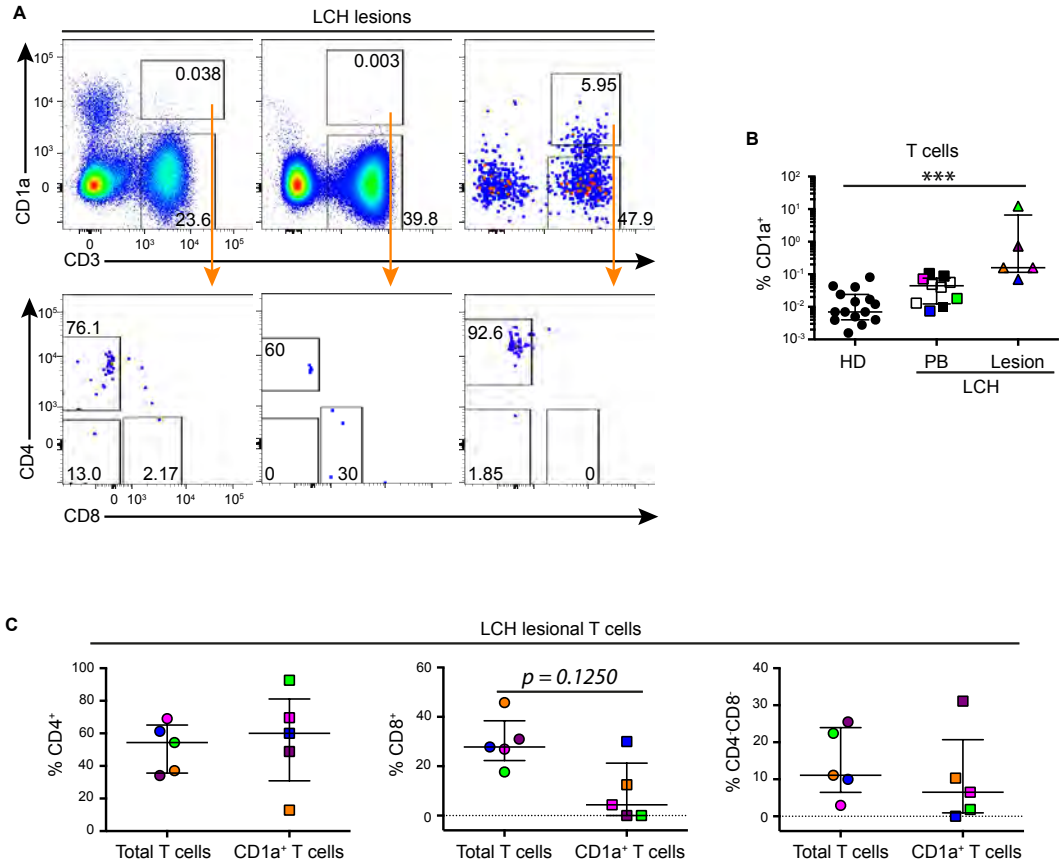


Figure 6.3. CD1a expression by T cells from LCH lesions.

*(**A**) Flow cytometry gating strategy for identifying CD1a$^+$ T cells (top row) and CD4 and CD8 subsets thereof from LCH lesions (bottom row). (**B**) Proportions of CD1a$^+$ T cells in total circulating T cells from healthy donors and from blood and lesions from LCH patients. Open/coloured squares indicate patients with active disease and closed/black squares indicate patients with non-active disease. (**C**) Proportions of CD4$^+$ T cells (left), CD8$^+$ T cells (centre) and CD4$^-$CD8$^-$ T cells (right) in the total T cell population and in matched CD1a$^+$ T cells from LCH lesions. A Kruskal-Wallis test with Dunn's multiple comparisons was conducted for (**B**) and two-tailed Wilcoxon matched-pairs signed rank tests were conducted for (**C**). ***p <0.001, error bars indicate median + interquartile range. Lesions and matched peripheral blood samples are colour coded on graphs.* ***HD =healthy donor, PB =peripheral blood.***

6.2.1.1. Subsets of CD1a$^+$ T cells in LCH lesions

Similar to the previous study by West et al.[31] this study investigated the CD4 and CD8 subset distribution of CD1a$^+$ T cells (**Figure 6.3A**) and found that CD1a$^+$ T cells were mostly CD4$^+$ (*median =60%*) in four of five specimens analysed. There were no significant differences in the proportions of CD4$^+$, CD8$^+$ or DN cells in the CD1a$^+$ T cell population when compared to the corresponding subsets in the total lesional T cell population, though there was a trend towards reduced CD8$^+$ cells in the CD1a$^+$ T cell population (*median =4.35%*) compared with the total T cell population (*median =27.80%*). The expression of CD4 by CD1a$^+$ T cells was highly variable (*range =12.90-92.60%*) when compared with the total T cell population from the same patients (*range =34.1-69.0%*) (**Figure 6.3C**).

6.2.1.2. CD1a expression by purified, stimulated T cells from LCH lesions

CD1a$^+$ T cells in LCH lesions might be an important factor in the development and/or progression of LCH, however the role of these cells is currently unknown. This study therefore investigated how CD1a$^+$ T cells respond to stimuli in their ability to produce cytokines. Purified lesional T cells from three individuals were stimulated with PMA and ionomycin (for methods see **Section 3.11.3**) and following this a small population of T cells expressing CD1a (*median =0.25%*) was identified (**Figure 6.4A**). The expression of CD4 and CD8 T cell co-receptors by CD1a$^+$ T cells was assessed again following stimulation (**Figure 6.4B**). Only two of three specimens analysed had sufficient numbers of CD1a$^+$ T cells to investigate further T cell subsets, however CD4 and CD8 expression appeared to change following stimulation and compared to matched total T cells (**Figure 6.4B**). There were more CD8$^+$ T cells in the lesions analysed in this assay, contrasting the findings of larger proportions of CD4$^+$CD8$^-$ expression for the unchallenged populations shown previously (**Figure 6.3 A** and **C**). Very few cells appeared to lack a co-receptor following stimulation, although differentiating between CD4$^+$ and DN populations following stimulation was difficult (**Figure 6.4B**) and this is a common problem due to downregulation of the CD4 co-receptor following stimulation[235]. Interestingly though, the CD1a$^+$ T cells from one lesional specimen displayed co-expression of CD4 and CD8 after stimulation (**Figure 6.4B**).

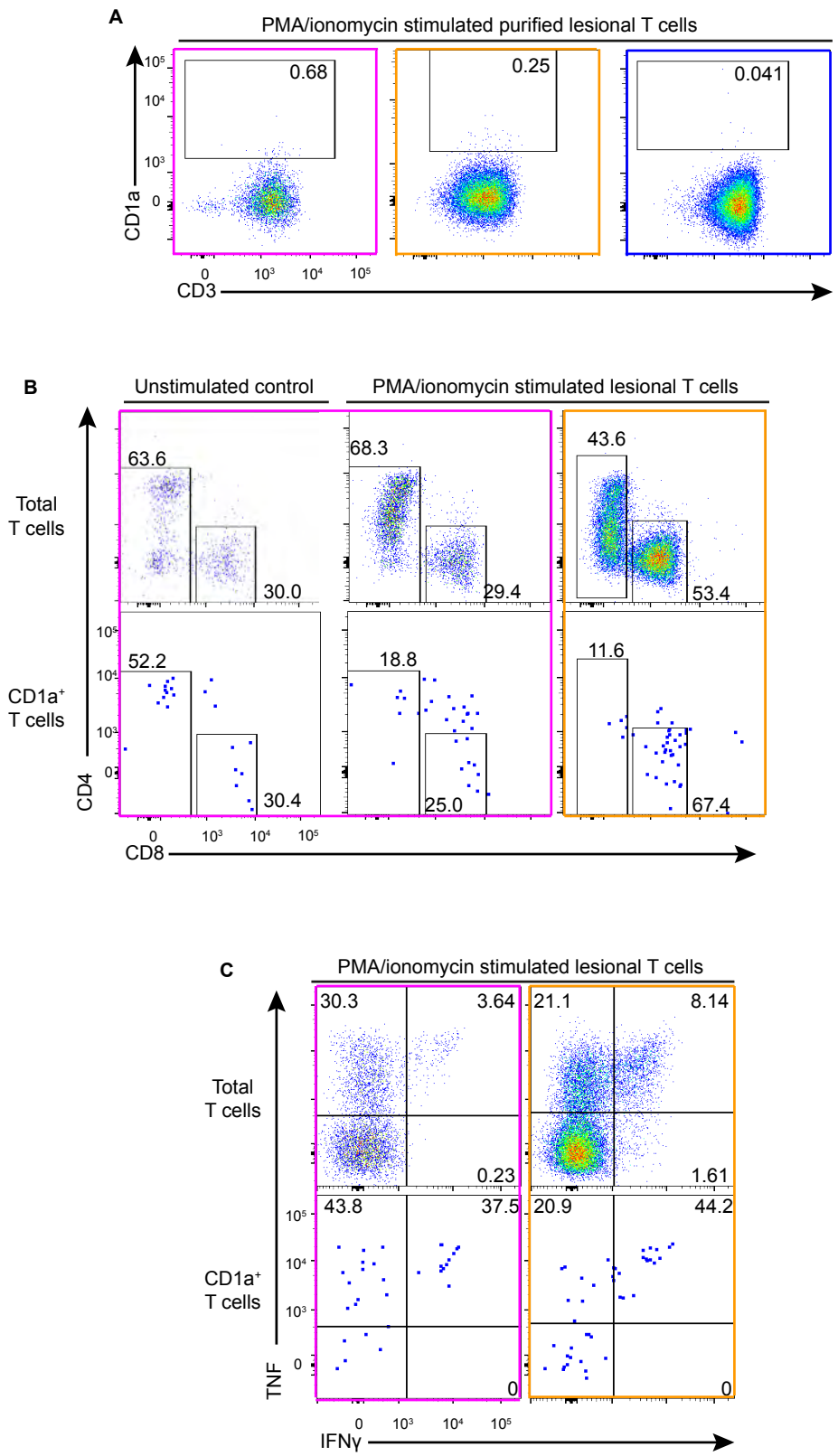


Figure 6.4. CD1a expression by purified, stimulated T cells from LCH lesions.

*(**A**) Post-stimulation analysis of CD1a expression by purified lesional T cells from LCH patients. (**B**) Post-stimulation analysis of CD4 and CD8 expression by total T cells and CD1a$^+$ T cells derived from (**A**). (**C**) Post-stimulation analysis of TNF and IFN-γ production by total T cells and CD1a$^+$ T cells derived from (**A**). Patient samples are colour coded to match graphs.*

We investigated TNF and IFN-γ production (**Figure 6.4C**) by the stimulated CD1a$^+$ T cells in the same lesional specimens and found that CD1a$^+$ T cells can produce these cytokines when challenged. As previously mentioned in **Chapter 4**, the production of IL-4 and IL-17A in total T cells from LCH lesions was negligible.

6.2.1.3. Purifying CD1a$^+$ T cells from LCH lesions

The results presented here confirm a population of CD1a$^+$ T cells found in LCH lesions. The population is small in frequency and somewhat difficult to clearly distinguish from the total T cell population, therefore it is best to study the population in isolation where possible. Experiments were thus designed to purify CD1a$^+$ T cells from LCH lesions for downstream gene expression studies to confirm *CD1A* expression and for further phenotypical and functional characterisation of the population. Lesional LCH tissue samples from five patients were labelled with antibodies directed towards CD1a and CD3 to isolate this population by FACS, however the post-FACS analysis of this population demonstrated that the population was not sufficiently pure to conduct downstream studies (**Table 6.1** and **Figure 6.5**). Importantly, alongside the attempted purification of CD1a$^+$ T cells, other immune cell populations were successfully isolated based on their expression of CD1a (LCH cells) (**Figure 4.23**) or CD3 along with other identifiers (Tregs and T responder cells) (**Figure 4.14**), therefore the antibodies used to identify CD1a$^+$ T cells were controlled for within the experiments.

Table 6.1. Results from FACS of CD1a$^+$ T cells.

LCH lesion/patient no.	Sort purity (%)	Yield
1	15.4	<375*
2	0.0	<150*
13	*NA*	<1*
14	0.0	<7*
18	*NA*	0*

**No cell count - estimate from FACS report only*

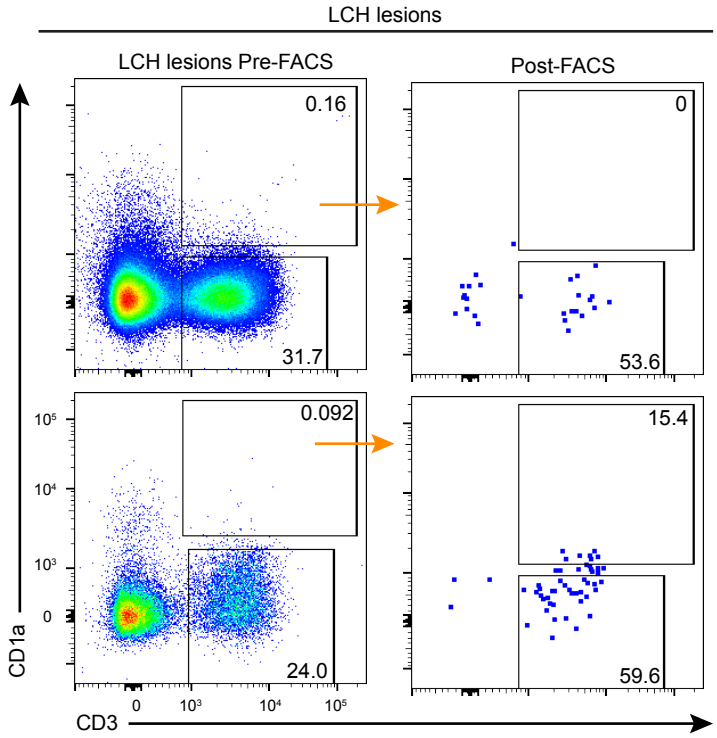


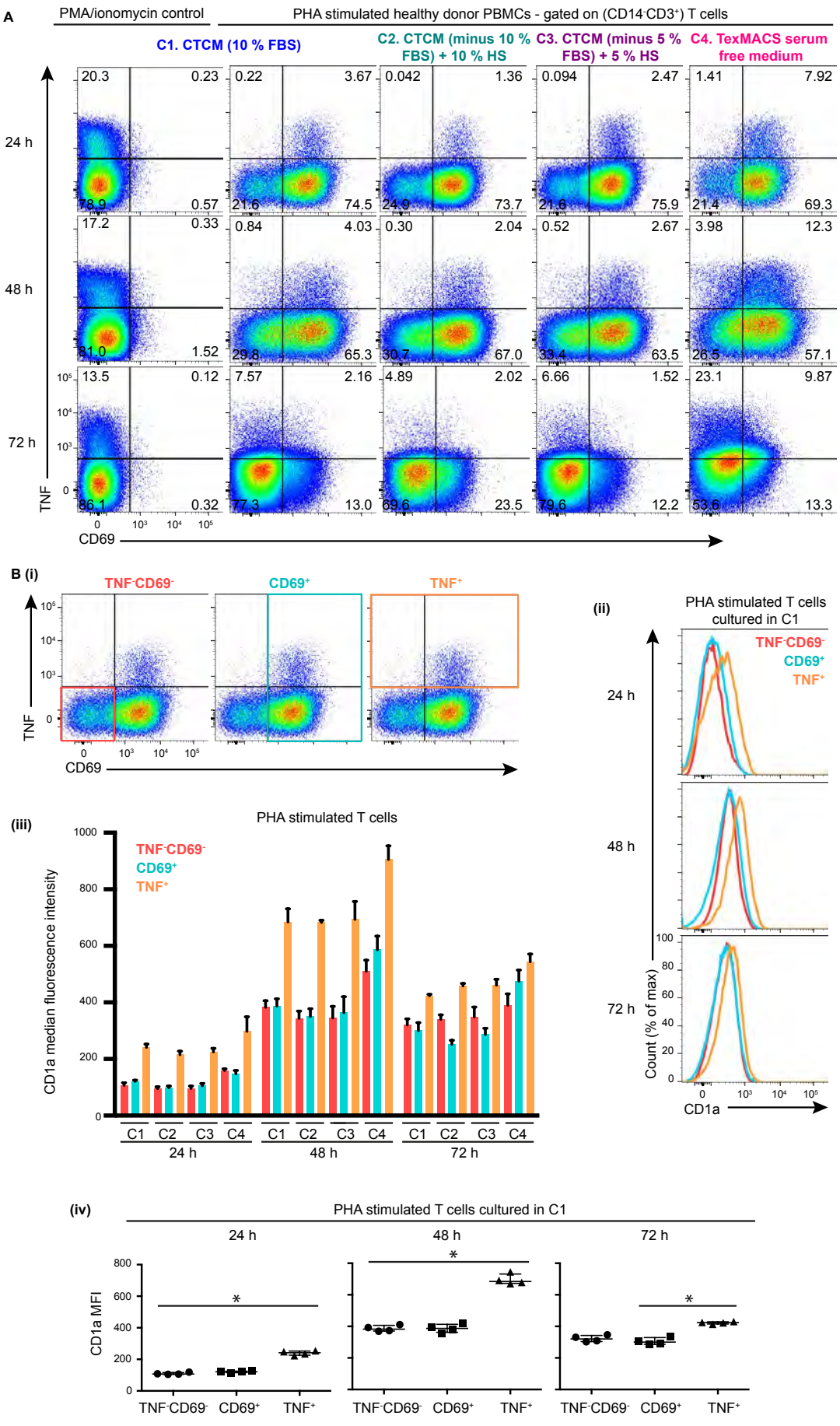
Figure 6.5. Purifying CD1a$^+$ T cells from LCH lesions.

Flow cytometry gating strategy for identifying and isolating CD1a$^+$ T cells (left) and post-FACS analysis (right).

6.2.3. The dynamics of CD1 expression by T cells following stimulation

Given the differences in the frequency of LCH lesion CD1a$^+$ T cells identified in this study compared with the previous study[31] and our knowledge that CD1a expression on cells other than T cells can vary under different temperature[229] and serum culture conditions[228], it is likely that CD1a expression by T cells is also intricately regulated. This study thus investigated the impact of culture conditions on T cell CD1a expression.

Healthy donor derived PBMCs were stimulated with PHA-L for a period of 72 hours under different serum culture conditions and T cell surface expression of CD1a, CD1b, CD1c and CD1d was assessed every 24 hours. Cells were cultured in media in the presence of FBS (C1), human serum (C2), FBS and human serum (C3), or in serum-free media (C4) (for complete methods see **Section 3.11.7**). Results demonstrate that T cells within the cultures were activated at each time point as determined by expression of activation marker CD69 and by TNF production (**Figure 6.6A**).


A
PMA/ionomycin control
PHA stimulated healthy donor PBMCs - gated on (CD14-CD3+) T cells
C1. CTCM (10 % FBS)
C2. CTCM (minus 10 % FBS) + 10 % HS
C3. CTCM (minus 5 % FBS) + 5 % HS
C4. TexMACS serum free medium
24 h
48 h
72 h
TNF
CD69
20.3 0.23 78.9 0.57
0.22 3.67 21.6 74.5
0.042 1.36 24.0 73.7
0.094 2.47 21.6 75.9
1.41 7.92 21.4 69.3
17.2 0.33 81.0 1.52
0.84 4.03 29.8 65.3
0.30 2.04 30.7 67.0
0.52 2.67 33.4 63.5
3.98 12.3 26.5 57.1
13.5 0.12 86.1 0.32
7.57 2.16 77.3 13.0
4.89 2.02 69.6 23.5
6.66 1.52 79.6 12.2
23.1 9.87 53.6 13.3
B (i)
TNF-CD69-
CD69+
TNF+
(ii)
PHA stimulated T cells cultured in C1
Count (% of max)
CD1a
(iii)
PHA stimulated T cells
CD1a median fluorescence intensity
C1 C2 C3 C4
24 h
48 h
72 h
(iv)
PHA stimulated T cells cultured in C1
CD1a MFI

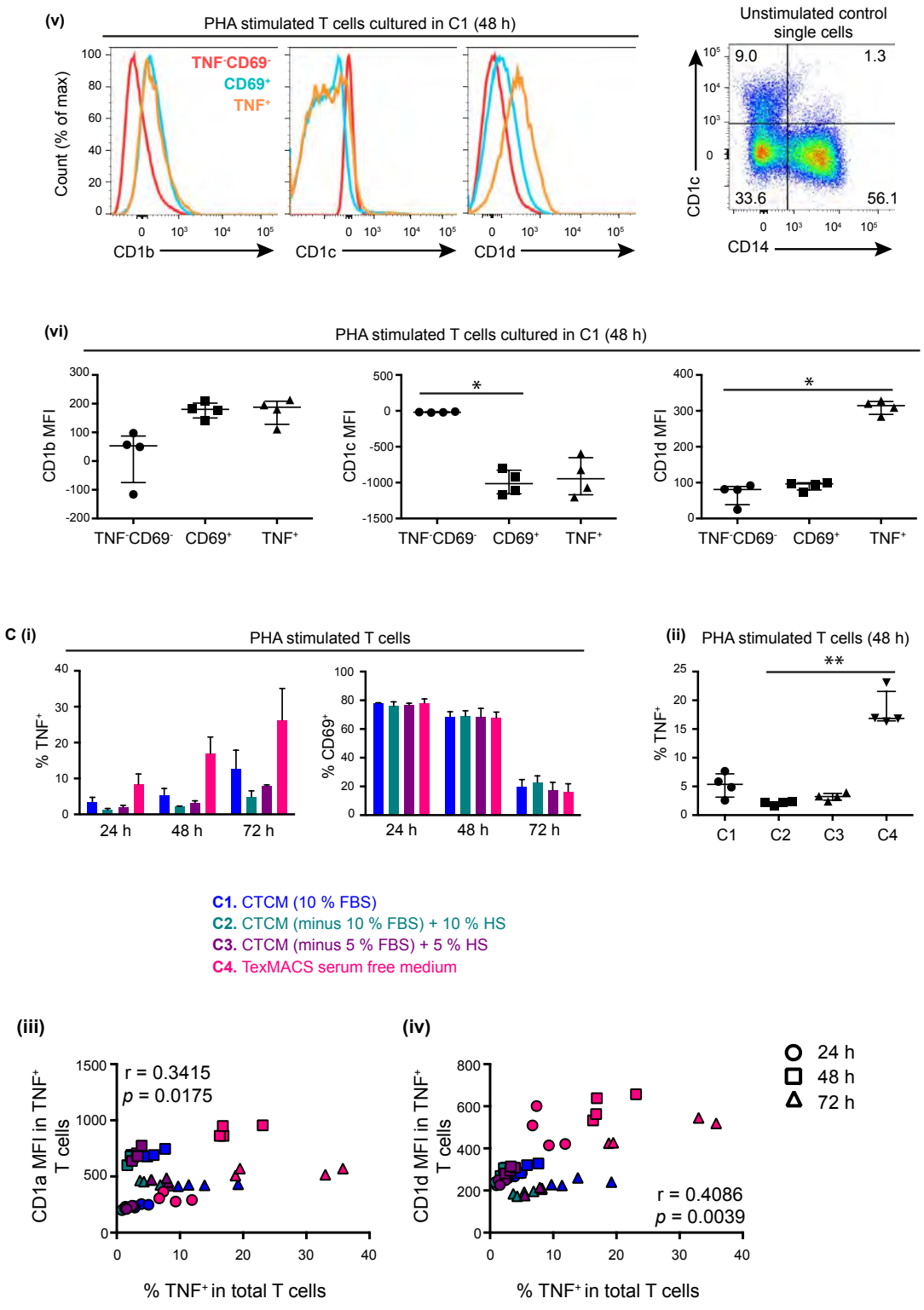


Figure 6.6. The dynamics of CD1 expression by T cells following PHA stimulation.

*(**A**) Representative flow cytometry plots from one individual displaying expression of activation markers by T cells within PHA-stimulated cultures of healthy donor derived PBMCs. PBMCs were cultured in media under a variety of serum conditions (C1, C2, C3 and C4; left to right)*

*and harvested for analysis at 24, 48 and 72 hours post-stimulation (top to bottom). Data are representative of four individual donors. (**B**) (**i**) Gating strategy for identifying TNF$^-$CD69$^-$ T cells (left), CD69$^+$(TNF$^{-/+}$) T cells (centre) and TNF$^+$(CD69$^{-/+}$) T cells (right) following PHA stimulation. (**ii**) Representative histograms from one individual display expression levels of CD1a on TNF$^-$CD69$^-$ T cells, CD69$^+$ T cells and TNF$^+$ T cells at 24 (top), 48 (middle), and 72 (bottom) hours post-stimulation under culture condition C1. Data are representative of four individual donors. (**iii**) Median fluorescence intensity (MFI) for CD1a on TNF$^-$CD69$^-$ T cells, CD69$^+$ T cells and TNF$^+$ T cells under culture conditions C1, C2, C3 and C4 at 24, 48 and 72 hours post-stimulation (n =4). (**iv**) MFI for CD1a on TNF$^-$CD69$^-$ T cells, CD69$^+$ T cells and TNF$^+$ T cells under culture condition C1 at 24, 48 and 72 hours post-stimulation. (**v**) Representative histograms from one individual display expression levels of (left to right) CD1b, CD1c and CD1d on TNF$^-$CD69$^-$ T cells, CD69$^+$ T cells and TNF$^+$ T cells at 48 hours post-stimulation under culture condition C1 and (far right) positive control for anti-CD1c antibody. (**vi**) MFI for CD1b (left), CD1c (centre) and CD1d (right) on TNF$^-$CD69$^-$ T cells, CD69$^+$ T cells and TNF$^+$ T cells under culture condition C1 at 48 hours post-stimulation. (**C**) (**i**) Percentage of TNF$^+$ cells (left) and CD69$^+$ cells (right) in total T cells under culture conditions C1, C2, C3 and C4 at 24, 48 and 72 hours post-stimulation (n =4). (**ii**) Percentage of TNF$^+$ cells in total T cells under culture conditions C1, C2, C3 and C4 at 48 hours post-stimulation. (**iii**) Correlation between the proportion of TNF$^+$ cells in total T cells and MFI for CD1a on TNF$^+$ T cells in PHA stimulated PBMCs. (**iv**) Correlation between the proportion of TNF$^+$ cells in total T cells and MFI for CD1d on TNF$^+$ T cells in PHA stimulated PBMCs. Friedman tests with Dunn's multiple comparisons were conducted for (**B**) (**iv**) and (**vi**) and (**C**) (**ii**). *p <0.05, **p <0.01, error bars indicate median + interquartile range. A Pearson's two-tailed correlation test was conducted for (**C**) (**iii**) and a Spearman's two-tailed non-parametric correlation test was conducted for (**C**) (**iv**) (95% confidence interval). **MFI =median fluorescence intensity, C1 – Complete tissue culture medium (CTCM; 10% FBS), C2 – CTCM (minus 5% FBS) + 5% human serum (HS), C3 – CTCM (minus 10% FBS) + 10% HS, C4 – TexMACS serum-free medium + 1x penicillin-streptomycin + 50 µM 2-mercaptoethanol.***

To investigate CD1a expression by activated T cells the MFI for CD1a was compared in three groups of cells by gating on (**1**) T cells negative for both activation markers (CD69 and TNF), (**2**) CD69$^+$ T cells and (**3**) TNF$^+$ T cells (**Figure 6.6B. i**). The MFI for CD1a was slightly higher in the TNF$^+$ T cell group compared to other groups at each time point (**Figure 6.6B. ii**) and this was true for each cell culture condition investigated (**Figure 6.6B. iii**). The MFI for CD1a was highest in T cells cultured in serum-free media when compared to T cells from serum supplemented cultures (**Figure 6.6B. iii**), which suggests that both human serum and FBS may partially inhibit CD1a expression by T cells. In the FBS supplemented culture (C1) the MFI for CD1a was significantly higher in TNF$^+$ T cells when compared to TNF$^-$CD69$^-$ T cells at 24 (*p =0.014, TNF$^+$ T cell MFI =241.5, TNF$^-$CD69$^-$ T cell MFI =107.0*) and 48 hours (*p =0.040, TNF$^+$ T cell MFI =684.5, TNF$^-$CD69$^-$ T cell MFI =382.5*) post-stimulation (**Figure 6.6B. iv**). At 72 hours post-stimulation the MFI for CD1a was significantly (*p =0.014*) higher in TNF$^+$ T cells (*MFI =423.5*) when compared to CD69$^+$ T cells (*MFI =300.5*) (**Figure 6.6B. iv**). Given the

uniformity of the MFI for CD1a between the TNF^-CD69^- (*24 hour MFI =107.0, 48 hour MFI =382.5, 72 hour MFI =320.0*) and the $CD69^+$ (*24 hour MFI =121.0, 48 hour MFI =387.0, 72 hour MFI =300.5*) T cell groups it is not likely that this detectable increase in fluorescence is due to blasting cells increasing in size.

Additionally, the expression of other CD1 molecules was investigated in TNF^-CD69^- T cells, $CD69^+$ T cells and TNF^+ T cells following PHA stimulation (**Figure 6.6B. v**) and at 48 hours post-stimulation there was a trend towards an increased MFI for CD1b in the $CD69^+$ (*MFI =180.0*) and TNF^+ (*MFI =187.5*) T cell groups when compared to TNF^-CD69^- T cells (*MFI =53.25*) (**Figure 6.6B. vi**). The TNF^-CD69^- T cell group had the highest fluorescence of CD1c (*MFI -18.5*) and this was significantly (*p =0.040*) greater than the $CD69^+$ T cell group (*MFI =-1015*), although all cell populations appeared mostly negative for expression of CD1c. Comparable to CD1a expression, at 48 hours post-stimulation there was a significant (*p =0.040*) increase in the MFI for CD1d in TNF^+ T cells (*MFI =314.15*) when compared to TNF^-CD69^- T cells (*MFI =80.95*) (**Figure 6.6B.vi**). These results indicate that CD1a and CD1d expression by T cells could be associated with TNF production.

Next the effect of different culture conditions was compared by measuring the proportions of TNF^+ T cells and $CD69^+$ T cells in total T cells for each group following stimulation (**Figure 6.6C. i**). Cells cultured in serum-free media had the highest proportion of TNF^+ T cells (*48 hour median: C4 =16.85%*) when compared to serum supplemented cell cultures (*48 hour median: C1 =5.36%, C2 =2.24%, C3 =3.25%*) (**Figure 6.6C. i** and **ii**), whilst the proportion of $CD69^+$ cells was consistent for each cell culture condition (**Figure 6.6C. i**). Given these results, this study set out to establish whether there is an association between CD1a expression by T cells and TNF production and identified a correlation between the frequency of TNF^+ T cells and the MFI for CD1a in that group (*r =0.3415, p =0.0175*) (**Figure 6.6C. iii**). This was also true for CD1d with a correlation between the frequency of TNF^+ T cells and the MFI for CD1d in the TNF^+ T cells (*r =0.4086, p =0.0039*) (**Figure 6.6C. iv**). In summary, the data presented here highlights that stimulation of T cells can induce CD1a and CD1d expression and moreover, the presence or absence of serum supplements in cell

cultures can influence CD1 expression by T cells. The results also demonstrate that both CD1a and CD1d expression by T cells is correlated to TNF production by those T cells.

6.2.3. CD1a-restricted T cells

6.2.3.1. Identification of endogenous lipid-loaded CD1a tetramer-binding T cells

To determine whether CD1a-restricted T cells are associated with LCH, this study investigated whether T cells that recognise endogenous lipid ligands bound to CD1a tetramers were present in peripheral blood and lesions from LCH patients. A rigorous gating strategy that excluded $CD14^+$ monocytes and $CD19^+$ B cells was used to identify endogenous lipid-loaded CD1a tetramer-binding T cells (referred to herein as CD1a tetramer$^+$ T cells) (**Figure 6.7A**). A fluorescence minus one control that omitted the CD1a tetramer was also included to demonstrate that the tetramer was responsible for the positive fluorescence signal detected in the PE channel (**Figure 6.7B**). These results show that CD1a tetramer$^+$ T cells can be identified using this technique in healthy donors (**Figure 6.7C**) and importantly, CD1a tetramer$^+$ T cells are identifiable and thus present in blood and lesions from LCH patients (**Figure 6.7D**). Furthermore, CD1a tetramer$^+$ T cells were classified into subsets based on their expression of CD4 and CD8 co-receptors (**Figure 6.7C** and **D**).

6.2.3.2. CD1a-restricted T cells in patients with LCH

Importantly, this study demonstrates that there are CD1a tetramer$^+$ T cells in lesions and peripheral blood from patients with LCH (**Figure 6.8A**). The results indicate that there is no significant difference in the proportion of CD1a tetramer$^+$ T cells in total T cells from LCH patient blood (*median =0.012%*) when compared to healthy donor blood (*median =0.005%*) (**Figure 6.8A**).

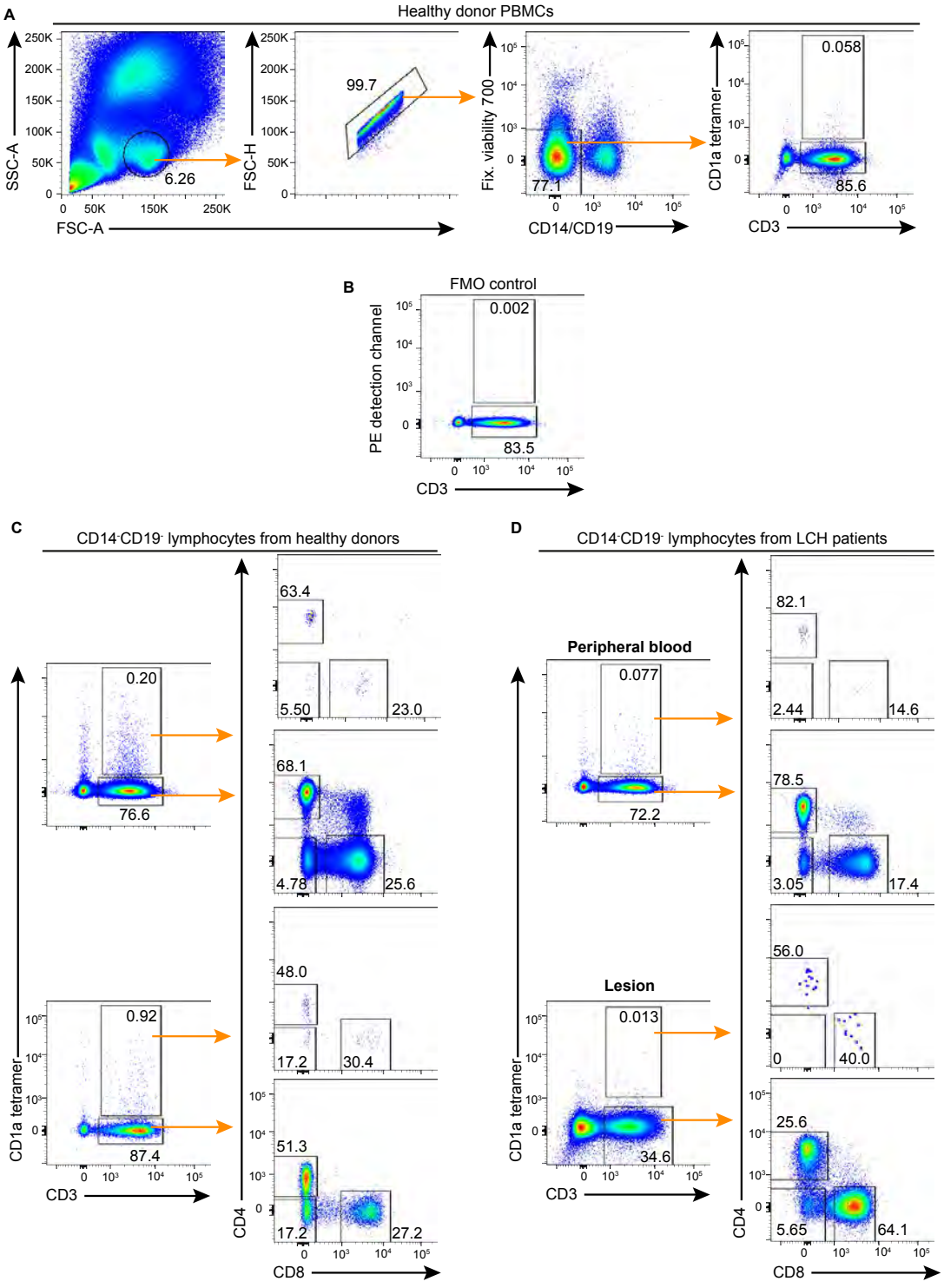


Figure 6.7. Identification of CD1a-restricted T cells.

*(**A**) Representative flow cytometry plots derived from healthy donor PBMCs demonstrating the hierarchical gating strategy used to identify CD1a tetramer^{+} T cells. Firstly, lymphocytes were identified (left), then doublets were excluded based on forward scatter parameters (second column), then dead cells, B cells and monocytes were excluded (third column) and CD1a tetramer^{+} T cells were identified by expression of CD3 and binding with endogenous lipid-loaded CD1a tetramer (right). (**B**) Fluorescence minus one (FMO) control for sample used in (**A**) to demonstrate that fluorescence in the PE detection channel is negative in the absence of*

*CD1a tetramer labelling. (**C**) Representative plots of lymphocytes from healthy donor derived PBMCs demonstrating the identification of CD1a tetramer$^+$ T cells and CD4 and CD8 subsets thereof following the application of the gating strategy from (**A**). CD4 and CD8 subset distributions for non-CD1a tetramer binding (tetramer negative) T cells are also included for comparison. (**D**) Representative plots of lymphocytes from blood and lesions from LCH patients demonstrating the identification of CD1a tetramer$^+$ T cells and CD4 and CD8 subsets thereof following the application of gating strategy from (**A**). CD4 and CD8 subset distributions for tetramer negative T cells are also included for comparison.*

Next, the proportions of CD4$^+$ (**Figure 6.8B**), CD8$^+$ (**Figure 6.8C**) and DN (**Figure 6.8D**) cells in CD1a tetramer$^+$ T cells were compared by examining peripheral blood from healthy donors (*CD4$^+$ median =50.00%, CD8$^+$ median =26.70%, DN median =12.20%*) and LCH patients (*CD4$^+$ median =50.85%, CD8$^+$ median =36.45%, DN median =5.39%*). There were no significant differences in the relative frequency detected in LCH patients compared to healthy donors. There was a greater range in the proportion of CD4$^+$ (*range =16.70-82.10%*) and CD8$^+$ (*range =6.67-66.70%*) cells in the CD1a tetramer$^+$ T cell population from LCH patient blood, thus this analysis should be expanded to investigate a larger cohort of patients. CD1a tetramer$^+$ T cells and subsets thereof from LCH lesions were not included in any statistical tests conducted because this population was only detectable in two of four specimens analysed. This is probably because of the small yields of total T cells from patient specimens. In the lesions where CD1a tetramer$^+$ T cells were identified, the frequency in total T cells (*median =0.032%*) was comparable to that seen in total T cells from the healthy donor and patient peripheral blood. Likewise, the proportion of CD4$^+$ (*median =61.95%*), CD8$^+$ (*median =30.70%*), and DN (*median =0.90%*) T cells in the lesional CD1a tetramer$^+$ T cell population were similar to those seen in healthy donors (*CD4$^+$ median =50%, CD8$^+$ median =26.70%, DN median =12.20%*) and patient (*CD4$^+$ median =61.95%, CD8$^+$ median =30.70%, DN median =0.895%*) peripheral blood. Although other lesional specimens had undetectable CD1a tetramer$^+$ T cells, it is important to note that the total T cell number yielded in the analysis of these specimens was 152 and 616 respectively. Thus, we can only deduce that the estimated frequency of CD1a tetramer$^+$ T cells in the total T cell population would be <0.66% and 0.17% respectively for these specimens.

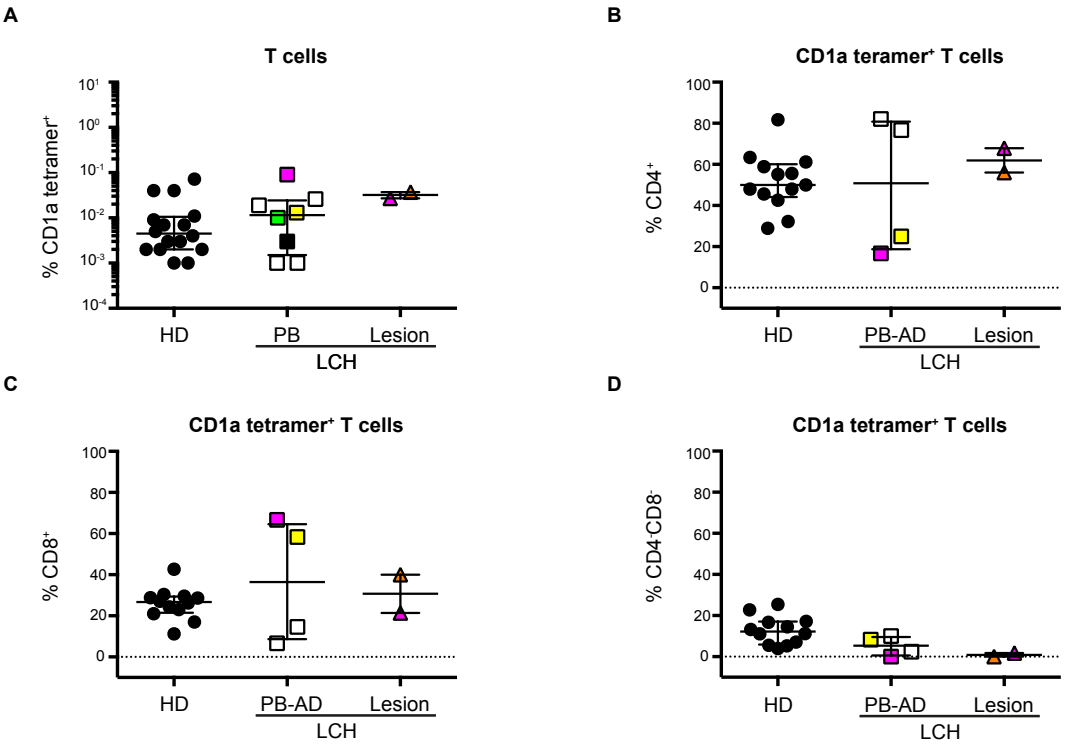


Figure 6.8. Proportions of CD1a-restricted T cells and CD4 and CD8 subsets.

*(**A**) Proportions of CD1a tetramer$^+$ T cells in total T cells from the peripheral blood from healthy donors and from blood and lesions from LCH patients. Black square in (**A**) indicates non-active LCH, all other LCH patients analysed had active disease. Proportions of CD4$^+$CD8$^-$ T cells (**B**), CD8$^+$CD4$^-$ T cells (**C**) and CD4$^-$CD8$^-$ T cells (**D**) in CD1a tetramer$^+$ T cells from blood from healthy donors and from blood and lesions from patients with active LCH. Two-tailed Mann Whitney tests were conducted for (**A**), (**B**), (**C**) and (**D**) (LCH lesion group excluded from tests). Error bars indicate median + interquartile range. Lesions and matched peripheral blood samples are colour coded on graphs.* ***HD =healthy donor, PB =peripheral blood, AD =active LCH.***

6.2.4. Expression of a Treg phenotype by CD1a-restricted T cells

The phenotype of CD1a-restricted T cells overall is not well established. In this study, healthy donor blood derived CD1a tetramer$^+$ T cells contained a population of CD4$^+$CD25$^+$CD127^{low} cells, consistent with a Treg phenotype (**Figure 6.9A**). Correspondingly, this phenotype was identified in CD1a tetramer$^+$ T cells from lesions and blood from LCH patients. Furthermore, the population identified in lesions also displayed some expression of CD56 (**Figure 6.9A**). For healthy donors, the proportion of Tregs in the CD1a tetramer$^+$ T cell population (*median =1.91%*) was significantly (*p =0.0078*) higher than the proportion of Tregs in the total T cell population (*median =1.03%*) (**Figure 6.9B**). Likewise, there was a trend towards an increased proportion of

Tregs in the CD1a tetramer^{+} T cell population from the blood (*median =4.81%*) and lesions (*median =26.30%*) from patients with active LCH when compared to the proportion in the total T cell population (*peripheral blood median =1.73%, lesion median =12.85%*) (**Figure 6.9B**). More LCH patient specimens need to be analysed to determine whether these differences are significant.

Upon examining CD4 expression specifically in lesions, matched specimens and median values displayed an upward trend in the frequency of CD4^{+}CD1a tetramer^{+} T cells that were CD25^{+}CD127^{low} (*median =38.55%*) compared to the frequency of total CD4^{+} T cells that were CD25^{+}CD127^{low} (*median =24.54%*) (**Figure 6.9C**). From another angle the results demonstrate that CD1a tetramer^{+} T cells were significantly enriched in the Treg population in the blood from healthy donors (*median =0.0615%*) and LCH patients (*median =0.0600%*) when compared to the proportions found in the total T cell population (*healthy donor p =0.0038, median =0.0045%) (LCH blood p =0.0479, median =0.0015%*) (**Figure 6.10A** and **B**). Similarly, there was a trend towards an increase in the proportion of CD1a tetramer^{+} T cells in the lesional Treg population (*median =0.6350%*) compared to the total lesional T cell population (*median =0.0320%*). As mentioned, a larger cohort of patients is required to continue this investigation, but the study already demonstrates that a substantial proportion of CD1a tetramer^{+} T cells from LCH lesions express the IL-2 receptor, CD25 and may be activated and/or have a regulatory role in lesions.

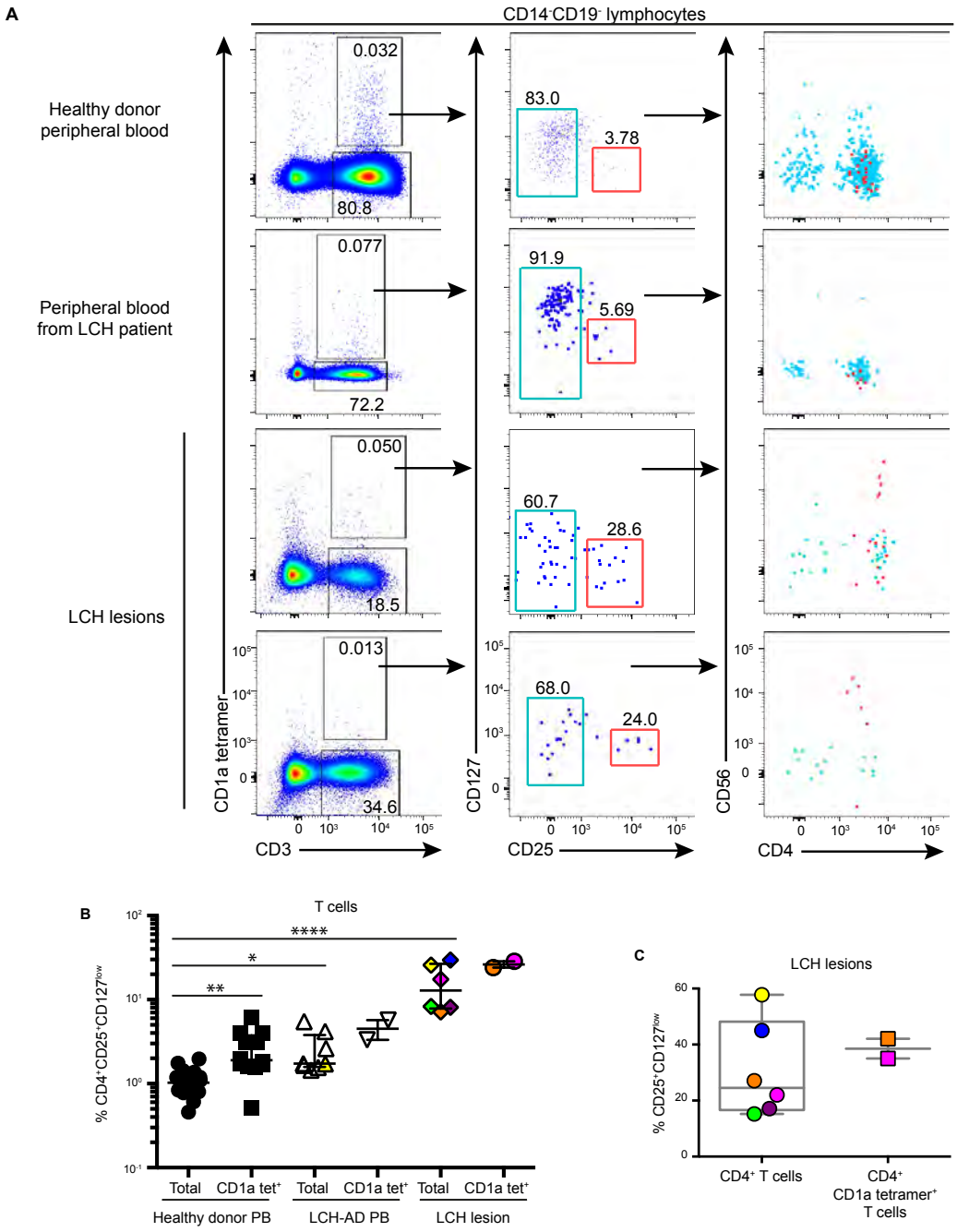


Figure 6.9. Expression of CD4, CD25, CD56 and low CD127 by CD1a-restricted T cells.

*(**A**) Representative flow cytometry plots derived from healthy donor PBMCs (top row), LCH patient peripheral blood (second row) and LCH lesions (bottom two rows) show identification of CD1a tetramer$^+$ T cells (left) and their expression of CD25 and CD127 (centre) and CD4 and CD56 (right). (**B**) Proportions of CD4$^+$CD25$^+$CD127^{low} T cells in total T cells and in CD1a tetramer$^+$ T cells from blood from healthy donors and from blood and lesions from patients with active LCH. (**C**) Proportions of CD25$^+$CD127^{low} T cells in CD4$^+$ T cells and CD4$^+$CD1a tetramer$^+$ T cells from LCH lesions. A Kruskal-Wallis test with Dunn's multiple comparisons was conducted for (**B**) (groups with only two values were excluded from test). ****p <0.0001, **p <0.01, *p <0.05, error bars indicate median + interquartile range. No statistical tests were conducted for (**C**) given only two values for one data set, box and whiskers plots indicate interquartile range and range with median. Lesions and matched peripheral blood samples are colour coded on graphs.* ***PB =peripheral blood, AD =active LCH.***

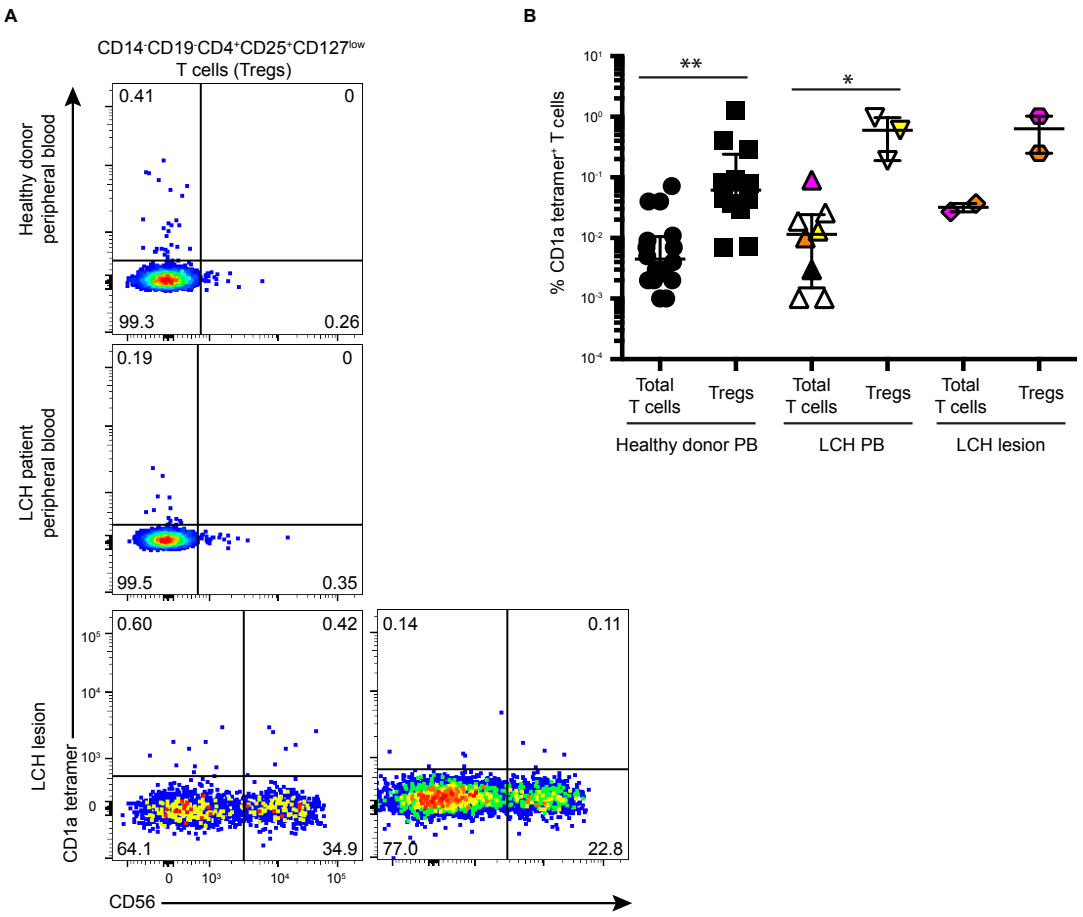


Figure 6.10. CD1a-restricted T cells amongst Tregs.

*(**A**) Representative plots derived from healthy donor PBMCs (top row), LCH patient peripheral blood (second row) and LCH lesions (bottom row) demonstrating the identification of CD1a tetramer$^+$ T cells in CD14$^-$CD19$^-$CD4$^+$CD25$^+$CD127^{low} T cells (Tregs). (**B**) Proportions of CD1a tetramer$^+$ T cells in Tregs and the total T cell population from blood from healthy donors, and from blood and lesions from LCH patients. A Kruskal-Wallis test with Dunn's multiple comparisons was conducted for (**B**) (groups with only two values were excluded from test). **p <0.01, *p <0.05, error bars indicate median + interquartile range. Lesions and matched peripheral blood samples are colour coded on graphs.* ***PB =peripheral blood.***

6.3. Discussion

This chapter explored the impact of CD1a expression in LCH by determining the phenotype and cytokine producing abilities of $CD1a^+$ T cells, by investigating how the expression of CD1 is regulated in T cells, and by identifying and characterising CD1a $tetramer^+$ T cells in LCH patients.

The identification of CD1a expression by a small population of T cells from LCH lesions primarily confirms the previous research that identified this novel T cell population[31]. The current study measured cell surface expression of CD1a by T cells in lesions from five LCH patients (*median =0.16%, range =0.07-12.42%*), however unlike the previous study, which analysed lesional tissues from six patients, there were no samples that had striking expression of CD1a on T cells. From the descriptive statistics of the previous study we can deduce that the proportion of T cells that expressed CD1a was highly variable (mean =8.3%, standard deviation =8.8, maximum =56.7%)[31]. In the current study, $CD1a^+$ T cells were mostly $CD4^+$, however the frequency of $CD4^+$ T cells in the $CD1a^+$ T cell population was more spread than that seen in the total T cell population from the same donors and this was consistent with the previous study[31]. The results presented here did not detect any significant difference in the proportion of $CD4^+$, $CD8^+$ or DN cells in $CD1a^+$ T cells when compared to the proportions seen in the total T cell population from the same individuals. There was a trend towards a decrease in the proportion of $CD8^+$ cells in the $CD1a^+$ T cell population compared to the proportion in the total T cell population, which should be further examined in a larger cohort to determine whether the difference is significant and if so, to establish why and potentially further elucidate the function of these cells.

We surprisingly identified co-expression of CD4 and CD8 in the $CD1a^+$ T cell population in one lesional specimen following stimulation. Extrathymic double positive T cells have previously been reported in healthy donors, in disease settings such as rheumatoid arthritis, and in response to viral challenge[236,237]. Whilst the function of mature T cells co-expressing CD4 and CD8 is not well established, it could represent a phenotypical and/or functional migration by T cells in response to stimuli, or a

powerful cell type that is capable of both Th functions and cytotoxicity[237], such as that seen in some unconventional T cell lineages.

Importantly, the results presented here established that $CD1a^+$ T cells can produce TNF and IFN-γ, indicating that they are capable of a Th1 type response, however their phenotype changed from predominantly $CD4^+$ to mostly $CD8^+$ following stimulation. This might be due to downregulation of CD1a on $CD4^+$ T cells and/or upregulation of CD1a on $CD8^+$ T cells. Alternatively, there may be downregulation of CD4 and subsequent upregulation of CD8 on $CD1a^+$ T cells, the latter of which ties in with the co-expression of CD4 and CD8 observed in one patient. This could be clarified in future experiments by purifying a population of $CD4^+CD1a^+$ T cells prior to stimulation, although purification of this population was beyond the scope of this study. Other Th responses by $CD1a^+$ T cells should also be investigated in the future, and given the potential increase in CD8 expression, the cytotoxic abilities of $CD1a^+$ T cells might be examined. Difficulties purifying the $CD1a^+$ T cell population arose in this study and might alternatively suggest that CD1a expression by T cells is downregulated upon binding to anti-CD1a antibody, although this is not likely, given that CD1a expression by LCH cells remains following FACS. It is most likely that the $CD1a^+$ T cell population is too sparse and too close in fluorescence intensity to the $CD1a^-$ T cell population to purify, especially since other populations were able to be purified successfully in parallel and using the same detection antibodies. Technologies such as single cell isolation might allow the purification of individual cells from LCH lesions in future studies, thus permitting transcriptional analyses. This may better establish the importance of $CD1a^+$ T cells and determine whether they play a role in LCH pathogenesis or progression.

This study investigated the dynamics of CD1a expression by T cells from healthy donor PBMCs following stimulation. The results demonstrated that CD1a expression was induced on T cells by adding PHA to cell cultures. CD1a expression was increased on the T cells that were producing TNF when compared to T cells lacking any signs of activation, and in some cases, when compared to T cells displaying a different marker of activation, namely the cell surface expression of CD69. Interestingly, there was a

positive correlation between CD1a expression on T cells and TNF production by those T cells. Induction of CD1d by T cells following stimulation has been reported previously[151] and similar to CD1a expression, there was an increase in cell surface CD1d expression by TNF^+ T cells when compared to T cells lacking activation markers. Further comparable to CD1a expression by T cells, there was a positive correlation between CD1d expression on TNF^+ T cells and TNF production by those T cells. These results suggest that CD1a and CD1d expression by T cells, and TNF production by T cells could be regulated by a common factor. For example, lipids captured and presented by CD1a and/or CD1d might be involved in the signalling process required to produce TNF. Otherwise, CD1a and CD1d expression by TNF^+ T cells may be an indicator of chronic stimulation, which would suggest that $CD1a^+$ T cells in LCH lesions could be an indicator of chronic activation. Since both the proportion of TNF^+ T cells and the fluorescence of CD1a by T cells was higher in serum-free media when compared to T cells cultured with serum additives, it is plausible that the addition of serum supplements to cell cultures provides lipids to the environment that partially inhibit CD1a expression and reduce the production of TNF. For example, we know that sphingomyelin can inhibit the binding of CD1a tetramer to a CD1-restricted T cell line[197]. Defining endogenous lipid ligands or exogenous antigens that bind with CD1a and allow us to manipulate both CD1 expression and TNF production by T cells could be beneficial in the context of regulating immune responses, for instance to enhance inflammation to target cancer cells or to diminish inflammation in autoimmune diseases. Additionally, the results from this study coupled with previous studies into factors that can impact CD1a expression demonstrate that inconsistencies such as temperature, environmental pollutants, and the addition of serum additives could lead to inconsistencies in identifying $CD1a^+$ T cells, therefore small technical differences in antibody labelling protocols, for example, could account for variations between the current study and the primary study[31]. The results presented here highlight that these mechanisms require addressing before further investigations into the LCH lesion derived T cell subset expressing CD1a.

In response to the expression of CD1a in LCH lesions, this study examined whether CD1a-restricted T cells were present in lesions. These results are the first to identify a

subset of CD1a tetramer$^+$ T cells that recognise endogenous lipid ligands in the lesions and in the peripheral blood from LCH patients. The proportions of T cells that were CD1a tetramer$^+$ within LCH patient derived specimens were comparable to those seen in healthy donors. Likewise, when investigating the expression of CD4 and CD8 subsets in CD1a tetramer$^+$ T cells, there were similar proportions in both LCH patients and healthy donors, although further studies on a large cohort of patients are necessary to confirm this.

An interesting finding regarding CD1a tetramer$^+$ T cells in both healthy donors and LCH patients was the considerable expression of IL-2 receptor CD25 along with expression of CD4 and low CD127 by this population. While this finding is preliminary and the sample size needs to be increased for more conclusive findings, it is certainly of note that approximately a quarter of all identified CD1a tetramer$^+$ T cells within LCH lesions displayed this phenotype along with varied CD56 expression. The Treg phenotype was represented at a higher frequency in the CD1a tetramer$^+$ T cell population when compared to the total T cell population in healthy donors and LCH patients alike, and reciprocally, CD1a tetramer$^+$ T cells were enriched in the Treg population when compared to the total T cell population. This finding evolves our understanding of basic CD1a-restricted T cell biology and has potential consequences for our understanding of the LCH microenvironment. It was established in **Chapter 4** that both CD56$^+$ and CD56$^-$ Tregs from LCH lesions can produce active TGF-β. Given their phenotype established by this study, CD1a tetramer$^+$ T cells could equally potentially harness a TGF-β producing ability. This should be tested in the future by measuring cytokine levels in the supernatant of a pure population of CD1a tetramer$^+$ T cells, or intracellularly within a mixed population. Since CD1a-restricted T cells are currently only identifiable in small populations such as the subset investigated in the current study, these assays may prove difficult.

It will be challenging to determine, but it is important to establish whether the expression of CD4, CD25 and low CD127 expression by CD1a tetramer$^+$ T cells denotes either a natural or induced Foxp3$^+$ Treg phenotype, or whether these cells are of an activated Th1 or Th22 phenotype, or even another phenotype that has not previously

been reported for this population. For example, studies have shown that upon chronic stimulation with α-galactosylceramide, NKT cells can shift to an exhaustion phenotype characterised by IL-10 production and expression of PD-1 and neuropilin 1[238]. Therefore, it is conceivable that chronic stimulation of CD1a-restricted T cells within LCH lesions could similarly lead to the production of IL-10 and expression of exhaustion markers. It is reasonable to envisage this because infiltration of activated conventional T cells within LCH lesions is associated with an IL-10 predominant rather than a TGF-β predominant or mixed IL-10/ TGF-β phenotype within lesions[100], whilst LCH cells were reported to display PD-L1, and lesional lymphocytes displayed PD-1[239]. This means that LCH cells and T cells have the potential to be interacting via the PD-1-PD-L1 axis.

Additionally, there is potential for other T cells from LCH patients, for example, the remainder of LCH lesional T cells that express CD25, to be CD1a-restricted too. This is especially possible since there is such high expression of CD1a in lesions, however this study only tested for T cell reactivity toward a group of endogenous lipid ligands presented by CD1a. The process of identifying the different antigens that CD1a-restricted T cells can recognise and respond to is underway[197]. T cell recognition of specific lipids presented by CD1a should be investigated in the context of LCH in addition to healthy donors. As our understanding of these cells progresses we should expect to determine whether T cell activation and/or regulation is CD1a dependent within the LCH lesional microenvironment. Further analyses could examine, for example, whether there are CD1a-DDM, -sphingomyelin, -sulfatide, -phosphatidylcholine, -LPC or -urushiol reactive T cells present within lesions or even blood from LCH patients.

In summary, this study confirmed the expression of CD1a by a subset of T cells in LCH lesions. Similar to the induction of CD1d, CD1a can be induced on T cells by stimulation, and both CD1a and CD1d expression by T cells is associated with TNF production by those T cells. In light of the results presented here, CD1a expression by T cells in LCH may be a marker of chronic stimulation, but further studies are required to investigate this. This study also established that CD1a tetramer^{+} T cells are present

in LCH patients, including within lesions. Additionally, the results presented here contribute to understanding the basic biology of CD1a-restricted T cells by demonstrating that those that recognise endogenous lipids are enriched in the $CD4^{+}CD25^{+}CD127^{low}$ T cell population. Some CD1a-restricted T cells could be suppressive in function, and within lesions this might be due to chronic stimulation by lipid antigens, although further investigations into the involvement of CD1a, lipids and CD1a-restricted T cells in LCH pathogenesis are necessary to elucidate this.

7.0. CONCLUDING REMARKS

The studies undertaken in this project investigated the relative frequency and function of a broad range of T cells that are potentially important for immune regulation in the context of LCH. This research has highlighted several abnormalities, of which, the most striking changes identified were those seen in MAIT cell and $Foxp3^+$ Treg populations from patients with active LCH. Additionally, these studies have built upon the previous understanding about the role of enigmatic LCH cells to better define their phenotype and the biological processes that they encompass. Finally, these studies have explored multiple potential roles for CD1a, one of the key diagnostic indicators for LCH, in the progression of this disease.

This research project investigated the role of both conventional and unconventional T cell subsets in patients with LCH. Overall, the relative frequencies of $CD4^+$ T cells and $Foxp3^+$ Tregs were increased in patients with active LCH compared to healthy donors and patients with non-active disease. Conversely, the relative frequencies of MAIT cells and $CD8^+$ T cells were decreased in patients with LCH compared to healthy donors. While the proportions were consistent between the blood and lesions for $CD4^+$ T cells, $CD8^+$ T cells and MAIT cells in patients with active LCH, $Foxp3^+$ Tregs were strikingly high within lesions, representing up to approximately 30% of total T cells. Despite abnormalities in the relative frequencies of T cell subsets in LCH patients, these studies have shown that when challenged, these cell populations are able to mount inflammatory responses in a similar manner to those from healthy donors. Importantly, this study established that $Foxp3^+$ Tregs from LCH lesions produce TGF-β when challenged, and this links the previous data identifying increased numbers of $Foxp3^+$ Tregs in lesions and TGF-β expression in lesions, and substantiates the concept that Tregs could be supressing the response of other immune cells, such as conventional T cells, which were incidentally lacking expression of CD25 and HLA-DR within the lesions.

While these cytokine responses were observed *in vitro*, it is important to note that responses could be hindered *in vivo* as suggested by the pilot study demonstrating that CD69 expression by T cells is reduced in the presence of PBMCs incubated with high concentrations of TGF-β. Further studies are now required to establish whether the LCH microenvironment affects the ability of T cells such as MAIT cells to elicit an appropriate immune response, which could conceivably clear lesions. Importantly, by characterising LCH cells, this study established that LCH cells express detectable levels of *MR1* and thus are likely able to present antigen to MAIT cells within LCH lesions. This is important to know if we wish to target or increase patients' MAIT cells as a future treatment strategy.

Additionally, this study was the first to identify expression of CD56 on $Foxp3^+$ Tregs specifically within lesions from patients with LCH. This population produced the immune suppressive cytokine TGF-β when challenged, indicating that $CD56^+Foxp3^+$ Tregs are suppressive, and further highlighting that they may be important in the progression of LCH. Along with increased CD56 expression by T cells within lesions (almost all of which co-expressed Foxp3) this study identified a reduced relative frequency of $CD8^+CD56^+$ T cells in patients with active disease, and a direct relationship between Tregs and $CD8^+CD56^+$ T cells. Together with the knowledge that TGF-β, which is present in lesions, can induce Foxp3 and CD25 expression in T cells, the findings presented here suggest that $CD4^+$ and/or $CD56^+$ T cells may be trafficking from the circulation to lesions, where they become induced Tregs, while $CD8^+CD56^+$ T cells may be inhibited in patients with active disease.

The characterisation of LCH cells highlighted an overrepresentation of genes involved in important biological processes and these processes could be involved in LCH pathogenesis. The enriched biological processes included exposure to endogenous antigens and regulating the recruitment, activation, and proliferation of T cells, which further suggests that antigen processing and presentation along with T cell recruitment, activation and proliferation are important mechanisms in LCH. Additionally, this study has highlighted that CD14 expression by LCH cells could suggest myeloid cell maturity and thus less disseminated disease activity, therefore

expression levels of this molecule could be an important and non-invasive prognostic indicator for future LCH patients.

While the results presented here have contributed to understanding LCH cells, there are still many further investigations needed to better understand these enigmatic cells. The suggestion that LCH cells have an enrichment of genes related to exposure to endogenous antigen is certainly of interest given the novel finding identifying CD1a-restricted T cells that recognise endogenous lipid ligands in LCH patients. Another valuable finding from this study was enrichment of the Treg phenotype in CD1a tetramer$^+$ T cells in healthy donors, which has contributed to our understanding of the basic biology and importance of this T cell lineage. There was a similar enrichment in patients with LCH, and this included the expression of CD56 by some of these cells. While it was beyond the scope of current studies, it is certainly conceivable that other Tregs from LCH patients could similarly recognise other lipid antigens or ligands that are presented by CD1a. Since the expression of CD1a on LCH cells is almost ubiquitous, this could be a mechanism by which LCH cells recruit antigen specific or CD4$^+$ and/or CD56$^+$ T cells to the microenvironment where the regulatory phenotype is then induced. Studies facilitating our understanding of CD1a-restricted T cell lipid antigens and ligands should be continued to better understand the basic biological functions of this highly specialised T cell lineage and their specific role in the context of LCH.

Albeit technically challenging, the impact of CD1a expression by T cells in LCH lesions was also investigated and CD1a$^+$ T cells produced the inflammatory cytokines, TNF and IFN-γ. Further studies should investigate whether this response can be extended to the production of other cytokines and whether the LCH microenvironment influences this. This study established that CD1a can be upregulated by T cells under the influence of PHA and that the level of CD1a expression was correlated to the production of TNF by those T cells, which was also true for CD1d expression. CD1a and CD1d expression by TNF$^+$ T cells could be an indicator of chronic stimulation and CD1a expression by T cells in LCH may be a marker of chronic activation. Additionally, this study found that both TNF production and fluorescence of CD1a by T cells was highest in serum free media, which suggests that serum may be inhibiting both factors, potentially via CD1a

interactions with lipids. Mechanisms influencing CD1a expression are quite broad and need to be studied further to fully elucidate these results.

The findings of this study disseminate fundamental information on abnormalities within several T cell lineages in LCH patients and importantly, the results have highlighted a new niche area of exploration in investigating the role of unconventional T cells in LCH patients. The data presented here suggest that LCH cells may be recruiting potentially antigen specific $CD4^+$ and/or $CD56^+$ T cells to the lesional microenvironment. LCH cells might then induce Foxp3 and CD25 expression in this population via TGF-β production and possibly also by presenting antigen for TCR recognition. Subsequent production of TGF-β by both LCH cells and $Foxp3^+$ Tregs could reduce the relative frequencies of $CD8^+$ T cell subsets including MAIT cells and $CD8^+CD56^+$ T cells and conceivably inhibit their functions *in vivo* too. Future studies should focus on understanding whether LCH cells can recruit antigen specific, $CD4^+$ or $CD56^+$ T cells to lesions and induce Foxp3 expression and TGF-β production by these populations to inhibit the proliferative ability and/or inflammatory response of other T cells within the environment. If we establish this as the mechanism by which LCH progresses, we can focus on inhibiting the pathway with specific immunotherapies to treat patients with LCH and improve their outcomes.

8.0. REFERENCES

1. Bonilla, F. A. & Oettgen, H. C. Adaptive immunity. *J. Allergy Clin. Immunol.* **125,** S33–40 (2010).
2. Akira, S., Uematsu, S. & Takeuchi, O. Pathogen recognition and innate immunity. *Cell* **124,** 783–801 (2006).
3. Dwivedy, A. & Aich, P. Importance of innate mucosal immunity and the promises it holds. *Int J Gen Med* **4,** 299–311 (2011).
4. Aderem, A. Phagocytosis and the inflammatory response. *J. Infect. Dis.* **187 Suppl 2,** S340–345 (2003).
5. Pardo, J., Balkow, S., Anel, A. & Simon, M. M. Granzymes are essential for natural killer cell-mediated and perf-facilitated tumor control. *Eur. J. Immunol.* **32,** 2881–2887 (2002).
6. Berke, G. The CTL's kiss of death. *Cell* **81,** 9–12 (1995).
7. Guilliams, M. *et al.* Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. *Nature Publishing Group* **14,** 571–578 (2014).
8. Hume, D. A. Macrophages as APC and the dendritic cell myth. *The Journal of Immunology* **181,** 5829–5835 (2008).
9. Randolph, G. J., Jakubzick, C. & Qu, C. Antigen presentation by monocytes and monocyte-derived cells. *Current Opinion in Immunology* **20,** 52–60 (2008).
10. Robinson, J. H. & Delvig, A. A. Diversity in MHC class II antigen presentation. *Immunology* **105,** 252–262 (2002).
11. Hart, D. N. Dendritic cells: unique leukocyte populations which control the primary immune response. *Blood* **90,** 3245–3287 (1997).
12. Ginhoux, F. & Merad, M. Ontogeny and homeostasis of Langerhans cells. *Immunol. Cell Biol.* **88,** 387–392 (2010).
13. Berres, M.-L., Allen, C. E. & Merad, M. Pathological consequence of misguided dendritic cell differentiation in histiocytic diseases. *Adv. Immunol.* **120,** 127–161 (2013).
14. Mittag, D. *et al.* Human dendritic cell subsets from spleen and blood are similar in phenotype and function but modified by donor health status. *The Journal of Immunology* **186,** 6207–6217 (2011).
15. Idoyaga, J. *et al.* Specialized role of migratory dendritic cells in peripheral tolerance induction. *J. Clin. Invest.* **123,** 844–854 (2013).
16. Steinman, R. M., Hawiger, D. & Nussenzweig, M. C. Tolerogenic dendritic cells. *Annu. Rev. Immunol.* **21,** 685–711 (2003).
17. Merad, M., Ginhoux, F. & Collin, M. Origin, homeostasis and function of Langerhans cells and other langerin-expressing dendritic cells. *Nature Publishing Group* **8,** 935–947 (2008).
18. Rossjohn, J. *et al.* T cell antigen receptor recognition of antigen-presenting molecules. *Annu. Rev. Immunol.* **33,** 169–200 (2015).
19. Alvarez, D., Vollmann, E. H. & Andrian, von, U. H. Mechanisms and consequences of dendritic cell migration. *Immunity* **29,** 325–342 (2008).

20. Merad, M. *et al.* Langerhans cells renew in the skin throughout life under steady-state conditions. *Nat. Immunol.* **3,** 1135–1141 (2002).
21. Andersen, M. H., Schrama, D., Thor Straten, P. & Becker, J. C. Cytotoxic T cells. *J. Invest. Dermatol.* **126,** 32–41 (2006).
22. Hall, B. M. T cells: soldiers and spies-the surveillance and control of effector T cells by regulatory T cells. *Clin J Am Soc Nephrol* **10,** 2050–2064 (2015).
23. Geginat, J. *et al.* The CD4-centered universe of human T cell subsets. *Seminars in Immunology* **25,** 252–262 (2013).
24. Liao, W., Lin, J.-X. & Leonard, W. J. Interleukin-2 at the crossroads of effector responses, tolerance, and immunotherapy. *Immunity* **38,** 13–25 (2013).
25. Herbst, S., Schaible, U. E. & Schneider, B. E. Interferon gamma activated macrophages kill mycobacteria by nitric oxide induced apoptosis. *PLoS ONE* **6,** e19105 (2011).
26. Woodward, E. A., Prêle, C. M., Nicholson, S. E., Kolesnik, T. B. & Hart, P. H. The anti-inflammatory effects of interleukin-4 are not mediated by suppressor of cytokine signalling-1 (SOCS1). *Immunology* **131,** 118–127 (2010).
27. Kouro, T. & Takatsu, K. IL-5- and eosinophil-mediated inflammation: from discovery to therapy. *Int. Immunol.* **21,** 1303–1309 (2009).
28. Martinez-Nunez, R. T., Louafi, F. & Sanchez-Elsner, T. The interleukin 13 (IL-13) pathway in human macrophages is modulated by microRNA-155 via direct targeting of interleukin 13 receptor alpha1 (IL13Rα1). *Journal of Biological Chemistry* **286,** 1786–1794 (2011).
29. Rizzo, F. M., Cives, M., Simone, V. & Silvestris, F. New insights into the molecular pathogenesis of Langerhans cell histiocytosis. *The Oncologist* **19,** 151–163 (2014).
30. Kannourakis, G. & Abbas, A. The role of cytokines in the pathogenesis of Langerhans cell histiocytosis. *Br. J. Cancer Suppl.* **23,** S37–40 (1994).
31. West, J. A. *et al.* Polyclonal T-cells express CD1a in Langerhans cell histiocytosis (LCH) lesions. *PLoS ONE* **9,** e109586 (2014).
32. Morimoto, A., Oh, Y., Shioda, Y., Kudo, K. & Imamura, T. Recent advances in Langerhans cell histiocytosis. *Pediatr Int* **56,** 451–461 (2014).
33. Stålemark, H. *et al.* Incidence of Langerhans cell histiocytosis in children: a population-based study. *Pediatr. Blood Cancer* **51,** 76–81 (2008).
34. Aricò, M. *et al.* Langerhans cell histiocytosis in adults. Report from the International Registry of the Histiocyte Society. *Eur. J. Cancer* **39,** 2341–2348 (2003).
35. Bechan, G. I., Egeler, R. M. & Arceci, R. J. Biology of Langerhans cells and Langerhans cell histiocytosis. *Int. Rev. Cytol.* **254,** 1–43 (2006).
36. Allen, C. E., Ladisch, S. & McClain, K. L. How I treat Langerhans cell histiocytosis. *Blood* **126,** 26–35 (2015).
37. Gadner, H. *et al.* Therapy prolongation improves outcome in multisystem Langerhans cell histiocytosis. *Blood* **121,** 5006–5014 (2013).
38. Yeh, E. A. *et al.* Evaluation and treatment of Langerhans cell histiocytosis patients with central nervous system abnormalities: current views and new vistas. *Pediatr. Blood Cancer* **65,** (2018).
39. Haupt, R. *et al.* Permanent consequences in Langerhans cell histiocytosis

patients: a pilot study from the Histiocyte Society-Late Effects Study Group. *Pediatr. Blood Cancer* **42,** 438–444 (2004).
40. Kotecha, R. *et al.* Clinical outcomes of radiation therapy in the management of Langerhans cell histiocytosis. *American Journal of Clinical Oncology* **37,** 592–596 (2014).
41. Haupt, R. *et al.* Langerhans cell histiocytosis (LCH): guidelines for diagnosis, clinical work-up, and treatment for patients till the age of 18 years. *Pediatr. Blood Cancer* **60,** 175–184 (2012).
42. Lahey, M. E. Histiocytosis X: comparison of three treatment regimens. *J. Pediatr.* **87,** 179–183 (1975).
43. Al-Anazi, K. A. *et al.* Successful outcome of Langerhans cell histiocytosis complicated by therapy-related myelodysplasia and acute myeloid leukemia: a case report. *Cases J* **1,** (2008).
44. Donadieu, J. *et al.* Cladribine and cytarabine in refractory multisystem Langerhans cell histiocytosis: results of an international phase 2 study. *Blood* **126,** 1415–1423 (2015).
45. Vaiselbuh, S. R., Bryceson, Y. T., Allen, C. E., Whitlock, J. A. & Abla, O. Updates on histiocytic disorders. *Pediatr. Blood Cancer* **61,** 1329–1335 (2014).
46. Allen, C. E., Merad, M. & McClain, K. L. Langerhans-cell histiocytosis. *N. Engl. J. Med.* **379,** 856–868 (2018).
47. Berres, M. L. *et al. BRAF-V600E* expression in precursor versus differentiated dendritic cells defines clinically distinct LCH risk groups. *Journal of Experimental Medicine* **211,** 669–683 (2014).
48. Sahm, F. *et al.* BRAFV600E mutant protein is expressed in cells of variable maturation in Langerhans cell histiocytosis. *Blood* **120,** e28–e34 (2012).
49. Badalian-Very, G., Vergilio, J.-A., Degar, B. A., Rodriguez-Galindo, C. & Rollins, B. J. Recent advances in the understanding of Langerhans cell histiocytosis. *British Journal of Haematology* **156,** 163–172 (2011).
50. Valladeau, J. *et al.* Langerin, a novel C-type lectin specific to Langerhans cells, is an endocytic receptor that induces the formation of Birbeck granules. *Immunity* **12,** 71–81 (2000).
51. Nezelof, C., Basset, F. & Rousseau, M. F. Histiocytosis X histogenetic arguments for a Langerhans cell origin. *Biomedicine* **18,** 365–371 (1973).
52. Willman, C. L. *et al.* Langerhans'-cell histiocytosis (histiocytosis X)-a clonal proliferative disease. *N. Engl. J. Med.* **331,** 154–160 (1994).
53. Yu, R. C., Chu, C., Buluwela, L. & Chu, A. C. Clonal proliferation of Langerhans cells in Langerhans cell histiocytosis. *Lancet* **343,** 767–768 (1994).
54. Busque, L. *et al.* Skewing of X-inactivation ratios in blood cells of aging women is confirmed by independent methodologies. *Blood* **113,** 3472–3474 (2009).
55. Liu, W. & Sun, X. Skewed X chromosome inactivation in diploid and triploid female human embryonic stem cells. *Hum. Reprod.* **24,** 1834–1843 (2009).
56. Egeler, R. M., Favara, B. E., van Meurs, M., Laman, J. D. & Claassen, E. Differential In situ cytokine profiles of Langerhans-like cells and T cells in Langerhans cell histiocytosis: abundant expression of cytokines relevant to disease and treatment. *Blood* **94,** 4195–4201 (1999).

57. Senechal, B. *et al.* Expansion of regulatory T cells in patients with Langerhans cell histiocytosis. *PLoS Med.* **4,** e253 (2007).
58. Egeler, R. M., van Halteren, A. G. S., Hogendoorn, P. C. W., Laman, J. D. & Leenen, P. J. M. Langerhans cell histiocytosis: fascinating dynamics of the dendritic cell-macrophage lineage. *Immunol. Rev.* **234,** 213–232 (2010).
59. Satoh, T. *et al.* B-RAF mutant alleles associated with Langerhans cell histiocytosis, a granulomatous pediatric disease. *PLoS ONE* **7,** e33891 (2012).
60. Badalian-Very, G. *et al.* Recurrent *BRAF* mutations in Langerhans cell histiocytosis. *Blood* **116,** 1919–1923 (2010).
61. Yousem, S. A., Dacic, S., Nikiforov, Y. E. & Nikiforova, M. Pulmonary Langerhans cell histiocytosis: profiling of multifocal tumors using next-generation sequencing identifies concordant occurrence of *BRAF* V600E mutations. *Chest* **143,** 1679–1684 (2013).
62. Nelson, D. S. *et al. MAP2K1* and *MAP3K1* mutations in Langerhans cell histiocytosis. *Genes Chromosomes Cancer* **54,** 361–368 (2015).
63. Chakraborty, R. *et al.* Mutually exclusive recurrent somatic mutations in *MAP2K1* and *BRAF* support a central role for ERK activation in LCH pathogenesis. *Blood* **124,** 3007–3015 (2014).
64. Brown, N. A. *et al.* High prevalence of somatic *MAP2K1* mutations in *BRAF V600E*-negative Langerhans cell histiocytosis. *Blood* **124,** 1655–1658 (2014).
65. Allen, C. E. *et al.* Cell-specific gene expression in Langerhans cell histiocytosis lesions reveals a distinct profile compared with epidermal Langerhans cells. *The Journal of Immunology* **184,** 4557–4567 (2010).
66. Davies, H. *et al.* Mutations of the *BRAF* gene in human cancer. *Nature* **417,** 949–954 (2002).
67. Dhomen, N. & Marais, R. New insight into *BRAF* mutations in cancer. *Curr. Opin. Genet. Dev.* **17,** 31–39 (2007).
68. Bubolz, A.-M. *et al.* Potential clinical implications of *BRAF* mutations in histiocytic proliferations. *Oncotarget* **5,** 4060–4070 (2014).
69. Cantwell-Dorris, E. R., O'Leary, J. J. & Sheils, O. M. $BRAF^{V600E}$: Implications for carcinogenesis and molecular therapy. *Molecular Cancer Therapeutics* **10,** 385–394 (2011).
70. Haroche, J. *et al.* Dramatic efficacy of vemurafenib in both multisystemic and refractory Erdheim-Chester disease and Langerhans cell histiocytosis harboring the *BRAF* V600E mutation. *Blood* **121,** 1495–1500 (2013).
71. Aricò, M. Langerhans cell histiocytosis in children: from the bench to bedside for an updated therapy. *British Journal of Haematology* **173,** 663–670 (2016).
72. Kansal, R. *et al.* Identification of the V600D mutation in Exon 15 of the *BRAF* oncogene in congenital, benign Langerhans cell histiocytosis. *Genes Chromosomes Cancer* **52,** 99–106 (2013).
73. Diamond, E. L. *et al.* Diverse and targetable kinase alterations drive histiocytic neoplasms. *Cancer Discov* **6,** 154–165 (2016).
74. Lee, L. H. *et al.* Real-time genomic profiling of histiocytoses identifies early-kinase domain BRAF alterations while improving treatment outcomes. *JCI Insight* **2,** e89473 (2017).
75. Mourah, S. *et al.* Recurrent *NRAS* mutations in pulmonary Langerhans cell

histiocytosis. *Eur. Respir. J.* **47,** 1785–1796 (2016).
76. Hamdorf, M., Berger, A., Schüle, S., Reinhardt, J. & Flory, E. PKCδ-induced PU.1 phosphorylation promotes hematopoietic stem cell differentiation to dendritic cells. *Stem Cells* **29,** 297–306 (2011).
77. Puig-Kröger, A. *et al.* Extracellular signal-regulated protein kinase signaling pathway negatively regulates the phenotypic and functional maturation of monocyte-derived human dendritic cells. *Blood* **98,** 2175–2182 (2001).
78. Berres, M.-L., Merad, M. & Allen, C. E. Progress in understanding the pathogenesis of Langerhans cell histiocytosis: back to histiocytosis X? *British Journal of Haematology* **169,** 3–13 (2015).
79. Collin, M., Bigley, V., McClain, K. L. & Allen, C. E. Cell(s) of origin of Langerhans cell histiocytosis. *Hematol. Oncol. Clin. North Am.* **29,** 825–838 (2015).
80. Braier, J. Is Langerhans cell histiocytosis a neoplasia? *Pediatr. Blood Cancer* (2016). doi:10.1002/pbc.26267
81. Simko, S. J. *et al.* Differentiating skin-limited and multisystem Langerhans cell histiocytosis. *J. Pediatr.* **165,** 990–996 (2014).
82. Grois, N., Prayer, D., Prosch, H., Lassmann, H. & Group, C. L. C.-O. Neuropathology of CNS disease in Langerhans cell histiocytosis. *Brain* **128,** 829–838 (2005).
83. McClain, K. L. *et al.* CNS Langerhans cell histiocytosis: Common hematopoietic origin for LCH-associated neurodegeneration and mass lesions. *Cancer* (2018). doi:10.1002/cncr.31348
84. Mass, E. *et al.* A somatic mutation in erythro-myeloid progenitors causes neurodegenerative disease. *Nature* **549,** 389–393 (2017).
85. Geissmann, F. *et al.* Differentiation of Langerhans cells in Langerhans cell histiocytosis. *Blood* **97,** 1241–1248 (2001).
86. Redd, L. *et al.* Langerhans cell histiocytosis shows distinct cytoplasmic expression of major histocompatibility class II antigens. *J Hematopathol* **9,** 107–112 (2016).
87. Andersson By, U., Tani, E., Andersson, U. & Henter, J.-I. Tumor necrosis factor, interleukin 11, and leukemia inhibitory factor produced by Langerhans cells in Langerhans cell histiocytosis. *J. Pediatr. Hematol. Oncol.* **26,** 706–711 (2004).
88. Annels, N. E. *et al.* Aberrant chemokine receptor expression and chemokine production by Langerhans cells underlies the pathogenesis of Langerhans cell histiocytosis. *J. Exp. Med.* **197,** 1385–1390 (2003).
89. Egeler, R. M., Favara, B. E., Laman, J. D. & Claassen, E. Abundant expression of CD40 and CD40-ligand (CD154) in paediatric Langerhans cell histiocytosis lesions. *Eur. J. Cancer* **36,** 2105–2110 (2000).
90. Chen, R. *et al.* Anti-programmed cell death (PD)-1 immunotherapy for malignant tumor: A systematic review and meta-analysis. *Transl Oncol* **9,** 32–40 (2016).
91. Farkona, S., Diamandis, E. P. & Blasutig, I. M. Cancer immunotherapy: the beginning of the end of cancer? *BMC Med* **14,** 73 (2016).
92. Yun, S., Vincelette, N. D., Green, M. R., Wahner Hendrickson, A. E. & Abraham, I. Targeting immune checkpoints in unresectable metastatic

cutaneous melanoma: a systematic review and meta-analysis of anti-CTLA-4 and anti-PD-1 agents trials. *Cancer Med* **5,** 1481–1491 (2016).
93. Res, P., Blom, B., Hori, T., Weijer, K. & Spits, H. Downregulation of CD1 marks acquisition of functional maturation of human thymocytes and defines a control point in late stages of human T cell development. *J. Exp. Med.* **185,** 141–151 (1997).
94. Hardman, C. S. *et al.* CD1a presentation of endogenous antigens by group 2 innate lymphoid cells. *Sci Immunol* **2,** (2017).
95. Craig, F. E. & Foon, K. A. Flow cytometric immunophenotyping for hematologic neoplasms. *Blood* **111,** 3941–3967 (2008).
96. Kim, W. Y., Kim, H., Jeon, Y. K. & Kim, C.-W. Follicular dendritic cell sarcoma with immature T-cell proliferation. *Hum. Pathol.* **41,** 129–133 (2010).
97. McClory, S. *et al.* Evidence for a stepwise program of extrathymic T cell development within the human tonsil. *J. Clin. Invest.* **122,** 1403–1415 (2012).
98. Tong, C., Jia, X., Jia, Y. & He, Y. Langerhans cell histiocytosis in Chinese adults: absence of BRAF mutations and increased $FOXP3^+$ regulatory T cells. *Int J Clin Exp Pathol* **7,** 3166–3173 (2014).
99. Battistella, M. *et al.* Neonatal and early infantile cutaneous Langerhans cell histiocytosis: comparison of self-regressive and non-self-regressive forms. *Arch Dermatol* **146,** 149–156 (2010).
100. Quispel, W. T., Stegehuis-Kamp, J. A., Santos, S. J., Egeler, R. M. & van Halteren, A. G. S. Activated conventional T-cells are present in Langerhans cell histiocytosis lesions despite the presence of immune suppressive cytokines. *Journal of Interferon & Cytokine Research* **35,** 831–839 (2015).
101. Alaibac, M. & Chu, A. C. T-lymphocytes bearing the γδ T-cell receptor in cutaneous lesions of Langerhans' cell histiocytosis. *Med. Pediatr. Oncol.* **21,** 347–349 (1993).
102. Seddiki, N. *et al.* Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J. Exp. Med.* **203,** 1693–1700 (2006).
103. Liu, W. *et al.* CD127 expression inversely correlates with FoxP3 and suppressive function of human $CD4^+$ T reg cells. *J. Exp. Med.* **203,** 1701–1711 (2006).
104. Hua, W. *et al.* Accumulation of $FoxP3^+$ T regulatory cells in the tumour microenvironment of human colorectal adenomas. *Pathol. Res. Pract.* **212,** 106–112 (2016).
105. Zhou, X., Tang, J., Cao, H., Fan, H. & Li, B. Tissue resident regulatory T cells: novel therapeutic targets for human disease. *Cell. Mol. Immunol.* 1–10 (2015). doi:10.1038/cmi.2015.23
106. Kingsley, C. I., Karim, M., Bushell, A. R. & Wood, K. J. $CD25^+CD4^+$ regulatory T cells prevent graft rejection: CTLA-4- and IL-10-dependent immunoregulation of alloresponses. *J Immunol* **168,** 1080–1086 (2002).
107. Nakamura, K., Kitani, A. & Strober, W. Cell contact-dependent immunosuppression by $CD4^+CD25^+$ regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *J. Exp. Med.* **194,** 629–644 (2001).
108. Godfrey, D. I., Uldrich, A. P., McCluskey, J., Rossjohn, J. & Moody, D. B. The

burgeoning family of unconventional T cells. *Nat. Immunol.* **16,** 1114–1123 (2015).
109. Dhodapkar, M. V. *et al.* A reversible defect in natural killer T cell function characterizes the progression of premalignant to malignant multiple myeloma. *J. Exp. Med.* **197,** 1667–1676 (2003).
110. Molling, J. W. *et al.* Low levels of circulating invariant natural killer T cells predict poor clinical outcome in patients with head and neck squamous cell carcinoma. *Journal of Clinical Oncology* **25,** 862–868 (2007).
111. Neparidze, N. & Dhodapkar, M. V. Harnessing CD1d-restricted T cells toward antitumor immunity in humans. *Ann. N. Y. Acad. Sci.* **1174,** 61–67 (2009).
112. Sundström, P. *et al.* Human mucosa-associated invariant T cells accumulate in colon adenocarcinomas but produce reduced amounts of IFN-γ. *The Journal of Immunology* **195,** 3472–3481 (2015).
113. Ling, L. *et al.* Circulating and tumor-infiltrating mucosal associated invariant T (MAIT) cells in colorectal cancer patients. *Sci. Rep.* **6,** (2016).
114. Shijubo, N., Shigehara, K., Tsutahara, S. & Abe, S. Increased level of circulating gamma/delta T cells in a patient with eosinophilic granuloma. *Chest* **105,** 967–968 (1994).
115. Rei, M., Pennington, D. J. & Silva-Santos, B. The emerging protumor role of γδ T lymphocytes: implications for cancer immunotherapy. *Cancer Research* **75,** 798–802 (2015).
116. Presti, Lo, E., Dieli, F. & Meraviglia, S. Tumor-infiltrating γδ T lymphocytes: pathogenic role, clinical significance, and differential programing in the tumor microenvironment. *Front Immunol* **5,** 975 (2014).
117. Berzins, S. P., Smyth, M. J. & Baxter, A. G. Presumed guilty: natural killer T cell defects and human disease. *Nat. Rev. Immunol.* **11,** 131–142 (2011).
118. Held, K. *et al.* αβ T-cell receptors from multiple sclerosis brain lesions show MAIT cell-related features. *Neurol Neuroimmunol Neuroinflamm* **2,** e107 (2015).
119. Wallace, M. E., Alcantara, M. B., Minoda, Y., Kannourakis, G. & Berzins, S. P. An emerging role for immune regulatory subsets in chronic lymphocytic leukaemia. *Int. Immunopharmacol.* **28,** 897–900 (2015).
120. van der Vliet, H. J. *et al.* Circulating $V\alpha24^+$ $V\beta11^+$ NKT cell numbers are decreased in a wide variety of diseases that are characterized by autoreactive tissue damage. *Clin. Immunol.* **100,** 144–148 (2001).
121. Iwamura, C. & Nakayama, T. Role of α-galactosylceramide-activated Vα14 natural killer T cells in the regulation of allergic diseases. *Allergol Int* **56,** 1–6 (2007).
122. McEwen-Smith, R. M., Salio, M. & Cerundolo, V. The regulatory role of invariant NKT cells in tumor immunity. *Cancer Immunology Research* **3,** 425–435 (2015).
123. Gherardin, N. A., Ritchie, D. S., Godfrey, D. I. & Neeson, P. J. OMIP-021: simultaneous quantification of human conventional and innate-like T-cell subsets. *Cytometry* **85,** 573–575 (2014).
124. Anderson, B., Teyton, L., Bendelac, A. & Savage, P. Stimulation of natural killer T cells by glycolipids. *Molecules* **18,** 15662–15688 (2013).
125. Chan, A. C. *et al. Ex-vivo* analysis of human natural killer T cells demonstrates

heterogeneity between tissues and within established $CD4^+$ and $CD4^-$ subsets. *Clin Exp Immunol* **172,** 129–137 (2012).
126. Pellicci, D. G. & Uldrich, A. P. All work and no Id2 makes a dull NKT cell. *Immunol. Cell Biol.* **94,** 619–620 (2016).
127. Smyth, M. J. *et al.* Differential tumor surveillance by natural killer (NK) and NKT cells. *J. Exp. Med.* **191,** 661–668 (2000).
128. Crowe, N. Y., Smyth, M. J. & Godfrey, D. I. A critical role for natural killer T cells in immunosurveillance of methylcholanthrene-induced sarcomas. *J. Exp. Med.* **196,** 119–127 (2002).
129. Tachibana, T. *et al.* Increased intratumor Vα24-positive natural killer T cells: a prognostic factor for primary colorectal carcinomas. *Clin. Cancer Res.* **11,** 7322–7327 (2005).
130. Najera Chuc, A. E., Cervantes, L. A. M., Retiguin, F. P., Ojeda, J. V. & Maldonado, E. R. Low number of invariant NKT cells is associated with poor survival in acute myeloid leukemia. *J. Cancer Res. Clin. Oncol.* **138,** 1427–1432 (2012).
131. Shaulov, A. *et al.* Peripheral blood progenitor cell product contains Th1-biased noninvariant CD1d-reactive natural killer T cells: implications for posttransplant survival. *Exp. Hematol.* **36,** 464–472 (2008).
132. Salio, M., Silk, J. D., Jones, E. Y. & Cerundolo, V. Biology of CD1- and MR1-restricted T cells. *Annu. Rev. Immunol.* **32,** 323–366 (2014).
133. Lynch, L. *et al.* Adipose tissue invariant NKT cells protect against diet-induced obesity and metabolic disorder through regulatory cytokine production. *Immunity* **37,** 574–587 (2012).
134. Gold, M. C. *et al.* Human mucosal associated invariant T cells detect bacterially infected cells. *PLoS Biol.* **8,** e1000407 (2010).
135. Loh, L. *et al.* Human mucosal-associated invariant T cells contribute to antiviral influenza immunity via IL-18-dependent activation. *Proc. Natl. Acad. Sci. U.S.A.* **113,** 10133–10138 (2016).
136. Salio, M. *et al.* Activation of human mucosal-associated invariant T cells induces CD40L-dependent maturation of monocyte-derived and primary dendritic cells. *The Journal of Immunology* **199,** 2631–2638 (2017).
137. Hinks, T. S. C. Mucosal-associated invariant T cells in autoimmunity, immune-mediated diseases and airways disease. *Immunology* **148,** 1–12 (2016).
138. Mekinian, A. *et al.* Mucosal-associated invariant (MAIT) cells are deficient in systemic sclerosis. *Scand J Immunol* **86,** 216–220 (2017).
139. Siddiqui, S., Visvabharathy, L. & Wang, C.-R. Role of group 1 CD1-restricted T cells in infectious disease. *Front Immunol* **6,** 337 (2015).
140. De Panfilis, G., Manara, G. C., Ferrari, C., Torresani, C. & Sansoni, P. Hairy cell leukemia cells express CD1a antigen. *Cancer* **61,** 52–57 (1988).
141. Cortelazzo, S., Ponzoni, M., Ferreri, A. J. M. & Hoelzer, D. Lymphoblastic lymphoma. *Crit. Rev. Oncol. Hematol.* **79,** 330–343 (2011).
142. Hussein, M. R. Evaluation of Langerhans' cells in normal and eczematous dermatitis skin by CD1a protein immunohistochemistry: preliminary findings. *J. Cutan. Pathol.* **35,** 554–558 (2008).
143. Oji, V. *et al.* Ichthyosis vulgaris: novel *FLG* mutations in the German

population and high presence of CD1a+ cells in the epidermis of the atopic subgroup. *Br. J. Dermatol.* **160,** 771–781 (2009).
144. Alshenawy, H. A. & Hasby, E. A. Immunophenotyping of dendritic cells in lesional, perilesional and distant skin of chronic plaque psoriasis. *Cell. Immunol.* **269,** 115–119 (2011).
145. de Jong, A. *et al.* CD1a-autoreactive T cells are a normal component of the human αβ T cell repertoire. *Nat. Immunol.* **11,** 1102–1109 (2010).
146. de Lalla, C. *et al.* High-frequency and adaptive-like dynamics of human CD1 self-reactive T cells. *Eur. J. Immunol.* **41,** 602–610 (2011).
147. Kasmar, A. G. *et al.* Cutting edge: CD1a tetramers and dextramers identify human lipopeptide-specific T cells ex vivo. *The Journal of Immunology* **191,** 4499–4503 (2013).
148. Carrera Silva, E. A. *et al.* $CD207^{+}CD1a^{+}$ cells circulate in pediatric patients with active Langerhans cell histiocytosis. *Blood* **130,** 1898–1902 (2017).
149. Quispel, W. T. *et al.* The presence of $CXCR4^{+}$ $CD1a^{+}$ cells at onset of Langerhans cell histiocytosis is associated with a less favorable outcome. *Oncoimmunology* **5,** e1084463 (2016).
150. de Jong, A. *et al.* CD1a-autoreactive T cells recognize natural skin oils that function as headless antigens. *Nat. Immunol.* **15,** 177–185 (2014).
151. Exley, M. *et al.* CD1d structure and regulation on human thymocytes, peripheral blood T cells, B cells and monocytes. *Immunology* **100,** 37–47 (2000).
152. Salamone, M. C., Rabinovich, G. A., Mendiguren, A. K., Salamone, G. V. & Fainboim, L. Activation-induced expression of CD1d antigen on mature T cells. *J. Leukoc. Biol.* **69,** 207–214 (2001).
153. Brigl, M. & Brenner, M. B. CD1: antigen presentation and T cell function. *Annu. Rev. Immunol.* **22,** 817–890 (2004).
154. Schutyser, E., Struyf, S. & Van Damme, J. The CC chemokine CCL20 and its receptor CCR6. *Cytokine Growth Factor Rev.* **14,** 409–426 (2003).
155. Charbonnier, A. S. *et al.* Macrophage inflammatory protein 3α is involved in the constitutive trafficking of epidermal Langerhans cells. *J. Exp. Med.* **190,** 1755–1767 (1999).
156. Dieu-Nosjean, M. C. *et al.* Macrophage inflammatory protein 3α is expressed at inflamed epithelial surfaces and is the most potent chemokine known in attracting Langerhans cell precursors. *J. Exp. Med.* **192,** 705–717 (2000).
157. Fleming, M. D. *et al.* Coincident expression of the chemokine receptors CCR6 and CCR7 by pathologic Langerhans cells in Langerhans cell histiocytosis. *Blood* **101,** 2473–2475 (2003).
158. Ouwehand, K. *et al.* CXCL12 is essential for migration of activated Langerhans cells from epidermis to dermis. *Eur. J. Immunol.* **38,** 3050–3059 (2008).
159. Ohl, L. *et al.* CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions. *Immunity* **21,** 279–288 (2004).
160. Sallusto, F. *et al.* Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *Eur. J. Immunol.* **28,** 2760–2769 (1998).
161. Dieu, M. C. *et al.* Selective recruitment of immature and mature dendritic

cells by distinct chemokines expressed in different anatomic sites. *J. Exp. Med.* **188,** 373–386 (1998).
162. Sozzani, S. Dendritic cell trafficking: more than just chemokines. *Cytokine Growth Factor Rev.* **16,** 581–592 (2005).
163. Hogstad, B. *et al.* RAF/MEK/extracellular signal-related kinase pathway suppresses dendritic cell migration and traps dendritic cells in Langerhans cell histiocytosis lesions. *Journal of Experimental Medicine* **215,** 319–336 (2018).
164. Jersmann, H. P. A. Time to abandon dogma: CD14 is expressed by non-myeloid lineage cells. *Immunol. Cell Biol.* **83,** 462–467 (2005).
165. de Saint-Vis, B. *et al.* A novel lysosome-associated membrane glycoprotein, DC-LAMP, induced upon DC maturation, is transiently expressed in MHC class II compartment. *Immunity* **9,** 325–336 (1998).
166. Dudek, A. M., Martin, S., Garg, A. D. & Agostinis, P. Immature, semi-mature, and fully mature dendritic cells: toward a DC-cancer cells interface that augments anticancer immunity. *Front Immunol* **4,** 438 (2013).
167. Sakaguchi, S., Yamaguchi, T., Nomura, T. & Ono, M. Regulatory T cells and immune tolerance. *Cell* **133,** 775–787 (2008).
168. Chaudhary, B. & Elkord, E. Regulatory T Cells in the tumor microenvironment and cancer progression: role and therapeutic targeting. *Vaccines (Basel)* **4,** (2016).
169. de Graaf, J. H., Tamminga, R. Y., Dam-Meiring, A., Kamps, W. A. & Timens, W. The presence of cytokines in Langerhans' cell histiocytosis. *J. Pathol.* **180,** 400–406 (1996).
170. Morimoto, A. *et al.* Inflammatory serum cytokines and chemokines increase associated with the disease extent in pediatric Langerhans cell histiocytosis. *Cytokine* **97,** 73–79 (2017).
171. Geissmann, F. *et al.* Transforming growth factor β1, in the presence of granulocyte/macrophage colony-stimulating factor and interleukin 4, induces differentiation of human peripheral blood monocytes into dendritic Langerhans cells. *J. Exp. Med.* **187,** 961–966 (1998).
172. Hutter, C. *et al.* Notch is active in Langerhans cell histiocytosis and confers pathognomonic features on dendritic cells. *Blood* **120,** 5199–5208 (2012).
173. Blanchard, N., Di Bartolo, V. & Hivroz, C. In the immune synapse, ZAP-70 controls T cell polarization and recruitment of signaling proteins but not formation of the synaptic pattern. *Immunity* **17,** 389–399 (2002).
174. Steinberg, M. *et al.* T-cell receptor-induced phosphorylation of the ζ chain is efficiently promoted by ZAP-70 but not Syk. *Blood* **104,** 760–767 (2004).
175. Francisco, L. M., Sage, P. T. & Sharpe, A. H. The PD-1 pathway in tolerance and autoimmunity. *Immunol. Rev.* **236,** 219–242 (2010).
176. Last'ovicka, J., Budinský, V., Spísek, R. & Bartůnková, J. Assessment of lymphocyte proliferation: CFSE kills dividing cells and modulates expression of activation markers. *Cell. Immunol.* **256,** 79–85 (2009).
177. Baecher-Allan, C., Wolf, E. & Hafler, D. A. MHC class II expression identifies functionally distinct human regulatory T cells. *The Journal of Immunology* **176,** 4622–4631 (2006).
178. Almehmadi, M., Flanagan, B. F., Khan, N., Alomar, S. & Christmas, S. E.

Increased numbers and functional activity of $CD56^+$ T cells in healthy cytomegalovirus positive subjects. *Immunology* **142,** 258–268 (2014).
179. Kelly-Rogers, J., Madrigal-Estebas, L., O'Connor, T. & Doherty, D. G. Activation-induced expression of CD56 by T cells is associated with a reprogramming of cytolytic activity and cytokine secretion profile *in vitro*. *Human Immunology* **67,** 863–873 (2006).
180. Takei, Y. *et al.* Killer cell immunoglobulin-like receptor 2DL4 is expressed in and suppresses the cell growth of Langerhans cell histiocytosis. *Oncotarget* (2017). doi:10.18632/oncotarget.16936
181. Peters, T. L., McClain, K. L. & Allen, C. E. Neither IL-17A mRNA nor IL-17A protein are detectable in Langerhans cell histiocytosis lesions. *Mol. Ther.* **19,** 1433–1439 (2011).
182. Coury, F. *et al.* Langerhans cell histiocytosis reveals a new IL-17A-dependent pathway of dendritic cell fusion. *Nat. Med.* **14,** 81–87 (2008).
183. Van Acker, H. H., Capsomidis, A., Smits, E. L. & Van Tendeloo, V. F. CD56 in the immune system: more than a marker for cytotoxicity? *Front Immunol* **8,** 892 (2017).
184. Gherardin, N. A. *et al.* Human blood MAIT cell subsets defined using MR1 tetramers. *Immunol. Cell Biol.* **96,** 507–525 (2018).
185. Lanza, F., Bi, S., Castoldi, G. & Goldman, J. M. Abnormal expression of N-CAM (CD56) adhesion molecule on myeloid and progenitor cells from chronic myeloid leukemia. *Leukemia* **7,** 1570–1575 (1993).
186. Alegretti, A. P. *et al.* The expression of CD56 antigen is associated with poor prognosis in patients with acute myeloid leukemia. *Rev Bras Hematol Hemoter* **33,** 202–206 (2011).
187. Gong, P. *et al.* Aberrant expression of CD56 on granulocytes and monocytes in myeloproliferative neoplasm. *J Hematopathol* **6,** 127–134 (2013).
188. Li, X. *et al.* Identification of a $FOXP3^+CD3^+CD56^+$ population with immunosuppressive function in cancer tissues of human hepatocellular carcinoma. *Sci. Rep.* **5,** 14757 (2015).
189. Kumari, S. *et al.* Leishmania donovani mediated higher expression of CCL4 induces differential accumulation of $CD4^+CD56^+$NKT and $CD8^+CD56^+$NKT cells at infection site. *Cytokine* (2018). doi:10.1016/j.cyto.2018.03.022
190. Janikashvili, N., Bonnotte, B., Katsanis, E. & Larmonier, N. The dendritic cell-regulatory T lymphocyte crosstalk contributes to tumor-induced tolerance. *Clin. Dev. Immunol.* **2011,** 430394 (2011).
191. Hirota, Y. *et al.* The expression and possible roles of chemokine CXCL11 and its receptor CXCR3 in the human endometrium. *J Immunol* **177,** 8813–8821 (2006).
192. Pittet, M. J., Speiser, D. E., Valmori, D., Cerottini, J. C. & Romero, P. Cutting edge: cytolytic effector function in human circulating $CD8^+$ T cells closely correlates with CD56 surface expression. *J Immunol* **164,** 1148–1152 (2000).
193. Hasumi, K., Aoki, Y., Wantanabe, R. & Mann, D. L. Clinical response of advanced cancer patients to cellular immunotherapy and intensity-modulated radiation therapy. *Oncoimmunology* **2,** e26381 (2013).
194. Bourgeois, E. A. *et al.* Bee venom processes human skin lipids for presentation by CD1a. *Journal of Experimental Medicine* **212,** 149–163

(2015).
195. Kim, J. H. *et al.* CD1a on Langerhans cells controls inflammatory skin disease. *Nat. Immunol.* **17,** 1159–1166 (2016).
196. Cheng, J. *et al.* Total synthesis of the *Mycobacterium tuberculosis* dideoxymycobactin-838 and stereoisomers: diverse CD1a-restricted T cells display a common hierarchy of lipopeptide recognition. *Chemistry* **23,** 1694–1701 (2017).
197. Birkinshaw, R. W. *et al.* αβ T cell antigen receptor recognition of CD1a presenting self lipid ligands. *Nat. Immunol.* **16,** 258–266 (2015).
198. Schwentner, R. *et al.* JAG2 signaling induces differentiation of $CD14^+$ monocytes into Langerhans cell histiocytosis-like cells. *J. Leukoc. Biol.* (2018). doi:10.1002/JLB.1A0318-098R
199. Yang, X. O. *et al.* Regulation of inflammatory responses by IL-17F. *Journal of Experimental Medicine* **205,** 1063–1075 (2008).
200. Jin, W. & Dong, C. IL-17 cytokines in immunity and inflammation. *Emerg Microbes Infect* **2,** e60 (2013).
201. Larregina, A. T. *et al.* Dermal-resident $CD14^+$ cells differentiate into Langerhans cells. *Nat. Immunol.* **2,** 1151–1158 (2001).
202. Lynch, G. W. *et al.* CD4 is expressed by epidermal Langerhans' cells predominantly as covalent dimers. *Exp. Dermatol.* **12,** 700–711 (2003).
203. Zhen, A. *et al.* CD4 ligation on human blood monocytes triggers macrophage differentiation and enhances HIV infection. *J. Virol.* **88,** 9934–9946 (2014).
204. Milne, P., Bigley, V., Gunawan, M., Haniffa, M. & Collin, M. $CD1c^+$ blood dendritic cells have Langerhans cell potential. *Blood* **125,** 470–473 (2015).
205. Milne, P. *et al.* Hematopoietic origin of Langerhans cell histiocytosis and Erdheim Chester disease in adults. *Blood* (2017). doi:10.1182/blood-2016-12-757823
206. Davis, A. L., McKenzie, J. L. & Hart, D. N. HLA-DR-positive leucocyte subpopulations in human skin include dendritic cells, macrophages, and CD7-negative T cells. *Immunology* **65,** 573–581 (1988).
207. Murakami, I. *et al.* Establishment of a Langerhans cell histiocytosis lesion cell line with dermal dendritic cell characteristics. *Oncol. Rep.* **33,** 171–178 (2015).
208. Gogusev, J. *et al.* DOR-1, a novel CD10+ stromal cell line derived from progressive Langerhans cell histiocytosis of bone. *Pediatr. Blood Cancer* **44,** 128–137 (2005).
209. Molling, J. W. *et al.* Generation and sustained expansion of mouse spleen invariant NKT cell lines with preserved cytokine releasing capacity. *Journal of Immunological Methods* **322,** 70–81 (2007).
210. Carolan, E. *et al.* Altered distribution and increased IL-17 production by mucosal-associated invariant T cells in adult and childhood obesity. *The Journal of Immunology* **194,** 5775–5780 (2015).
211. Reantragoon, R. *et al.* Antigen-loaded MR1 tetramers define T cell receptor heterogeneity in mucosal-associated invariant T cells. *Journal of Experimental Medicine* **210,** 2305–2320 (2013).
212. Novak, J., Dobrovolny, J., Novakova, L. & Kozak, T. The decrease in number and change in phenotype of mucosal-associated invariant T cells in the

elderly and differences in men and women of reproductive age. *Scand J Immunol* **80,** 271–275 (2014).
213. Kumar, V. & Ahmad, A. Role of MAIT cells in the immunopathogenesis of inflammatory diseases: new players in old game. *Int. Rev. Immunol.* 1–21 (2017). doi:10.1080/08830185.2017.1380199
214. Kurioka, A. *et al.* Shared and distinct phenotypes and functions of human CD161++ Vα7.2+ T cell subsets. *Front Immunol* **8,** 1031 (2017).
215. Attaf, M., Legut, M., Cole, D. K. & Sewell, A. K. The T cell antigen receptor: the Swiss army knife of the immune system. *Clin Exp Immunol* **181,** 1–18 (2015).
216. Fergusson, J. R. *et al.* CD161 defines a transcriptional and functional phenotype across distinct human T cell lineages. *Cell Rep* **9,** 1075–1088 (2014).
217. O'Neil-Andersen, N. J. & Lawrence, D. A. Differential modulation of surface and intracellular protein expression by T cells after stimulation in the presence of monensin or brefeldin A. *Clinical and Vaccine Immunology* **9,** 243–250 (2002).
218. Chan, A. C. *et al.* Immune characterization of an individual with an exceptionally high natural killer T cell frequency and her immediate family. *Clin Exp Immunol* **156,** 238–245 (2009).
219. Lourda, M. *et al.* Detection of IL-17A-producing peripheral blood monocytes in Langerhans cell histiocytosis patients. *Clin. Immunol.* **153,** 112–122 (2014).
220. Zeng, Z. *et al.* Crystal structure of mouse CD1: an MHC-like fold with a large hydrophobic binding groove. *Science* **277,** 339–345 (1997).
221. Porcelli, S., Morita, C. T. & Brenner, M. B. CD1b restricts the response of human $CD4^{-}8^{-}$ T lymphocytes to a microbial antigen. *Nature* **360,** 593–597 (1992).
222. Zajonc, D. M. *et al.* Structural basis for CD1d presentation of a sulfatide derived from myelin and its implications for autoimmunity. *J. Exp. Med.* **202,** 1517–1526 (2005).
223. Teyton, L. Role of lipid transfer proteins in loading CD1 antigen presenting molecules. *J. Lipid Res.* (2018). doi:10.1194/jlr.R083212
224. Van Rhijn, I., Godfrey, D. I., Rossjohn, J. & Moody, D. B. Lipid and small-molecule display by CD1 and MR1. *Nature Publishing Group* **15,** 643–654 (2015).
225. Jayawardena-Wolf, J., Benlagha, K., Chiu, Y. H., Mehr, R. & Bendelac, A. CD1d endosomal trafficking is independently regulated by an intrinsic CD1d-encoded tyrosine motif and by the invariant chain. *Immunity* **15,** 897–908 (2001).
226. Al-Amodi, O. *et al.* CD1a-expressing monocytes as mediators of inflammation in ulcerative colitis. *Inflamm. Bowel Dis.* **24,** 1225–1236 (2018).
227. Laupeze, B. *et al.* Polycyclic aromatic hydrocarbons affect functional differentiation and maturation of human monocyte-derived dendritic cells. *J Immunol* **168,** 2652–2658 (2002).
228. Leslie, D. S. *et al.* Serum lipids regulate dendritic cell CD1 expression and function. *Immunology* **125,** 289–301 (2008).
229. Yu, R. C., Kolettas, E., Kamalati, T., Chu, A. & Buluwela, L. Stable expression

of CD1a molecule in human epithelial cell lines shows temperature-dependent expression and affects cell morphology and growth. *Arch. Dermatol. Res.* **289,** 352–359 (1997).
230. Shamshiev, A. *et al.* Presentation of the same glycolipid by different CD1 molecules. *J. Exp. Med.* **195,** 1013–1021 (2002).
231. Agea, E. *et al.* Human CD1-restricted T cell recognition of lipids from pollens. *J. Exp. Med.* **202,** 295–308 (2005).
232. Godfrey, D. I., Le Nours, J., Andrews, D. M., Uldrich, A. P. & Rossjohn, J. Unconventional T cell targets for cancer immunotherapy. *Immunity* **48,** 453–473 (2018).
233. Mori, L., Lepore, M. & De Libero, G. The immunology of CD1- and MR1-restricted T cells. *Annu. Rev. Immunol.* **34,** 479–510 (2016).
234. Lepore, M. *et al.* A novel self-lipid antigen targets human T cells against $CD1c^+$ leukemias. *Journal of Experimental Medicine* **211,** 1363–1377 (2014).
235. Grishkan, I. V., Ntranos, A., Calabresi, P. A. & Gocke, A. R. Helper T cells down-regulate CD4 expression upon chronic stimulation giving rise to double-negative T cells. *Cell. Immunol.* **284,** 68–74 (2013).
236. Quandt, D., Rothe, K., Scholz, R., Baerwald, C. W. & Wagner, U. Peripheral CD4CD8 double positive T cells with a distinct helper cytokine profile are increased in rheumatoid arthritis. *PLoS ONE* **9,** e93293 (2014).
237. Nascimbeni, M., Shin, E.-C., Chiriboga, L., Kleiner, D. E. & Rehermann, B. Peripheral $CD4^+CD8^+$ T cells are differentiated effector memory cells with antiviral functions. *Blood* **104,** 478–486 (2004).
238. Stradner, M. H., Cheung, K. P., Lasorella, A., Goldrath, A. W. & D'Cruz, L. M. Id2 regulates hyporesponsive invariant natural killer T cells. *Immunol. Cell Biol.* **94,** 640–645 (2016).
239. Gatalica, Z. *et al.* Disseminated histiocytoses biomarkers beyond BRAFV600E: frequent expression of PD-L1. *Oncotarget* **6,** 19819–19825 (2015).

APPENDIX A – SORT PURITIES, CELL COUNTS, CT AND ΔCT VALUES

Table A1. Gene expression by conventional T cells (excluding Tregs) following purification and transcriptome amplification.

Donor description	Sort purity (%)	WTA start cell number	Ct *RPLP0*	ΔCt *LIF*	ΔCt *LIFR*	ΔCt *IL2RA*	ΔCt *CD40LG*	ΔCt *TNF*
HD 1	99.5	980	23.43	+3.53	+7.13	+5.12	+4.04	+11.12
HD 2	99.3	980	23.00	+7.50	+1.49	-0.71	+2.60	+2.06
HD 3	99.9	1000	24.08	+2.69	+25.92	+2.74	+4.30	+25.92
HD 4	99.3	1000	31.82	+2.31	+18.19	+3.49	+18.19	+4.04
HD 5	99.9	1000	>50	*NA*[1]	*NA*[1]	*NA (Ct =34.16)*	*NA*[1]	*NA*[1]
HD 6	99.2	1000	>50	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]
LCH 1 PB	94.3	210	>50	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]
LCH 14 PB	99.9	956	25.18	+1.32	+1.08	+4.43	+0.09	-1.27
LCH 15 PB	98.3	1000	>50	*NA*[1]	*NA (Ct =22.27)*	*NA*[1]	*NA*[1]	*NA*[1]
LCH 16 PB	100	1000	>50	*NA*[1]	*NA*[1]	*NA (Ct =28.65)*	*NA*[1]	*NA*[1]
LCH 17 PB	99.7	1000	>50	*NA (Ct =33.92)*	*NA (Ct =29.89)*	*NA*[1]	*NA (Ct =30.76)*	*NA*[1]
LCH 1 lesion	96.8	630	23.58	+1.67	+1.09	+1.47	+2.69	+4.01
LCH 2 lesion	99.4	950	27.71	*NA*[1]	-3.01	-2.46	*NA*[1]	*NA*[1]
LCH 13 lesion	100	<40*	>50	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]
LCH 14 lesion	100	<250*	>50	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]
LCH 18 lesion	*NA*	0	-	-	-	-	-	-

**No cell count – estimate from FACS report only*

[1]Where gene expression was undetectable (Ct >50)

ΔCt =change in cycle threshold compared to reference gene (RPLPO)

Green font indicates successful gene amplification.

Table A2. Gene expression by Tregs following purification and transcriptome amplification.

Donor description	Sort purity (%)	WTA start cell number	Ct *RPLP0*	ΔCt *TGFB1*	ΔCt *NCAM1*	ΔCt *IL2RA*	ΔCt *CTLA4*	ΔCt *HLA-DRA*	ΔCt *FOXP3*	ΔCt *CD40LG*	ΔCt *LIFR*	ΔCt *IL10*	ΔCt *TNF*
HD 1	95.2	280	>50	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]	*NA (Ct =34.02)*	*NA*[1]	-
HD 2	95.2	420	31.42	-0.42	+2.63	+1.23	-0.35	-0.70	+2.39	+2.58	-1.45	-1.87	+1.8
HD 3	96.4	280	36.84	-0.73	-7.21	-4.79	-2.50	*NA*[1]	*NA*[1]	-0.44	-1.41	-9.16	*NA*[1]
HD 4	96.6	1000	34.10	-0.04	-0.23	*NA*[1]	*NA*[1]	*NA*[1]	+0.32	+6.95	*NA*[1]	+2.60	*NA*[1]
HD 5	87.7	863	36.40	*NA*[1]	*NA*[1]	-5.39	+3.65	*NA*[1]	+4.23	*NA*[1]	*NA*[1]	*NA*[1]	+3.48
HD 6	94.8	910	33.22	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]	+6.36	*NA*[1]	-4.17	*NA*[1]	*NA*[1]
LCH 1 PB	*NA*	<90*	>50	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]	-
LCH 14 PB	90.7	<1000*	>50	*NA (Ct =47.32)*	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]	*NA (Ct =34.4)*	*NA (Ct =42.59)*	*NA*[1]	*NA*[1]	-
LCH 15 PB	82.0	1000	40.20	-17.03	*NA*[1]	-11.49	*NA*[1]	*NA*[1]	-8.74	*NA (Ct =42.27)*	*NA*[1]	+0.65	*NA*[1]
LCH 16 PB	90.3	<225*	>50	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]	-
LCH 17 PB	92.2	<350*	>50	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]	*NA (Ct =41.56)*	*NA*[1]	*NA (Ct =39.51)*	*NA*[1]	-
LCH 1 lesion	94.0	<1000*	30.66	-3.92	+3.64	-0.45	-2.39	*NA*[1]	+5.68	+0.88	+2.61	+2.93	-3.08
LCH 2 lesion	97.3	140	40.14	*NA*[1]	-9.64	-13.96	*NA*[1]	*NA*[1]	+1.43	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]
LCH 13 lesion	*NA*	<6*	-	-	-	-	-	-	-	-	-	-	-
LCH 14 lesion	80.0	<25*	>50	*NA (Ct =47.37)*	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]	*NA*[1]	-
LCH 18 lesion	*NA*	0	-	-	-	-	-	-	-	-	-	-	-

**No cell count – estimate from FACS report only*

[1]Where gene expression was undetectable (Ct >50)

ΔCt =change in cycle threshold compared to reference gene (RPLPO)

Green font indicates successful gene amplification.

Table A3. Gene expression by myeloid cells following purification and transcriptome amplification.

Donor description	Sort purity (%)	WTA start cell number	Ct *RPLP0*	ΔCt *LIF*	ΔCt *LIFR*	ΔCt *CD80*	ΔCt *CD86*	ΔCt *CD1A*	ΔCt *CD1B*	ΔCt *CD1C*	ΔCt *CD1D*	ΔCt *MR1*	ΔCt *CD274*	ΔCt *PDCD1LG2*	ΔCt *CD40*	ΔCt *CLEC2D*	ΔCt TNF
HD 1	96.8	1000	29.10	+18.52	+6.44	+1.26	+15.14	-0.1	+5.01	+0.13	NA[1]	NA[2]	+11.89	-1.60	NA[1]	+7.57	+0.87
HD 2	90.5	1000	29.20	+5.77	+1.11	+1.53	+8.76	-1.92	-0.76	-1.48	-1.65	+1.93	NA[1]	+0.13	+1.52	+2.59	+7.65
HD 3	99.2	1000	38.00	-2.07	NA[1]	-6.57	NA[1]	NA[1]	-2.31	NA[1]	-6.74	NA[1]	*-0.69*	*-7.14*	*-8.72*	NA[1]	NA[1]
HD 4	96.2	1000	>50	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]
HD 5	99.0	1000	>50	NA[1]	NA[1]	*NA (Ct =38.11)*	NA[1]	NA[1]	-7.53	NA[2]	NA[1]	NA[2]	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]
HD 6	98.6	1000	>50	NA[1]	NA[1]	NA[1]	NA[1]	NA[2]	NA[1]	*NA (Ct =37.96)*	NA[1]	*NA (Ct =36.45)*	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]
LCH 1 PB	94.4	840	>50	NA[1]	NA[1]	NA[1]	NA[1]	NA[2]	NA[1]	NA[2]	NA[1]	NA[2]	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]
LCH 14 PB	97.7	1000	26.33	+10.94	+11.51	+6.23	+8.02	+5.06	+7.53	+7.11	+9.31	+3.65	NA[1]	+4.92	+2.25	+12.44	+5.13
LCH 15 PB	97.5	1000	31.78	NA[1]	+5.05	NA[1]	NA[1]	+5.04	+8.47	NA[2]	NA[1]	NA[2]	+5.39	NA[1]	NA[1]	NA[1]	NA[1]
LCH 16 PB	?	840	>50	NA[1]	NA[1]	NA[1]	NA[1]	NA[2]	*NA (Ct =28.13)*	NA[2]	NA[1]	NA[2]	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]
LCH 17 PB	92.8	280	>50	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]
LCH 1 lesion	90.3	920	33.29	+1.58	-2.22	-6.43	+6.29	-1.78	-9.88	NA[2]	-5.69	*-2.24*	+7.27	+3.69	+4.38	+5.87	+3.11
LCH 2 lesion	96.9	420	39.62	NA[1]	-5.76	+0.26	-0.25	NA[2]	NA[1]	-1.55	NA[1]	*-7.97*	NA[1]	NA[1]	*+0.72*	NA[1]	NA[1]
LCH 13 lesion	100	<125*	>50	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]
LCH 14 lesion	91.0	<375*	42.16	NA[1]	NA[1]	NA[1]	NA[1]	NA[2]	NA[1]	NA[2]	NA[1]	NA[2]	NA[1]	NA[1]	NA[1]	NA[1]	NA[1]
LCH 18 lesion	*NA*	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

**No cell count – estimate from FACS report only*

[1]Where gene expression was undetectable (Ct >50)

[2]Primer dimers occurred before 50 cycles, therefore Ct values up to 50 were not able to be determined

ΔCt =change in cycle threshold compared to reference gene (RPLPO)

Green font indicates successful gene amplification.

APPENDIX B – MANUSCRIPTS

SCIENTIFIC REPORTS

OPEN

Altered Populations of Unconventional T Cell Lineages in Patients with Langerhans Cell Histiocytosis

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Langerhans cell histiocytosis (LCH) lesions are defined by the presence of CD1a$^+$/CD207$^+$ myeloid cells, but many other immune cells are present including unconventional T cells, which have powerful immunoregulatory functions. Unconventional T cell lineages include mucosal-associated invariant T (MAIT) cells, type I natural killer T (NKT) cells and gamma-delta (γδ) T cells, which are associated with many inflammatory conditions, although their importance has not been studied in LCH. We characterized their phenotype and function in blood and lesions from patients with LCH, and identified a deficiency in MAIT cell frequency and abnormalities in the subset distributions of γδ T cells and NKT cells. Such abnormalities are associated with immune dysregulation in other disease settings and are therefore potentially important in LCH. Our study is the first to recognize alterations to MAIT cell proportions in patients with LCH. This finding along with other abnormalities identified amongst unconventional T cells could potentially influence the onset and progression of LCH, thereby highlighting potential targets for new immune based therapies.

Langerhans cell histiocytosis (LCH) is a rare disease that most frequently affects children but can also occur in adults[1]. LCH is characterized by inflammatory lesions affecting one or more organs. Osseous and cutaneous tissue are the sites most frequently affected while liver, spleen and hematopoietic involvement are associated with an increased mortality risk[2]. All LCH lesions contain myeloid lineage cells that express CD1a and CD207[2–4]. These 'LCH cells' form the characteristic LCH microenvironment alongside a cellular infiltrate of T cells, macrophages, eosinophils, neutrophils, B cells, plasma cells and multinucleated giant cells[3,5]. Immune cells are fundamental to the inflammation and subsequent organ damage seen in LCH, but the role of different lineages is not well understood and they have not been specifically targeted in therapies. Recent advances in our understanding of immune regulation coupled with the development of new immunotherapies suggests that immune cells within LCH lesions may be potential targets for new treatments.

Patients with LCH often have mutations in the mitogen-activated protein kinase (MAPK) cell signalling pathway[6–12] and show signs of immune dysregulation[5,13–15], although the nature of these defects and their significance to the etiology of LCH is not fully understood. The composition and inflammatory characteristics of LCH lesions suggest a localised dysregulation of immune cells and a link between innate and adaptive immunity at the site of inflammation in LCH remains to be elucidated. The characteristic presence of LCH cells implies their involvement in the pathogenesis of this disease and it is possible that they promote T cell signalling that leads to potent cytokine release within lesions. This hypothesis is consistent with the large number of activated T cells within LCH lesions[5,13,16].

Interestingly, there are also signs of immune abnormalities outside of the lesions of patients with LCH. For example, there are reports of CD1a$^+$ LCH-like cells with myeloid characteristics[1,17,18] and increased Foxp3$^+$

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Patient Code	Specimen description	Sex	Age at diagnosis	Tissues affected	Treatment prior to specimen	Age at specimen	Disease status at specimen	Sequelae, other comments
1	Bone lesion	M	68	Bone	Previous irradiation at site of and excision of hip lesion	68	Active (AD)	Diabetes insipidus from age 55
1	Matched blood							
2	Pulmonary lesion	M	40	Lung	Nil	40	AD	Mild pulmonary fibrosis, smoker
2	Matched blood							
3	Involved lymph node	M	9	Bone, lymph node	Nil	9	AD	Skull defect
4	Blood	M	18 months	Skin	Nil	13	Non-active (NAD)	
5	Blood	M	53	Bone, lung, bone marrow	Vinblastine/prednisolone	64	NAD	Mild pulmonary fibrosis
6	Blood	M	39	Lung	Vinblastine/prednisolone	52	NAD	
7	Blood	F	60	Skin	Methotrexate/prednisolone	64	NAD	Leg scarring
8	Blood	F	36	Bone	Lesion excision	37	NAD	
9	Blood	F	25	Bone	Vinblastine/prednisolone	42	NAD	Ataxia at time of specimen
10	Blood	M	41	Skin	Nil	41	AD	
11	Blood	F	39	Bone, lung	Nil	39	AD	
12	Blood	F	34	Lung	Biopsy and vinblastine/prednisolone	54	NAD	Long term pulmonary fibrosis
13	Bone lesion	M	2	Bone	Nil	2	AD	Mutation in BRAFV600, CNS-risk lesion
13	Matched blood							
14	Blood	M	8 months	Skin, lymph node, liver	Nil	22 months	AD	No detectable mutation in BRAFV600
15	Blood	M	10	Bone, skin	Cytarabine, prednisolone and vinblastine (ceased 6 months prior to specimen)	12	AD	Diabetes insipidus, mutation in BRAFV600
16	Blood	M	67	Bone, skin	Nil	67	NAD	

Table 1. Clinical details of study participants.

regulatory T cells (Tregs)[14] in the circulation of patients with active LCH that suggest altered immune regulation in LCH. Other lineages of immune cells with known regulatory functions have not been well-studied in LCH patients. Prime candidates to investigate are unconventional T cells such as mucosal associated invariant T (MAIT) cells, gamma delta (γδ) T cells and type I natural killer T cells (referred to herein as NKT cells), which are all capable of rapid inflammatory cytokine responses that can trigger and potentiate innate and adaptive immune responses. These unconventional T cells are already proposed to play fundamental roles in regulating aspects of tumor immunity, infection and autoimmunity[19–23], piquing our curiosity about their role in LCH. One study found γδ T cells at a high frequency in LCH lesions[24] and there is overexpression of *CD1D* in LCH cells compared with skin-resident Langerhans cells (LCs)[25]. NKT cells respond to lipids presented by the major histocompatibility complex (MHC)-like molecule, CD1d, hence higher expression of *CD1D* in lesions suggests a potential role for NKT cells in LCH. MAIT cells were first associated with anti-microbial responses, but have more recently been found to have an altered function in colorectal tumors[22,23] and type 2 diabetes[26], and they are associated with several autoimmune diseases[27,28]. Like NKT cells, MAIT cells have not been studied in LCH, but expression of CD161 is a defining characteristic of MAIT cells, and it is noteworthy that the gene encoding for the C-type lectin receptor for CD161 (*CLEC2D*) is also overexpressed by LCH cells compared with skin-resident LCs[25]. A unique characteristic of unconventional T cells is their ability to produce large amounts of T helper (Th) 1 (TNF/IFNγ), Th2 (IL-4), Th10 (IL-10), or Th17 (IL-17) cytokines[29–31] (also see review by Godfrey *et al.*[32]) depending on the setting. Consequently, unconventional T cells are a logical target of investigation in an inflammatory setting such as LCH. We therefore characterized the frequency and function of unconventional T cell lineages in blood and tissue lesions from patients with LCH and healthy individuals. We hypothesize that the immune dysregulation accompanying LCH progression is associated with abnormalities in unconventional T cells and that one or more of these lineages may represent a viable target for new treatments.

Results

Clinical details of patients with LCH. Our cohort included 11 male and 5 female patients with LCH aged from 22 months to 68 years (Table 1). The most commonly affected tissues were bone, skin and lung. With the exception of one patient, donors with active disease (AD) had no known prior treatment with steroids or chemotherapy at the time of specimen collection, but the majority of those with non-active disease (NAD) had received treatments (Table 1).

Circulatory MAIT cell proportion is decreased in patients with LCH. Unconventional T cells were stringently identified according to the following criteria: MAIT cells were classified as $CD3^+V\alpha7.2\ TCR^+CD161^+$ lymphocytes, γδ T cells as $CD3^+$γδ TCR^+ and NKT cells as $CD3^+$PBS44-loaded CD1d-tetramer$^+$ lymphocytes. Specimens analyzed were from the blood of healthy donors and patients with LCH, and from lesions from patients with LCH (Fig. 1a). The frequency of MAIT cells expressed as a proportion of total T cells was significantly lower in blood ($p = 0.0010$) and lesions ($p = 0.0353$) from patients with LCH compared to healthy donors (Fig. 1b). The measure of MAIT cell frequency can sometimes be confounded by downregulation of CD161, however the proportion of $V\alpha7.2\ TCR^+CD161^-$ T cells did not increase in blood and lesions from LCH donors (Fig. 1b). No substantial difference was seen in the proportions of γδ T cells or NKT cells across the groups (Fig. 1b). Interestingly, there was greater variability in the proportion of circulatory NKT cells in patients with LCH (*range = 0.000% to 8.68%*) compared to healthy donors (*range = 0.005% to 0.871%*) (Fig. 1b). Blood samples from patients with LCH were further stratified into AD and NAD, however there were no differences in the proportions of circulatory MAIT cells, γδ T cells or NKT cells (Fig. 1c). Thus, while no discernible differences were observed in unconventional T cells between samples from AD and NAD donors, MAIT cells were reduced in samples from LCH donors compared to healthy blood donors.

Comparing MAIT cell identification methods and proportions across age groups. We identified MAIT cells as $CD3^+V\alpha7.2\ TCR^+CD161^+$ lymphocytes, however a recently developed alternative is the use of 5-OP-RU-loaded MR1 tetramer[33]. We conducted an analysis to confirm that both approaches identify the same cells. Indeed, we found that $V\alpha7.2\ TCR^+CD161^+$ MAIT cells were almost exclusively 5-OP-RU-loaded MR1 tetramer$^+$ and 5-OP-RU-loaded MR1 tetramer$^+$ MAIT cells were almost all $V\alpha7.2\ TCR^+CD161^+$ (Fig. 2a), and importantly, that both approaches yielded similar data in analysis of LCH samples (Fig. 2b).

We also noted that MAIT cell frequency can fall with age in healthy individuals[34,35], so it was important to establish if the abnormalities we observed in LCH patients may have been influenced by age. MAIT cell proportions were plotted against age and a line of best fit was determined for healthy donors and for patients with LCH between the ages 37 to 68 (Fig. 2c). For healthy donors, a Spearman's non-parametric correlation test (95% confidence interval) determined that MAIT cell proportion was not significantly ($p = 0.31$) correlated to age ($r = -0.3$), while in patients with LCH, MAIT cell proportion was significantly ($p = 0.01$) negatively correlated to age ($r = -0.78$) (Fig. 2c). Although there was no significant correlation for healthy donors, we do not exclude the possibility that an association may have been identified with an increased cohort and indeed, our data did suggest a slight trend. Importantly, however, there remained a significant ($p = 0.0015$) decrease in the proportion of MAIT cells in total T cells when we compared the peripheral blood from patients with LCH age 37 to 68 compared to healthy donors age 37 to 68 (Fig. 2d). Therefore age related changes in MAIT cell frequency do not account for the abnormalities we have observed in patients with LCH.

$CD4^+V\alpha7.2\ TCR^+CD161^+$ T cells are increased in patients with LCH. Given there are functionally distinct subsets of unconventional T cells, we next tested the relative frequency of several MAIT, NKT and γδ T cell subpopulations. MAIT cells were primarily identified as $V\alpha7.2\ TCR^+CD161^+$ T cells and we examined CD8 and CD4 subsets (Fig. 3a) and found that patients with LCH had an increased proportion of blood $CD4^+V\alpha7.2\ TCR^+CD161^+$ T cells, and a similar trend was observed in lesions, although this was not significant (Fig. 3b). No differences were observed upon further stratification of LCH blood samples into AD and NAD (Fig. 3b). Although $CD4^+V\alpha7.2\ TCR^+CD161^+$ T cells are not exclusively 5-OP-RU-MR1 tetramer binding[35], Eomes, PLZF and CCR4 expression is comparable in these populations irrespective of the gating method, thus $CD4^+V\alpha7.2\ TCR^+CD161^+$ T cells are phenotypically similar to $CD4^+$ 5-OP-RU antigen specific MAIT cells[36].

Unusual γδ T cell and type I NKT cell phenotype in patients with LCH. Typically γδ T cells are $CD8^-CD4^-$, however there are known transcriptional differences between $CD161^+$ and $CD161^-$ γδ T cells[37]. Therefore we compared the proportions of $CD8^-CD4^-$, $CD8^+(CD4^-)$ and $CD4^+(CD8^-)$ γδ T cells and $CD8^-CD161^-$, $CD8^+CD161^-$ $CD8^-CD161^+$ $CD8^+CD161^+$ γδ T cell subsets in patients with LCH (Fig. 4a). We found a significant ($p = 0.0465$) increase in the proportion of $CD4^+(CD8^-)$ γδ T cells from LCH lesions compared with healthy donors (Fig. 4b) but no discernible differences were detected in the CD161 subsets between the healthy donor and LCH patient groups (Fig. 4c).

For NKT cells we investigated CD8 and CD4 subsets and CD56 expression (Fig. 5a) and found that while there were similar proportions of circulatory $CD56^+$ NKT cells (Fig. 5b) and $CD8^+$ NKT cells (Fig. 5c) between patients with LCH and healthy donors there were trends toward a higher proportion of $CD4^+$ and a lower proportion of $CD8^-CD4^-$ NKT cells in blood from patients with AD compared to NAD (Fig. 5c). Interestingly we observed reduced circulatory $CD8^+CD161^-$ NKT cells in patients with AD compared to healthy donors (Fig. 5d).

Activation status of unconventional T cells in patients with LCH. LCH lesions are thought to harbor activated T cells[8,13,16] and we were therefore assessed CD25 expression in LCH donors. We unexpectedly did not identify any considerable CD25 expression by either MAIT cells or NKT cells in LCH lesions (Fig. 6a) and most $CD25^+$ T cells within LCH lesions were $CD4^+Foxp3^+$ Tregs (Fig. 6b).

Expression of *MR1* by LCH cells. While γδ T cells can be stimulated by antigen independently of MHC or MHC-like molecules, CD1d and MR1 are necessary for antigenic stimulation of NKT cells and MAIT cells respectively. It is already known that *CD1D* is expressed by LCH cells[25], but MR1 has not been directly studied in the context of LCH. *MR1* is ubiquitously expressed but it was possible that a defect could impact MAIT cells, so we tested for the expression of *MR1* by purified LCH ($CD1a^+$) cells using qPCR. We found that *MR1* was expressed by LCH cells (delta Ct from reference gene *RPLP0* values: −2.24 and −7.97). These results suggest that LCH cells can express MR1 and could potentially present MR1-restricted antigens to MAIT cells in lesions.

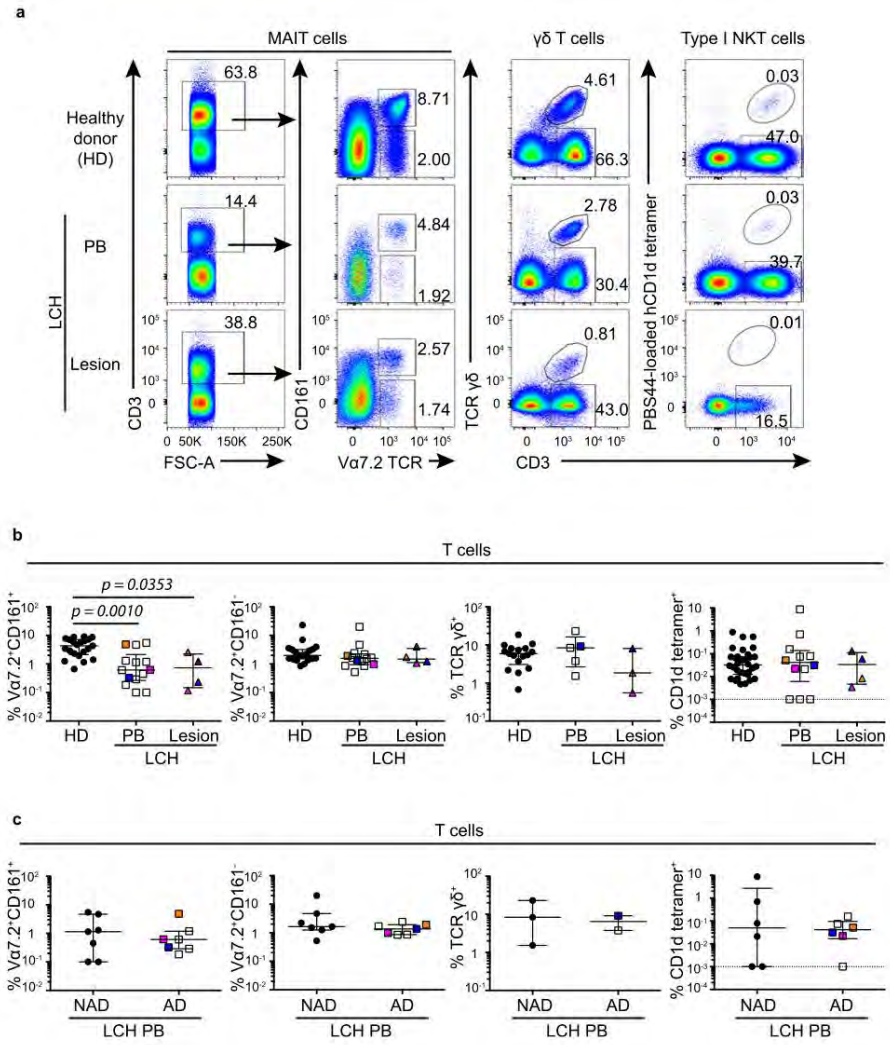

Figure 1. Circulatory and lesional unconventional T cell proportions in LCH. (**a**) Gating strategy for identifying MAIT cells (1st and 2nd columns), γδ T cells (3rd column) and type I NKT cells (4th column) from circulating lymphocytes in healthy donors (1st row) and patients with LCH (2nd row), and from lesional lymphocytes in patients with LCH (3rd row). (**b**) Proportions of MAIT cells and Vα7.2 TCR$^+$CD161$^-$ T cells, γδ T cells and type I NKT cells in total T cells. (**c**) Proportions of MAIT cells, Vα7.2 TCR$^+$CD161$^-$ T cells, γδ T cells and type I NKT cells in circulating T cells from patients with LCH (LCH-PB) stratified by active disease (AD) and non-active disease (NAD). Kruskal-Wallis tests with Dunn's multiple comparisons were conducted for (**b**) and Mann Whitney tests for (**c**), error bars indicate median + interquartile range, 10^{-3} on logarithmic scale indicates 'undetectable'.

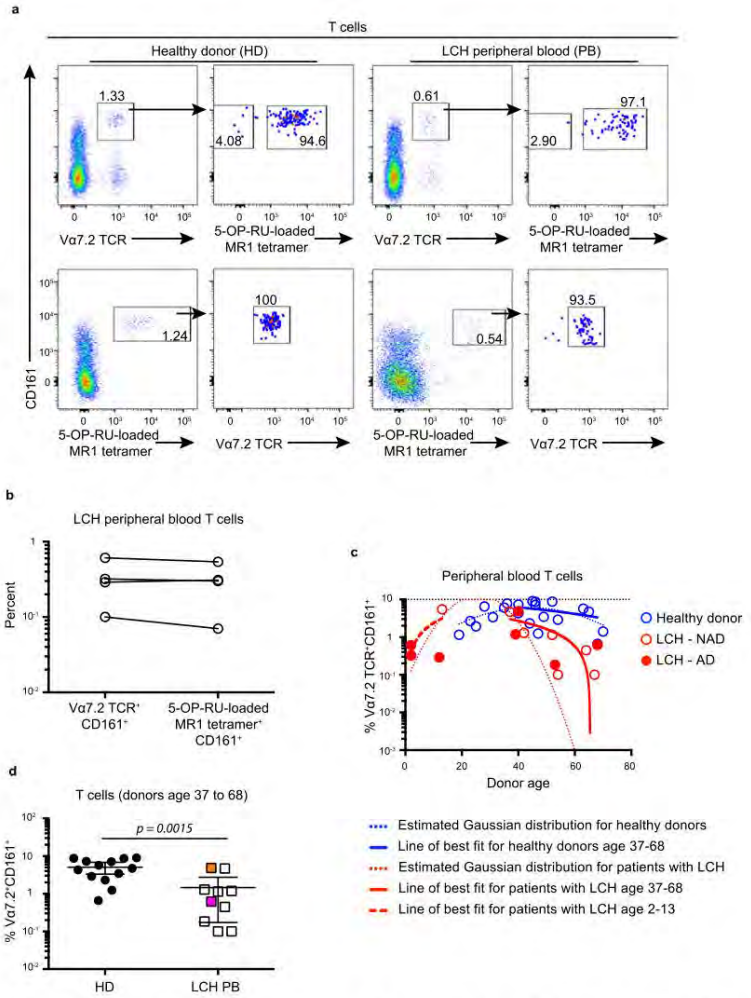

Figure 2. Comparing MAIT cell proportions across different identification methods and age groups. (**a**) Gating strategy for identifying 5-OP-RU-loaded MR1 tetramer$^+$ cells within the Vα7.2 TCR$^+$CD161$^+$ T cell population (top row) and Vα7.2 TCR$^+$ cells within the 5-OP-RU-loaded MR1 tetramer$^+$CD161$^+$ T cell population (bottom row) in peripheral blood from healthy donors (left) and patients with LCH (right). Data are representative of at least 3 individual donors per group. (**b**) Matched proportions of Vα7.2 TCR$^+$CD161$^+$ T cells and 5-OP-RU-loaded MR1 tetramer$^+$CD161$^+$ T cells in total T cells from the peripheral blood of patients with LCH. (**c**) Proportions of MAIT cells in peripheral blood T cells from healthy donors (blue circles) and patients with active (AD) LCH (closed red circles) and non-active (NAD) LCH (open red circles) with imposed Gaussian distributions for each data set (dotted lines) and a line of best fit for healthy donors (solid blue line) and LCH patients (solid red line) aged 37–68, and for LCH patients aged 2–13 (red dashed line). (**d**) Proportions of MAIT cells in total T cells from the peripheral blood from healthy donors aged 37 to 68 and patients with LCH aged 37 to 68. An unpaired t Test with Welch's correction was conducted for (**d**), error bars indicate mean + 95% confidence interval.

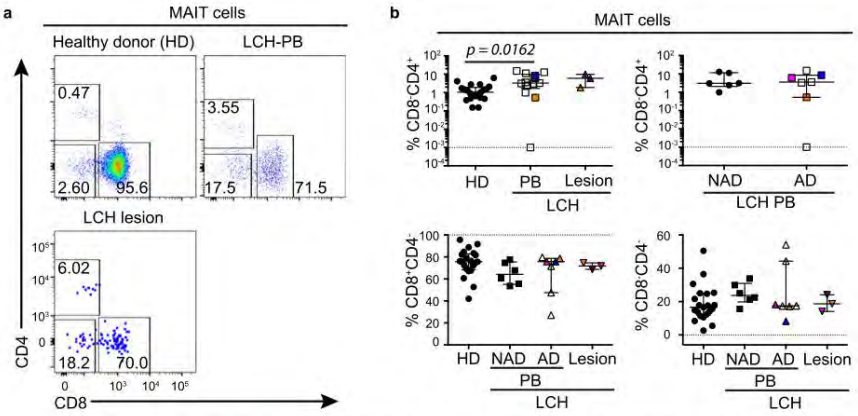

Figure 3. MAIT cell subsets in LCH. (**a**) Gating strategy for identifying CD8 and CD4 cells in MAIT cells from the peripheral blood from healthy donors (top left) and LCH patients (PB) (top right), and in lesional MAIT cells from patients with LCH (bottom left). (**b**) Proportions of $CD4^+$, $CD8^+$, and $CD8^-CD4^-$ cells in MAIT cells from the peripheral blood from healthy donors (HD), patients with non-active LCH (PB-NAD) and active LCH (PB-AD) and in lesional MAIT cells from patients with active LCH. Kruskal-Wallis tests with Dunn's multiple comparisons were conducted for (**b**) except where only two groups were compared (Mann Whitney test), error bars indicate median + interquartile range, 10^{-3} on logarithmic scale indicates 'undetectable'.

Importantly, MAIT cells can also be activated independently of antigen by IL-12 and IL-18, which is noteworthy because there are elevated levels of IL-18 in the peripheral blood from patients with LCH[38].

Unconventional T cells from patients with LCH can produce Th1 cytokines. Next, we investigated the functional capacity of unconventional T cells from LCH patients as it was important to establish if these cells could mount a normal cytokine response to stimulation. We stimulated T cells with PMA and ionomycin and examined TNF and IFNγ production by MAIT cells (Fig. 7a), γδ T cells (Fig. 7b) and NKT cells (Fig. 7c). MAIT cells, NKT cells and γδ T cells from patients with LCH were able to produce TNF and IFNγ, albeit in small proportions from one donor in particular (Fig. 7d). IL-4 and IL-17A cytokine production was negligible by all T cells in LCH and healthy donors (data not shown). Our data suggest that although decreased in proportion, the MAIT cells in patients with LCH are functionally competent. It is important to note, however that more subtle defects may become evident with different forms of activation.

Relative frequency of circulatory MAIT cells during AD and NAD and the effects of treatment. Given our observation that MAIT cells are reduced in the circulation of patients with LCH, and their known immunoregulatory ability, we conducted a longitudinal analysis of MAIT cells at different stages of disease and treatment following lesion excision and during chemotherapy in two patients at a number of time points for a period of 20 to 25 months (Fig. 8). We observed minor changes in the MAIT cell proportion and total lymphocyte count of one patient with AD who was receiving vinblastine or methotrexate and prednisolone. More interestingly, the MAIT cell frequency of the other patient increased during treatment with vinblastine and prednisolone and decreased at cessation of treatment when the patient had NAD. Furthermore, this patient's MAIT cell proportion increased when the disease reactivated and treatment recommenced. Whether the changes in MAIT cell proportion were disease specific or treatment specific was not established, but this is an indication that MAIT cell frequency could potentially be associated with LCH disease activity.

Discussion

Unconventional T cells include several immunoregulatory lineages but have been difficult to study due to low numbers and a lack of reagents to enable stringent identification and functional analysis. Improved knowledge about these cells and our access to important reagents enabled us to characterize MAIT cells, NKT cells and γδ T cells and determine if there were defects in their frequency or cytokine production.

MAIT cells were reduced in proportion in the blood from patients with LCH and the lesional proportions were consistent with that seen in the blood from patients. Interestingly, the proportion of circulating MAIT cells was similar in patients with AD and NAD, which suggests that a MAIT cell deficiency may precede the onset of AD and could be a potential predisposing factor for LCH. Of note, longitudinal analysis of one patient revealed a substantial rise in MAIT cell frequency after treatment and a subsequent fall when treatment ceased and the disease was non-active. Fluctuations in MAIT cell proportion, which were remarkably at approximately 17% of total

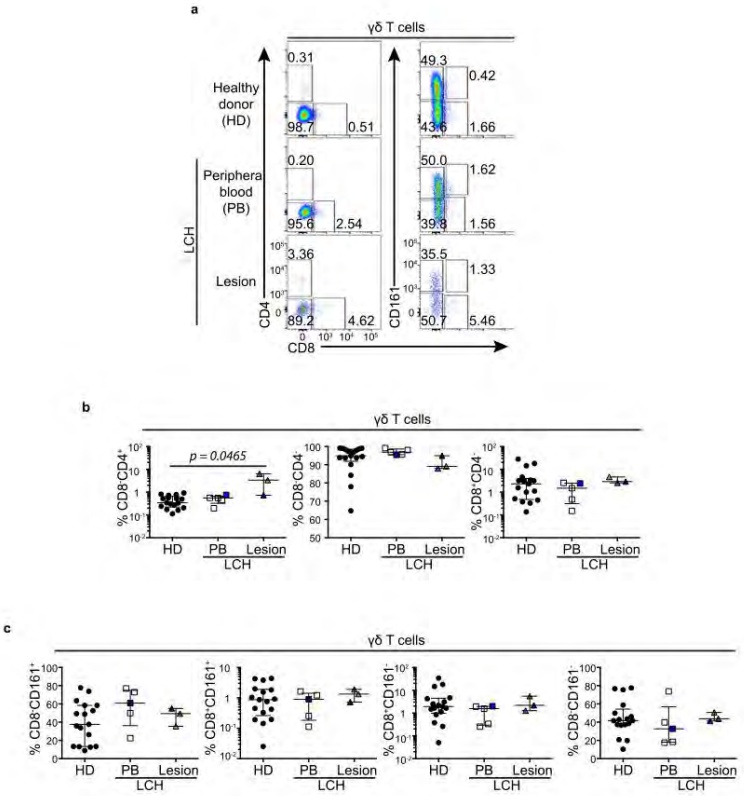

Figure 4. γδ T cell subsets in LCH. (**a**) Gating strategy for identifying γδ T cell subsets. (**b**) Proportions of $CD4^+CD8^-$, $CD8^-CD4^-$ and $CD8^+CD4^-$ cells in γδ T cells from the peripheral blood from healthy donors (HD) and patients with LCH (PB) and in lesional γδ T cells from patients with active LCH. (**c**) Proportions of $CD8^-CD161^+$, $CD8^+CD161^+$, $CD8^+CD161^-$ and $CD8^-CD161^-$ cells in γδ T cells from the peripheral blood from healthy donors (HD) and patients with LCH (PB) and in lesional γδ T cells from patients with active LCH. Kruskal-Wallis tests with Dunn's multiple comparisons were conducted for (**b**,**c**), error bars indicate median + interquartile range.

T cells for three separate time points, will be interesting to pursue in a cohort of LCH donors. Albeit in a single donor, the changes are consistent with our hypothesis that increasing MAIT cell frequency could be beneficial in the resolution of LCH. It is vital to emphasise that the result is a very preliminary observation. We did not observe an increase in γδ T cells reported by Alaibac and Chu[24], although an important difference in our study was that we assayed lesions from various tissue sites, whereas the earlier study was limited to cutaneous lesions.

Unconventional T cells include several functionally distinct lineages and it was noteworthy that there were changes in subset proportions of γδ T cells and NKT cells as well as in the relative frequency of MAIT cells. γδ T cells do not usually express CD4, but we unexpectedly identified an increase in the proportion of lesional $CD4^+$ γδ T cells. Determining the cause and functional significance of this is outside the scope of this study, but it was interesting that we also detected an increase in the proportion of $CD4^+$Vα7.2 TCR^+CD161^+ T cells in patients with LCH. As with γδ T cells, the $CD4^+$Vα7.2 TCR^+CD161^+ T cells are usually a minor subset, but their increase in LCH parallels a similar observation reported for patients with colorectal cancer[23]. It is important to note, however, that this increase in $CD4^+$Vα7.2 TCR^+CD161^+ T cells may be due to the overall reduction in MAIT cells.

The frequency of circulating NKT cells in healthy donors usually ranges from 0.01 to 0.1% of overall T cells, but is rarely greater than 1%[39]. Surprisingly, NKT cell frequency in patients with LCH was more variable and even extended to a patient where NKT cells comprised almost 9% of T cells in blood. A previous case study of

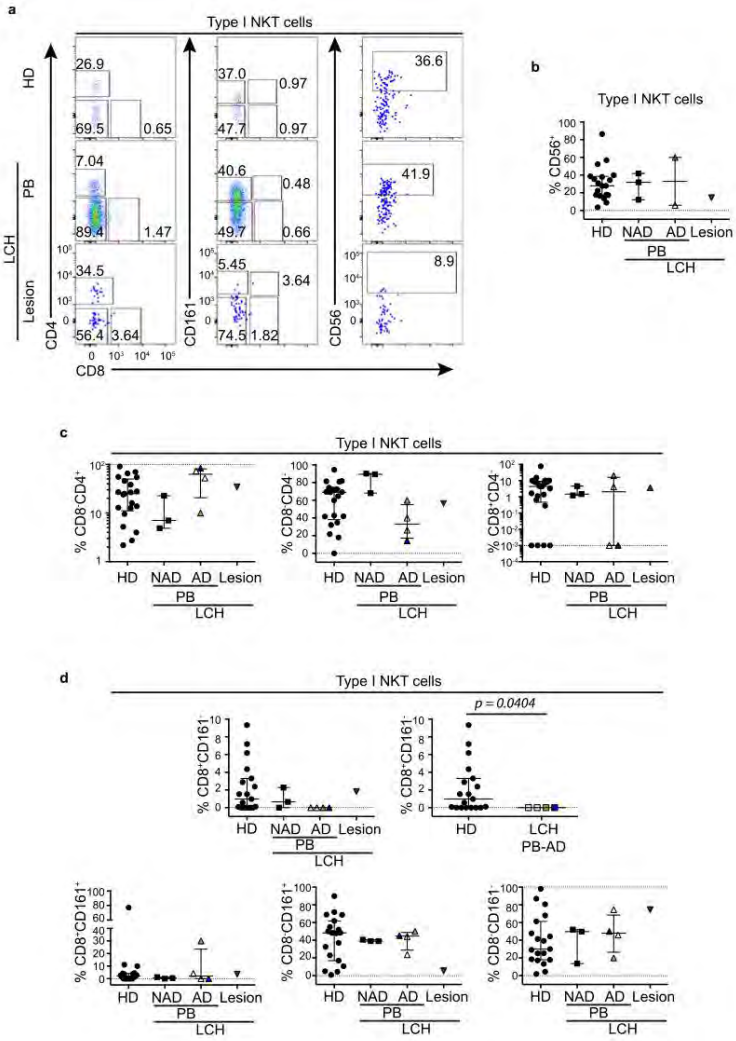

Figure 5. NKT cell subsets in LCH. (**a**) Gating strategy for identifying type I NKT cell subsets in peripheral blood from healthy donors (HD) and patients with LCH (PB), and in lesions from patients with LCH. (**b**) Proportions of $CD56^+$ cells in type I NKT cells. (**c**) Proportions of $CD4^+CD8^-$, $CD8^-CD4^-$ and $CD8^+CD4^-$ cells in type I NKT cells. (**d**) Proportions of $CD8^+CD161^-$, $CD8^+CD161^+$, $CD8^-CD161^+$ and $CD8^-CD161^-$ cells in type I NKT cells. Kruskal-Wallis tests with Dunn's multiple comparisons were conducted for (**b**–**d**) (LCH lesion group excluded due to sample size) except where only two groups were compared (Mann Whitney test), error bars indicate median + interquartile range, 10^{-3} on logarithmic scale indicates 'undetectable'.

an individual with 5% NKT cells was considered exceptional[39] and it is noteworthy that the patient in our study had NAD at the time of sample collection, which followed a spontaneous disease resolution without treatment. Because NKT cells are immunoregulatory in nature, it would be interesting to determine if the high NKT cell

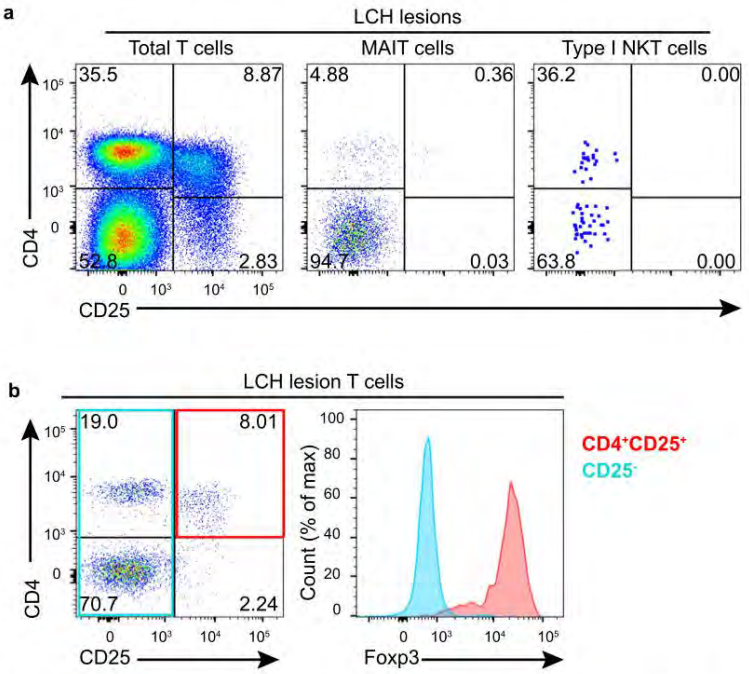

Figure 6. Activation status of T cells in patients with LCH. (**a**) Representative plots demonstrate that most $CD25^+$ T cells in LCH lesions are $CD4^+$ (left) and there is little to no CD25 expression by MAIT cells (center) or type I NKT cells (right). (**b**) Representative plots demonstrate that most $CD25^+$ T cells in lesions are $CD4^+Foxp3^+$. Data are representative of at least 4 individual donors in (**a**) and 3 individual donors in (**b**).

frequency was pre-existing or accompanied this remission, which may indicate a direct role for NKT cells. In striking contrast were several patients with LCH that had undetectable NKT cells. This too is unusual in healthy individuals and such a deficit could be expected to have a large impact on immune regulation and potentially the progression of LCH. The factors controlling NKT cell frequency in humans are poorly understood but once determined, it will be interesting to assess if they are defective in LCH patient groups.

We investigated whether the frequency abnormalities of unconventional T cells extended to their functional capacity, as this has previously been reported for many patient groups, including with cancer[23,40]. Each T cell lineage that we studied from patients with LCH retained the capability to produce Th1 cytokines after stimulation. High levels of IL-17A have been reported in the blood of patients with LCH[41] and more controversially[25,42] in lesions[43]. Our findings of negligible IL-17A production by T cells from patients with LCH indicates that T cells in LCH do not respond with rapid IL-17A release, although we cannot rule out that an *in vivo* stimulus could cause IL-17A production.

Having identified these immune abnormalities, it will be essential for future studies to determine their origin and whether they precede LCH, or are caused by it. An intriguing question is whether unconventional T cells are directly interacting with LCH cells and if so, whether this results in T cell activation, or affects the LCH cells. For example, in addition to cytokine release, activated MAIT cells express CD40L[44], which can mature LCH cells *in vitro* and increase their allostimulatory capacity[45]. This implies that LCH cells could mature and present antigen more effectively in the presence of activated MAIT cells, which could promote inflammation and reduce Treg induction by immature myeloid cells, in turn resolving lesions.

We have shown for the first time that the frequency of unconventional T cells, especially MAIT cells, is abnormal in patients with LCH. MAIT cell frequencies appear similar in the periphery of patients with AD and NAD, although an increase in proportion was observed upon treatment in one patient that suggested MAIT cells could be associated with LCH onset and progression, although further studies are required to confirm this. Changes in unconventional T cell subset distributions and frequency in patients with LCH are consistent with the established role of unconventional T cells in immune dysregulation. It will be especially informative to determine the direct

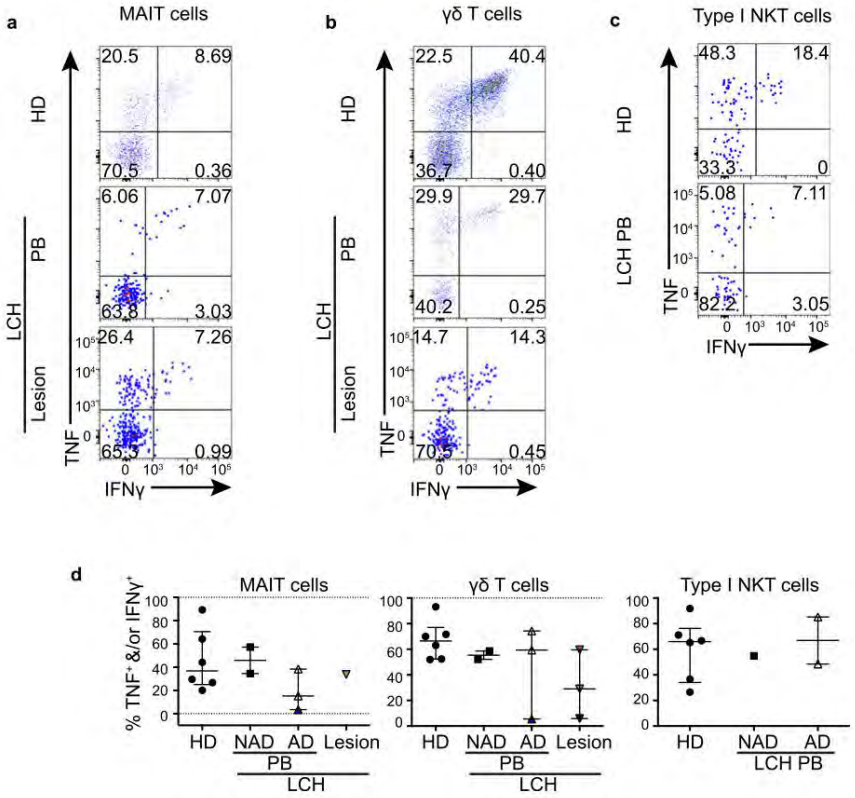

Figure 7. Th1 cytokine production by unconventional T cells. Gating strategy for post stimulation identification of TNF and IFNγ producing MAIT cells (**a**), γδ T cells (**b**) and type I NKT cells (**c**) from the peripheral blood from healthy donors (HD) and patients with LCH (PB), and from lesional T cells from patients with active LCH. (**d**) Proportions of TNF^+ and/or $IFN\gamma^+$ T cells in MAIT cells, γδ T cells and type I NKT cells from the peripheral blood from healthy donors (HD), patients with non-active LCH (PB-NAD) and active LCH (PB-AD) and in lesional T cells from patients with active LCH. Kruskal-Wallis tests with Dunn's multiple comparisons were conducted for (**d**) (groups with only one value excluded), except where only two groups were compared (Mann Whitney test), error bars indicate median + interquartile range, 10^{-3} on logarithmic scale indicates 'undetectable'.

roles of these cells in LCH and whether the abnormalities we have identified are a cause or consequence of the disease. Our analysis strengthens earlier reports of immune dysfunction in LCH by showing the abnormalities extend beyond $Foxp3^+$ Tregs to unconventional T cell lineages that could be equally important factors in LCH. Their distinctive functions and the development of specific agonists make unconventional cells promising candidates as biomarkers or targets of immune based therapies, but more detailed analysis is now required for translation to clinical applications.

Materials and Methods

Human blood and tissue. This research was approved by the Ballarat Health Services and Saint John of God Ballarat Hospital Human Research Ethics Committee (HREC) (HREC/15/BHSSJOG/5 and HREC/10/BHSSJOG/57) and Federation University Australia HREC (A08-100) and the methods were carried out in accordance with the approved guidelines. Patients, and/or parents of children with LCH where appropriate, provided written informed consent. Healthy donor peripheral blood mononuclear cells (PBMCs) were obtained from the Australian Red Cross Blood Service.

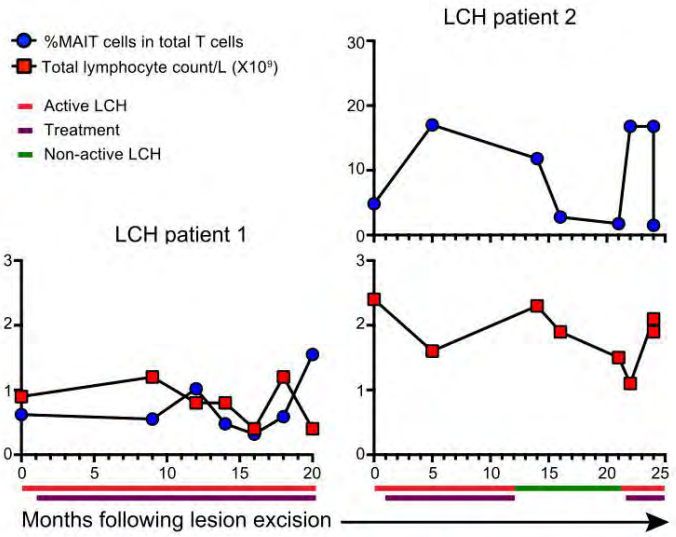

Figure 8. Relative frequency of circulatory MAIT cells over time. Graphs show proportions of MAIT cells in total T cells and the corresponding total lymphocyte counts over a period of months following lesion excision and during chemotherapy. Patient 1 received vinblastine or methotrexate and prednisolone for the duration of the period. Patient 2 received vinblastine and prednisolone.

Healthy donor PBMCs were isolated with Histopaque-1077 (Sigma-Aldrich). PBMCS were isolated from the peripheral blood from patients with LCH using Histopaque-1077 or Lymphoprep (Axis-Shield PoC AS, Oslo, Norway), otherwise white blood cells were isolated using an in house red cell lysis buffer. Tissue was processed using MACS tumour dissociation kit for humans (Miltenyi Biotec) as per manufacturer's instructions. Different methods were used to achieve a single cell suspension for lesions and for some LCH peripheral blood samples compared to healthy donors, therefore flow cytometry analysis was conducted relative to the $CD3^+$ (T cell) population or sub-populations thereof. LCH lesions and matched peripheral blood samples are colour coded for tracking throughout the figures.

Surface antibody staining. Cells were stained with viability dye 7-aminoactinomycin D (7-AAD; BD Pharmingen) and anti-human cell surface antibodies from the following list: CD1a-BV605 (SK9; BD Biosciences),CD3-Pacific Blue (HIT3a; BioLegend), CD3-BV605 (HIT3a; BD Horizon), CD3-BV650 (UCHT1; BD Horizon), CD4-PE-Cy7 (SK3; BD Pharmingen), CD4-BV650 or -BV711 (SK3; BD Horizon), CD8-BV510 (RPA-T8; BD Horizon), CD25-PE-Cy7 (M-A251; BD Pharmingen), CD56-BV786 (NCAM16.2; BD Horizon), CD127-BV421 (HIL-7R-M21; BD Horizon), CD161-APC or -PE/Dazzle 594 (HP-3G10; BioLegend), TCR Vα7.2-FITC (3C10; BioLegend), and TCRγ/δ-1-PE-Cy7 (11F2; BD Biosciences).

For identification of NKT cells, cells were stained with PBS44 loaded human CD1d tetramers. PBS44-CD1d monomers were kindly donated by Dale Godfrey (Dept. Microbiology and Immunology, Peter Doherty Institute, University of Melbourne, Parkville, Australia). In some instances, MAIT cells were identified using 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) loaded human MR1 tetramers. 5-OP-RU-MR1 monomers were a kind gift from the McCluskey laboratory (Dept. Microbiology and Immunology, Peter Doherty Institute, University of Melbourne, Parkville, Australia). CD1d and MR1 monomers were tetramerized by conjugation to PE streptavidin (BD Pharmingen).

Foxp3 transcription factor staining. Following surface staining the eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher Scientific) was used as per manufacturer's instructions.

Flow cytometry and fluorescence activated cell sorting. Analyses were performed using either a BD FACS Aria II cell sorter or BD LSR Fortessa. All cell sorting was performed using a BD FACS Aria II cell sorter and data analyzed using FlowJo software (Treestar). All analyses excluded dead cells and doublets.

RNA extraction, cDNA synthesis, transcriptome amplification and qPCR. Cells were purified by FACS (purity >90%), then the REPLI-g WTA Single Cell Kit (QIAGEN) for transcriptome amplification of small cell populations (<1000) was used to extract and reverse transcribe total RNA and amplify cDNA directly from

cells, as per manufacturer's instructions using a thermal cycler (Applied Biosystems Gene Amp® PCR System 2700). RT_2 SYBR® Green qPCR Mastermix (QIAGEN), Nuclease-Free Water (QIAGEN) and RT_2 qPCR Primer Assays for *MR1* (QIAGEN) and reference gene *RPLP0* (QIAGEN) were combined with cDNA as per manufacturer's instructions. No-template control samples were included for each component, substituting cDNA volumes with nuclease free water. Aliquots of each component were added to 0.1 mL strip tubes (QIAGEN) in triplicate and capped then placed in the Rotor-Disk 72 Rotor (QIAGEN) and placed in the Rotor-Gene Q (QIAGEN) for qPCR cycling. Conditions for amplification, annealing, extension and melt curve analysis were as per QIAGEN RT_2 qPCR primer assay instructions. Melt curve analysis was performed to confirm that specific product was amplified as indicated by the data sheet for each primer assay.

Unconventional T cell stimulation assay. $CD3^+$ T cells were sorted with a purity above 95%, except from two LCH peripheral blood samples, which were above 92%, and rested overnight at 37 °C in TexMACS Medium (Miltenyi Biotec) with 10% Fetal Bovine Serum (Sigma-Aldrich) and 1x Penicillin-Streptomycin (Sigma-Aldrich), then stimulated for 5 hours with PMA (10 ng/mL) and ionomycin (1 μg/mL) in the immediate presence of GolgiPlug (BD Biosciences).

Fixation, permeabilization and intracellular cytokine staining. Following stimulation assays and surface staining, cells were fixed and permeabilized using BD Cytofix/Cytoperm kit (BD Biosciences) as per manufacturer's instructions. Cells were then stained for intracellular cytokines from the following list: IL-4-APC (BD Pharmingen), TNFα-APC-Cy7 (MAb11; BioLegend), IFNγ-BV711 (B27; BD Horizon) IL-17A-BV786 (N49-653; BD Horizon).

Statistics. Data were analyzed using GraphPad Prism (GraphPad Software). Three donor matched blood and LCH lesions were available for the initial characterization of MAIT cells and NKT cells and one for the characterization of γδ T cells and the PMA and ionomycin stimulation of unconventional T cells. D'Agostino and Pearson omnibus normality tests (alpha = 0.05), were used to select parametric or non-parametric analysis. Differences were considered statistically significant at an alpha level of 0.05.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

References

1. Morimoto, A., Oh, Y., Shioda, Y., Kudo, K. & Imamura, T. Recent advances in Langerhans cell histiocytosis. *Pediatr Int* **56**, 451–461 (2014).
2. Allen, C. E., Ladisch, S. & McClain, K. L. How we treat Langerhans cell histiocytosis. *Blood* **126**, 26–35 (2015).
3. Rizzo, F. M., Cives, M., Simone, V. & Silvestris, F. New insights into the molecular pathogenesis of Langerhans cell histiocytosis. *The Oncologist* **19**, 151–163 (2014).
4. Vaiselbuh, S. R., Bryceson, Y. T., Allen, C. E., Whitlock, J. A. & Abla, O. Updates on histiocytic disorders. *Pediatr. Blood Cancer* **61**, 1329–1335 (2014).
5. Kannourakis, G. & Abbas, A. The role of cytokines in the pathogenesis of Langerhans cell histiocytosis. *Br. J. Cancer Suppl.* **23**, S37–40 (1994).
6. Sahm, F. *et al.* BRAFV600E mutant protein is expressed in cells of variable maturation in Langerhans cell histiocytosis. *Blood* **120**, e28–e34 (2012).
7. Satoh, T. *et al.* B-RAF mutant alleles associated with Langerhans cell histiocytosis, a granulomatous pediatric disease. *PLoS ONE* **7**, e33891 (2012).
8. Badalian-Very, G. *et al.* Recurrent *BRAF* mutations in Langerhans cell histiocytosis. *Blood* **116**, 1919–1923 (2010).
9. Yousem, S. A., Dacic, S., Nikiforov, Y. E. & Nikiforova, M. Pulmonary Langerhans cell histiocytosis: profiling of multifocal tumors using next-generation sequencing identifies concordant occurrence of *BRAF* V600E mutations. *Chest* **143**, 1679–1684 (2013).
10. Nelson, D. S. *et al. MAP2K1* and *MAP3K1* mutations in Langerhans cell histiocytosis. *Genes Chromosomes Cancer* **54**, 361–368 (2015).
11. Chakraborty, R. *et al.* Mutually exclusive recurrent somatic mutations in *MAP2K1* and *BRAF* support a central role for ERK activation in LCH pathogenesis. *Blood* **124**, 3007–3015 (2014).
12. Brown, N. A. *et al.* High prevalence of somatic *MAP2K1* mutations in *BRAF V600E*-negative Langerhans cell histiocytosis. *Blood* **124**, 1655–1658 (2014).
13. Egeler, R. M., Favara, B. E., van Meurs, M., Laman, J. D. & Claassen, E. Differential *In situ* cytokine profiles of Langerhans-like cells and T cells in Langerhans cell histiocytosis: abundant expression of cytokines relevant to disease and treatment. *Blood* **94**, 4195–4201 (1999).
14. Senechal, B. *et al.* Expansion of regulatory T cells in patients with Langerhans cell histiocytosis. *PLoS Med.* **4**, e253 (2007).
15. Egeler, R. M., van Halteren, A. G. S., Hogendoorn, P. C. W., Laman, J. D. & Leenen, P. J. M. Langerhans cell histiocytosis: fascinating dynamics of the dendritic cell-macrophage lineage. *Immunol. Rev.* **234**, 213–232 (2010).
16. Quispel, W. T., Stegehuis-Kamp, J. A., Santos, S. J., Egeler, R. M. & van Halteren, A. G. S. Activated conventional T-cells are present in Langerhans cell histiocytosis lesions despite the presence of immune suppressive cytokines. *Journal of Interferon & Cytokine Research* **35**, 831–839 (2015).
17. Quispel, W. T. *et al.* The presence of $CXCR4^+$ $CD1a^+$ cells at onset of Langerhans cell histiocytosis is associated with a less favorable outcome. *Oncoimmunology* **5**, e1084463 (2016).
18. Carrera Silva, E. A. *et al.* $CD207^+CD1a^+$ cells circulate in pediatric patients with active Langerhans cell histiocytosis. *Blood*, https://doi.org/10.1182/blood-2017-05-782730 (2017).
19. Dhodapkar, M. V. *et al.* A reversible defect in natural killer T cell function characterizes the progression of premalignant to malignant multiple myeloma. *J. Exp. Med.* **197**, 1667–1676 (2003).
20. Molling, J. W. *et al.* Generation and sustained expansion of mouse spleen invariant NKT cell lines with preserved cytokine releasing capacity. *Journal of Immunological Methods* **322**, 70–81 (2007).
21. Neparidze, N. & Dhodapkar, M. V. Harnessing CD1d-restricted T cells toward antitumor immunity in humans. *Ann. N. Y. Acad. Sci.* **1174**, 61–67 (2009).
22. Sundström, P. *et al.* Human mucosa-associated invariant T cells accumulate in colon adenocarcinomas but produce reduced amounts of IFN-γ. *The Journal of Immunology* **195**, 3472–3481 (2015).

23. Ling, L. *et al.* Circulating and tumor-infiltrating mucosal associated invariant T (MAIT) cells in colorectal cancer patients. *Sci. Rep.* **6**, (2016).
24. Alaibac, M. & Chu, A. C. T-lymphocytes bearing the gamma delta T-cell receptor in cutaneous lesions of Langerhans' cell histiocytosis. *Med. Pediatr. Oncol.* **21**, 347–349 (1993).
25. Allen, C. E. *et al.* Cell-specific gene expression in Langerhans cell histiocytosis lesions reveals a distinct profile compared with epidermal Langerhans cells. *The Journal of Immunology* **184**, 4557–4567 (2010).
26. Carolan, E. *et al.* Altered distribution and increased IL-17 production by mucosal-associated invariant T cells in adult and childhood obesity. *The Journal of Immunology* **194**, 5775–5780 (2015).
27. Hinks, T. S. C. Mucosal-associated invariant T cells in autoimmunity, immune-mediated diseases and airways disease. *Immunology* **148**, 1–12 (2016).
28. Mekinian, A. *et al.* Mucosal-associated invariant (MAIT) cells are deficient in systemic sclerosis. *Scand J Immunol* **86**, 216–220 (2017).
29. McEwen-Smith, R. M., Salio, M. & Cerundolo, V. The regulatory role of invariant NKT cells in tumor immunity. *Cancer Immunology Research* **3**, 425–435 (2015).
30. Anderson, B., Teyton, L., Bendelac, A. & Savage, P. Stimulation of natural killer T cells by glycolipids. *Molecules* **18**, 15662–15688 (2013).
31. Pellicci, D. G. & Uldrich, A. P. All work and no Id2 makes a dull NKT cell. *Immunol. Cell Biol.* **94**, 619–620 (2016).
32. Godfrey, D. I., Uldrich, A. P., McCluskey, J., Rossjohn, J. & Moody, D. B. The burgeoning family of unconventional T cells. *Nat. Immunol.* **16**, 1114–1123 (2015).
33. Reantragoon, R. *et al.* Antigen-loaded MR1 tetramers define T cell receptor heterogeneity in mucosal-associated invariant T cells. *Journal of Experimental Medicine* **210**, 2305–2320 (2013).
34. Novak, J., Dobrovolny, J., Novakova, L. & Kozak, T. The decrease in number and change in phenotype of mucosal-associated invariant T cells in the elderly and differences in men and women of reproductive age. *Scand J Immunol* **80**, 271–275 (2014).
35. Gherardin, N. A. *et al.* Human blood MAIT cell subsets defined using MR1 tetramers. *Immunol. Cell Biol.* https://doi.org/10.1111/imcb.12021 (2018).
36. Kurioka, A. *et al.* Shared and Distinct Phenotypes and Functions of Human CD161++ Vα7.2+ T Cell Subsets. *Front Immunol* **8**, 1031 (2017).
37. Fergusson, J. R. *et al.* CD161 defines a transcriptional and functional phenotype across distinct human T cell lineages. *Cell Rep* **9**, 1075–1088 (2014).
38. Morimoto, A. *et al.* Inflammatory serum cytokines and chemokines increase associated with the disease extent in pediatric Langerhans cell histiocytosis. *Cytokine* **97**, 73–79 (2017).
39. Chan, A. C. *et al.* Immune characterization of an individual with an exceptionally high natural killer T cell frequency and her immediate family. *Clin Exp Immunol* **156**, 238–245 (2009).
40. Berzins, S. P., Smyth, M. J. & Baxter, A. G. Presumed guilty: natural killer T cell defects and human disease. *Nature Publishing Group* **11**, 131–142 (2011).
41. Lourda, M. *et al.* Detection of IL-17A-producing peripheral blood monocytes in Langerhans cell histiocytosis patients. *Clin. Immunol.* **153**, 112–122 (2014).
42. Peters, T. L., McClain, K. L. & Allen, C. E. Neither IL-17A mRNA nor IL-17A protein are detectable in Langerhans cell histiocytosis lesions. *Mol. Ther.* **19**, 1433–1439 (2011).
43. Coury, F. *et al.* Langerhans cell histiocytosis reveals a new IL-17A-dependent pathway of dendritic cell fusion. *Nat. Med.* **14**, 81–87 (2008).
44. Salio, M. *et al.* Activation of human mucosal-associated invariant T cells induces CD40L-dependent maturation of monocyte-derived and primary dendritic cells. *The Journal of Immunology* **199**, 2631–2638 (2017).
45. Geissmann, F. *et al.* Differentiation of Langerhans cells in Langerhans cell histiocytosis. *Blood* **97**, 1241–1248 (2001).

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Author Contributions

T.v.B.G., E.K., J.-I.H. and G.K. recruited patients with LCH and provided tissue samples and clinical information. S.B. provided healthy donor control tissues. J.M., S.B. and G.K. designed experiments. J.M. performed experiments, analyzed results, prepared figures and wrote the manuscript. S.B. and G.K. critically revised the manuscript. T.v.B.G., E.K., D.G.P. and J.-I.H. reviewed the manuscript. S.B. and G.K. led the investigation.

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Review Article

A potentially important role for T cells and regulatory T cells in Langerhans cell histiocytosis

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ABSTRACT

Langerhans cell histiocytosis is characterized by lesions containing inflammatory immune cells, including myeloid cells and T cells. Patient mortality remains unacceptably high and new treatment options are required. Several LCH studies have identified aberrant frequencies of T cell subsets with potential immune regulatory properties. High numbers of Foxp3$^+$ regulatory T cells and gamma-delta T cells have been reported in patients with LCH, although, the cause of their presence or their significance is not yet clear. This review describes the current understanding of how LCH develops and progresses, focusing on the growing evidence that regulatory T cell subsets may be important and discussing the exciting potential for harnessing these cells to treat LCH using immune based therapies.

1. Introduction

Langerhans cell histiocytosis (LCH) is characterized by lesions containing myeloid derived dendritic cells (DCs) that coexpress CD1a and CD207 and are referred to as LCH cells. Studies on these presumed pathogenic LCH cells have produced a reasonable understanding of the basic biology and origins of this disease and led to improved treatment options for many patients, although morbidity, mortality and associated sequelae still persist. The lesional microenvironment contains an inflammatory infiltrate of T cells, macrophages, eosinophils, neutrophils, B cells, plasma cells and multinucleated giant cells [1, 2], whose importance is not well defined (Fig. 1). The cellular infiltrate and the cytokine milieu they produce are collectively central to the inflammatory microenvironment and resultant organ damage, so while the LCH cells are important, it is highly likely that other immune cells within LCH lesions are contributors to the disease and may be potential targets for new immune based treatments.

The inflammatory microenvironment of LCH lesions suggests that failure of immune regulation could be an important factor in LCH development and progression. T cell subsets with a regulatory function including Foxp3$^+$ regulatory T cells (Tregs) and gamma-delta (γδ) T cells have already been identified as having abnormal frequencies in LCH patients, and other unconventional T cells with regulatory roles, including type I natural killer T (NKT) cells, mucosal-associated invariant T (MAIT) cells and CD1a-restricted T cells have not been studied at all. These so-called immuno-regulatory cells influence other immune cells through potent cytokine release and cell-cell contact, including regulating the recruitment, activation or function of immune cells such as cytotoxic and/or helper T cells (Fig. 2). They are therefore prime candidates for influencing the inflammatory setting of LCH lesions. There are several specialized lineages of T cells with regulatory functions that can promote or inhibit antigen-specific and antigen-independent immune responses in cancer, infection and autoimmunity, and their influential roles make them appealing immunotherapy targets in diseases where immune dysregulation is a factor. The precise role of these cells in LCH remains poorly understood, but they could conceivably promote or inhibit the development of lesions. A better understanding of the role of regulatory T cell subsets in LCH will improve our understanding of the causes and progression of inflammatory lesions in LCH and determine the potential for using these cells in novel immune based therapies.

1.1. Langerhans cell histiocytosis

LCH is a rare and multifaceted disease of unknown cause [3, 4]. The disease occurs mostly in children [3] with an incidence of 4–5 cases per million per year in those under 15 years with more frequent detection in the 0–3 year age group [4]. LCH also affects 1–2 adults per million each year (most commonly young adults) [4]. LCH lesions are caused by an inflammatory infiltrate, which includes (amongst other immune cells)

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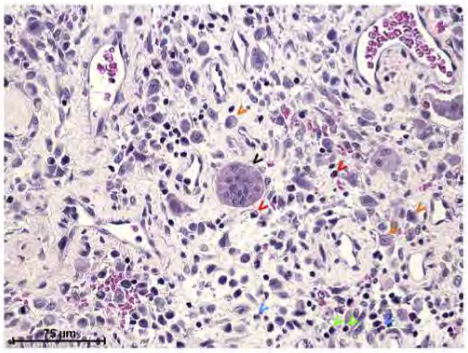

Fig. 1. Histology of LCH lesions. A hematoxylin and eosin stained lesion characteristic of LCH lesions, containing a variety of immune cells including histiocytes (orange arrows), lymphocytes (green arrows), neutrophils (blue arrows), eosinophils (red arrows) and multinucleated giant cells (black arrow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

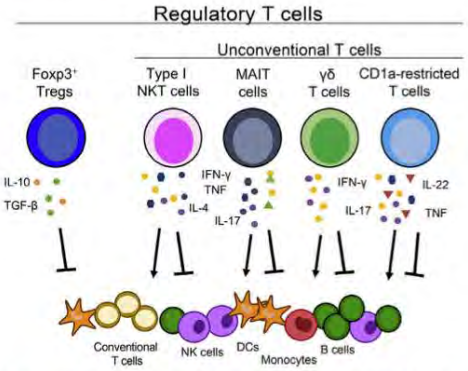

Fig. 2. Regulatory T cells have the ability to inhibit or promote the response other immune cells. Regulatory T cells including FoxP3$^+$ Tregs and unconventional T cells such as type I NKT cells, MAIT cells, γδ T cells and CD1a-restricted T cells have the ability to influence the recruitment, activation and functional status of other immune cells such as conventional T cells, natural killer (NK) cells, DCs, monocytes and B cells through cytokine release and cell-cell contact (not shown).

presumed pathogenic LCH cells, and T cells [5], which are the focus of this review. Lesions can be unifocal or multifocal within one or more organs. The clinical outcome for LCH is diverse, ranging from a single lesion with a good prognosis to disseminated disease and mortality, with many intermediate stages. The liver, spleen and bone marrow are considered high risk organs [6], as lesional infiltration of these sites can result in organ dysfunction. For patients with risk organ involvement, the mortality rate is approximately 15% [7]. Other commonly occurring sites for lesions are within osseous and cutaneous tissue, although infiltration of these organs has a better prognosis than those involving 'risk organs'.

1.1.1. Treatment options for LCH

Treatment of LCH varies depending on the severity and location of lesions. Low risk patients may only require observation, while those with higher risk usually undergo surgical removal of lesions and/or systemic or topical administration of steroids in conjunction with chemotherapy [8]. A combination of prednisone and vinblastine [9] has been used to treat LCH since 1966 with an overall response rate of 60% [10]. The mechanism of this treatment is not completely understood, but could be due to an immunosuppressive impact on the LCH cells within the microenvironment. Radiation is typically a last resort treatment, particularly for children [9], and while it can improve or stabilize lesion-related symptoms in most LCH patients, short- and long-term morbidity is often observed [8]. Etoposide chemotherapy has been used for systemic disease but has increased toxicity and may contribute to the development of myelodysplastic syndrome [11, 12]. A trial of cladribine and cytosine-arabinoside salvage therapy for 'risk-organ' LCH patients with sub-optimal responses to standard treatment showed an excellent response rate, although all patients experienced severe toxicity [13]. Most recently BRAF inhibitors have been trialled in LCH (discussed further under section 'RAS/RAF/MEK/ERK cell signaling pathway mutations'). Better treatment options are still required, along with the need for earlier detection to reduce life-long sequelae in patients with LCH. For example, pituitary lesions often cause central diabetes insipidus, and neurological problems such as neurodegenerative central nervous system disease is common with central nervous system infiltration [4].

1.1.2. Etiology of LCH

LCH is a histiocytic disease, meaning it is one of a group of rare and varied proliferative disorders derived from DCs and macrophages [14]. Cells of the histiocyte lineage include antigen-processing and presenting cells such as monocytes, macrophages, DCs and Langerhans cells (LCs) [1]. LCH was first reported around 1900 and was originally referred to as Hand-Schüller-Christian disease and Letterer-Siwe disease [6]. The disease was unified as histiocytosis X in 1953, with the 'X' representing a cell of unknown origin [1, 15–17]. LCH cells were subsequently described as the progeny of migrating mature epidermal LCs [18], however most recently it was hypothesized that the cells rather arise from an early bone marrow derived or myeloid precursor [17].

LCH is diagnostically defined by immunohistochemistry detection of S100$^+$ and CD1a$^+$ (or CD207$^+$) LCH cells [1, 6, 14]. Whether the LCH cells are neoplastic or the result of an immune dysfunction is still somewhat contentious. Historically LCH cells were identified as clonal using X-inactivation studies [19, 20], however several groups have highlighted the shortcomings of using this approach to confirm clonality [21, 22]. Immune dysregulation has also been suggested as a potential cause for LCH because of the inflammatory nature of lesions [2, 23–25]. The most recent etiologically relevant finding is the association of RAS/RAF/MEK/ERK cell signaling pathway mutations by the detection of *BRAF V600E* and *MAP2K1* protein kinase gene mutations within LCH cells [16, 26–31]. This association gives weight to the 'Misguided Myeloid DC Precursor' hypothesis [15, 32–34] that describes LCH cells as immature CD1a$^+$ DCs [4] derived from early myeloid precursors [32, 35].

1.1.3. Significance of RAS/RAF/MEK/ERK cell signaling pathway mutations in LCH

The detection of RAS/RAF/MEK/ERK cell signaling mutations in LCH cells suggests that LCH is an inflammatory myeloid neoplasm and aligns with the 'Misguided Myeloid DC Precursor' hypothesis [4, 14, 36, 37], although opinions vary in the LCH field [38]. *BRAF V600E* accounts for 90% of all *BRAF* mutations in human cancers [39] and is also the most common *BRAF* mutation identified to date in LCH lesions [16, 26–28]. Approximately 50% of LCH lesions reportedly contained the *BRAF V600E* mutation in LCH cells [40]. BRAF is a member of the RAS/RAF/MEK/ERK cell signaling pathway, and *BRAF V600E* substitutes

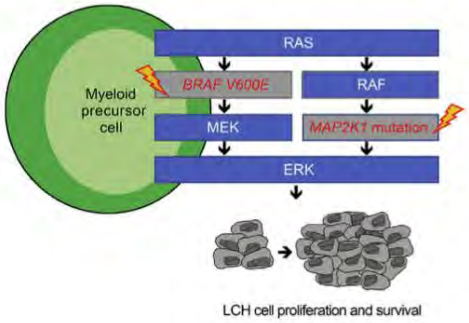

Fig. 3. RAS/RAF/MEK/ERK cell signaling pathway mutations in LCH. Myeloid precursor cells with various mutations in the MAPK pathway result in LCH cell proliferation and survival.

amino acid valine in position 600 with glutamic acid [26], stimulating downstream signal transduction of MEK and ERK protein kinases constitutively [6, 41]. This can in turn activate transcription factors to promote uncontrolled cell division. The detection of *BRAF V600E* in LCH may be helpful in the treatment of this disease. Patients treated with targeted therapy to inhibit the BRAF protein show encouraging early results [42], although the long-term effects of these drugs, which could conceivably result in severe consequences when administered to children, is currently unknown [43].

Mutations in other protein kinase genes affecting the RAS/RAF/MEK/ERK signaling pathway have also been discovered in LCH cells. *MAP2K1* mutations were detected in *BRAF* wild type LCH lesions, meaning that the *BRAF* and *MAP2K1* mutations were found in mutually exclusive lesions [29–31] (Fig. 3). This accounts for a further 27.5% of LCH lesions harboring MAPK pathway mutations and suggests that MAPK pathway mutations are reported in almost 80% of LCH cases. All mutations in the *MAP2K1* gene result in a similar outcome to *BRAF V600E*, and are associated with constitutive signal transduction and oncogenesis [29, 30]. Other *BRAF* mutations detected in LCH lesions [16, 26, 44] include a *BRAF* gene fusion and small in-frame *BRAF* deletions in lesions lacking *BRAF V600E* and *MAP2K1* mutations [45] suggesting that there may be other MAPK pathway mutations in wild type lesions that are yet to be detected. These findings could potentially account for the constitutive activation of ERK that is seen LCH cells [27, 34, 45, 46]. This constitutive activation within LCH cells could affect immune regulation in LCH lesions. RAS/RAF/MEK/ERK cell signaling pathway mutations are known to affect DC differentiation [47, 48] and maturation [48] and if this occurs within LCH lesions, T cell responses may be directly affected within the microenvironment through cell signaling. Given this, treatment with BRAF inhibitors, which target LCH cells, may also have a considerable effect on the T cells within LCH lesions.

1.2. T cells potentially involved in the immune regulation of LCH

LCH cells make up 36–58% of lesional cells [1] and share common features with epidermal LCs and activated LCs in their expression of CD1a, CD207 and T cell costimulatory molecules, however a distinguishing feature compared to LCs is their lower surface expression of HLA-DR. [49, 50] The LCH microenvironment typically contains pro-inflammatory cytokines consisting of high levels of tumor necrosis factor (TNF), interferon gamma (IFNγ), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-1beta (IL-1β) and IL-2 [2, 14, 23, 32]. Kannourakis and Abbas reported that cytokines detected within LCH lesions reflect local T cell activation (along with other inflammatory leukocytes) [2]. In line with this, Egeler et al. [23] demonstrated that both T cells and LCH cells within LCH lesions produced high levels of cytokines. Collectively these studies indicate that both T cells and LCH cells are contributing to the lesional inflammation.

The *in vitro* addition of T cell costimulatory molecule CD40L-transfected fibroblasts to LCH cells was demonstrated to induce surface expression of HLA-DR and CD86 on LCH cells and also improved the allogeneic response of T cells towards LCH cells [49], suggesting that the addition of CD40L, and most likely the engagement of CD40 on LCH cells, induces LCH cell maturation. This also indicates that the inflammatory nature of LCH facilitates a microenvironment in which immune regulatory cell responses such as that of T cells may be inhibited.

T cells are heavily involved in anti-tumor immune responses, however tumors can evade the immune system by producing inhibitory signals. If we consider LCH to be neoplastic disease, we can think of the disease in terms of LCH cells making adaptations that result in an immune system evasion. T cells have a central role in cell-mediated immunity and their interactions with antigen presenting cells are an important component of immune regulation. Antibodies to T cell costimulatory molecules such as CTLA-4 and PD-1 have been used as immunotherapy drugs, which have shown a good clinical response in the treatment of various cancers [51–53]. It is known that LCH cells have reduced surface expression of receptors HLA-DR, CD83, CD86 and CD208, all with important roles in antigen-dependent T cell activation [49, 50]. Although immune cells such as T cells are being recruited to the site and contributing to the inflammation, they may be unable to completely recognize and destroy LCH cells within the microenvironment because of reduced receptor-ligand interactions. Investigating T cells with a regulatory function may identify how inflammation is established and maintained in LCH lesions and how LCH cells are evading the immune system. Defining this process is likely to lead to new possibilities for using targeted therapies to manipulate the immune system into tumor immunity.

1.2.1. CD1a$^+$ T cells

The Kannourakis group identified a previously unreported subset of T cells expressing CD1a in LCH, although the function of these cells was not described [3]. CD1a is not normally expressed by mature T cells, however it is expressed by immature thymocytes and downregulated in the latter stages of T cell development. Abnormal CD1a expression on immature T cells in malignancies [54, 55] and on extrathymic T cells in tonsil tissue [56] have been reported, however the presence of polyclonal CD1a$^+$ T cells within LCH lesions is the only published example of CD1a expression by mature T cells [3]. These cells were not detected in the blood of patients with LCH or in peripheral blood and tonsil from healthy donors and instead appear restricted to the lesions where inflammation may have induced them [3]. The functional significance of CD1a$^+$ T cells has not been determined and their functional capacity has not been studied. They are intriguing because there is already evidence of increased frequency of specialized T cell subsets in patients with LCH and one or all of these lineages has the potential to affect the pathogenesis and progression of LCH, although functional studies are required to fully investigate their importance. For example, there are several reports of increased levels of Foxp3$^+$ Tregs in blood and lesions from patients with LCH [24, 32, 57–59], and unconventional γδ T cells have also been found in high numbers in cutaneous LCH [60]. The significance of these cells and the potential involvement of other unconventional T cell subsets is discussed next.

1.2.2. Foxp3$^+$ Tregs

Foxp3$^+$ Tregs are a regulatory T cell subset defined by the expression of forkhead box P3 (Foxp3) transcription factor. Foxp3$^+$ Tregs are identified by their expression of CD3, CD4, CD25 and Foxp3, although a surrogate definition of CD3$^+$CD4$^+$CD25$^+$CD127^{low} lymphocytes can be used, which allows for functional analysis of this subset [61]. Tregs

help to maintain immunologic tolerance [62] by suppressing T cell activation through direct cell-cell contact and by secreting inhibitory cytokines TGF-β and IL-10 [63–65]. This can lead to Tregs promoting tumor development by inhibiting anti-tumor immune responses [61, 63].

Increased frequencies of Foxp3$^+$ Tregs have been identified in LCH lesions in several studies [32, 57–59]. Senechal et al. [4, 24] found that 18% of lesional T cells in children's LCH tissue samples were CD4$^+$CD25$^+$ and confirmed in 9 of 11 samples that these cells 'strongly expressed' Foxp3 and observed that they were in close proximity to the LCH cells. A caveat to this study is that the authors relied on CD25 to identify Tregs, which is a less stringent approach than using anti-Foxp3 monoclonal antibodies, because activated T cells can also express CD25. A supporting study, however, did identify CD3$^+$Foxp3$^+$ Tregs in high numbers in LCH lesions (22–30% of T cells), and determined a positive correlation between the number of Tregs within lesions and the total lymphocyte infiltration density [57]. Allen et al. [32] reported an increase in the expression of *FOXP3* in T cells from LCH lesions compared with T cells isolated from patient matched peripheral blood. They also identified an increase in the expression of *CTLA4* in T cells, which can promote the suppressive function of Tregs [32]. The presence of high numbers of Tregs within LCH lesions suggests a potential role for these cells in the pathogenesis of the disease.

Immature DCs have previously been shown to promote Treg production and immune tolerance [66]. As LCH cells are considered to be functionally immature DCs [4], Tong et al. [57] proposed that LCH cells could potentially promote Treg production and immune tolerance. This could explain the increased number of Tregs within LCH lesions and subsequent suppression of the immune response of T cells and other immune cells against LCH cells, which would account for LCH cell survival, and infiltrating immune cells within lesions (Fig. 4). Quispel et al. [59] showed that increases in activated conventional T cells within LCH lesions correlate with a further increase of Foxp3$^+$ Tregs and suggest that that Tregs could be increased in response to activated T cells in an attempt to counteract the immune response of the conventional T cells. It is important to determine whether Tregs have a suppressive role in LCH to increase our understanding of immune regulation in LCH.

Senechal et al. [24] also detected a significant increase in the absolute number and frequency of Tregs (CD3$^+$CD4$^+$CD25$^+$Foxp3$^+$) in the blood of patients with newly diagnosed LCH compared with age-matched controls. The presence of elevated levels of Tregs in the blood of patients with active LCH indicates that the lesion microenvironment alone may not be a complete representation of this disorder and there may be more widespread, potentially systemic causes or effects taking place.

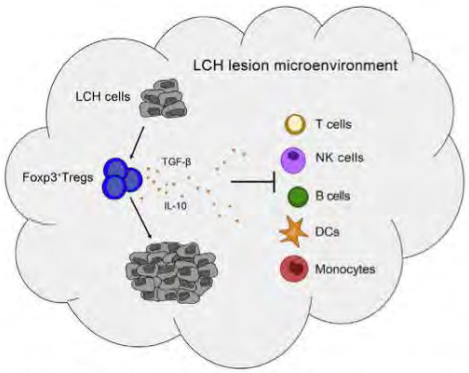

Fig. 4. LCH cells may promote Tregs. LCH cells are potentially responsible for promoting Tregs within LCH lesions, accounting for the high proportions of Tregs identified in LCH. Tregs may subsequently inhibit the immune response of other cells within the microenvironment, thus promoting LCH cell survival.

1.2.3. Unconventional T cells

A number of unconventional T cell subsets such as γδ T cells, type I NKT cells (referred to herein as NKT cells) and MAIT cells are involved in tumor immunity [67–71]. While conventional T cell subsets make up a large and important component of the immune system, the interest in unconventional T cells is growing (reviewed in Godfrey et al. [72]). It is well established that conventional T cells recognize and respond to peptide antigens presented by MHC molecules, however unconventional T cells recognize and respond to a variety of non-peptide antigens such as lipids and vitamin B metabolites presented by MHC-like molecules such as CD1 and MHC-related protein 1 (MR1) respectively (reviewed in Rossjohn et al. [73]). Some subsets of unconventional T cells have limited T cell receptor diversity and are capable of rapid antigen-specific and non-specific immune responses. Unconventional T cells are associated with the immune regulation of cancer, infection, and autoimmunity, and could be involved in LCH.

One research study demonstrated a high frequency of γδ T cells (30% of total T cells) in 3/6 cutaneous LCH lesions [60]. A caveat to this study is that the proportions of γδ T cells within LCH lesions were not compared to any control group such as healthy peripheral blood or skin. A separate case study found an LCH patient to have increased circulating γδ T cells (25%) in comparison to 50 healthy controls (< 10%) although whether this is a proportion of lymphocytes or total T cells is unclear [74]. γδ T cells usually constitute around 5% of peripheral T cells yet they can provide tumor immunity through direct killing and indirectly through IFNγ release [75]. For example, cytotoxic γδ T cells isolated from patients with various types of cancer could be expanded and shown to kill tumor cell lines and primary cancers *in vitro* [76], however their precise role is unclear as IL-17-producing γδ T cells have also been suggested as tumor promoting [75]. Given that γδ T cells have both tumor-promoting and -protective properties, it is important to better define their role in LCH. Reports of increased circulating and lesional γδ T cells associated with LCH are certainly of interest, however need further investigation. Increases in γδ T cells in LCH could be tumor-promoting or -protective, although stronger evidence is needed to determine whether increases in γδ T cells are definitivelty associated with LCH.

It is surprising that CD1d-restricted NKT cells and MR1-resrticted MAIT cells have never been examined in the context of LCH, especially because immune dysregulation is a suggested cause for the disease. NKT cells and MAIT cells are increasingly associated with diseases characterized by immune dysregulation [70, 71, 77–79] and it is feasible that they could contribute to LCH progression through deficiencies and thus failure to regulate. Indeed there are already many examples where NKT cell and MAIT cell defects are associated with localised tissue inflammation, including psoriasis, allergies and tumors [67, 69–71, 78, 80, 81].

NKT cells make up 0.01–0.5% of human T cells [82–85] and produce a wide variety of cytokines with the ability to bias the immune response towards Th1 (IFNγ), Th2 (IL-4), Th10 (IL-10) or Th17 (IL-17) responses [82, 84, 86]. This means they have the potential to modify an immune microenvironment [82, 87, 88], suggesting a potentially powerful role in tumor immunity. Reduced NKT cell frequency correlates with poor survival in acute myeloid leukemia [89–91] and head and neck cancer [92, 93], while increased frequency in colon cancer and hematological malignancies is linked to better prognosis [89, 91, 94].

MAIT cells, like NKT cells, are capable of powerful and rapid immune responses. MAIT cells respond to infection by secreting inflammatory cytokines IFNγ and TNF [95, 96], both of which are present

in LCH lesions. MAIT cells also upregulate CD40L upon activation, which can in turn mature DCs and has been shown to mature LCH cells [49, 97]. To date, most studies on MAIT cells have focused on microbial infection, although it is increasingly apparent they are also present in some tumors [70, 71] and other sites of inflammation [98, 99] and thus should be looked at in the context of LCH. Like NKT cells, MAIT cells have not been studied in LCH, but expression of CD161 is a defining characteristic of MAIT cells and it is noteworthy that the gene encoding for the C-type lectin receptor for CD161, *CLEC2D*, is overexpressed by LCH cells compared with skin-resident LCs [32].

Given the presence of high levels of CD1a expression in LCH lesions, there is a capacity to activate or inhibit CD1a-restrcited T cells in LCH. To date little is known about the relatively newly discovered CD1a-restricted T cells, although CD1a expression is altered in several disease models [100] including aberrant expression in cancers such as hairy cell leukemia [101] and some T lymphoblastic lymphomas [102]. CD1a is also linked with autoimmune skin diseases such as atopic eczema [103, 104] and psoriasis [105], thus CD1a-restricted T cells may be implicated in the pathology of many disease states, including cancer and autoimmunity. CD1a-restricted T cells have been detected in the blood of healthy individuals using a variety of endogenous and exogenous lipid ligands [106–108]. The cells can express skin homing receptors CCR4, CCR6 and CCR10, and produce IL-22 [106], IFNγ and TNF [106–108]. These cells may play a vital role in skin immunity, however the circulatory presence of these cells also infers a potential role for CD1a-restricted T cells more systemically.

1.3. Summary and perspective

Foxp3$^+$ Tregs, unconventional T cells and CD1a$^+$ T cells are important avenues of investigation with respect to LCH because they or subsets thereof have already been identified as being altered in or unique to LCH. Now with advances in technology there is scope to further determine the role of those already implicated and other T cell subsets in the disease. These immunoregulatory subsets to date have not been well studied likely due to the rarity of samples. There is a definite under-representation of functional studies in the LCH field. While many of the current LCH studies are appropriately focused on mutations within LCH cells, it is important to look at all aspects of immunity and include T cells because of the various ligand-receptor interactions between DCs and T cells. Studies on Tregs, unconventional T cells and CD1a$^+$ T cells may therefore help us to better understand the nature of the perplexing microenvironment that characterizes this disease.

T cells with a regulatory function are already implicated in a wide variety of diseases such as cancer and autoimmunity, although some have not been studied at all in LCH. They are a vital component of the immune system and play a key role in immune cell homeostasis, mostly through the production of a diverse range of cytokines. By better understanding the role of T cells with a regulatory function in LCH we may be able to determine whether LCH lesions result from an immune system imbalance or evasion, and improve treatment options for LCH.

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Declaration of interest

None.

Authorship

J.M.M wrote the manuscript and created the figures, which were critically reviewed by S.P.B. and G.K.

References

[1] F.M. Rizzo, M. Cives, V. Simone, F. Silvestris, New insights into the molecular pathogenesis of Langerhans cell histiocytosis, Oncologist 19 (2) (2014) 151–163.
[2] G. Kannourakis, A. Abbas, The role of cytokines in the pathogenesis of Langerhans cell histiocytosis, Br. J. Cancer Suppl. 23 (1994) S37–S40.
[3] J.A. West, S.L. Olsen, J.M. Mitchell, et al., Polyclonal T-cells express CD1a in Langerhans cell histiocytosis (LCH) lesions, PLoS One 9 (10) (2014) e109586.
[4] A. Morimoto, Y. Oh, Y. Shioda, K. Kudo, T. Imamura, Recent advances in Langerhans cell histiocytosis, Pediatr. Int. 56 (4) (2014) 451–461.
[5] G.I. Bechan, R.M. Egeler, R.J. Arceci, Biology of Langerhans cells and Langerhans cell histiocytosis, Int. Rev. Cytol. 254 (2006) 1–43.
[6] C.E. Allen, S. Ladisch, K.L. Mcclain, How we treat Langerhans cell histiocytosis, Blood 126 (1) (2015) 26–35.
[7] H. Gadner, M. Minkov, N. Grois, et al., Therapy prolongation improves outcome in multisystem Langerhans cell histiocytosis, Blood 121 (25) (2013) 5006–5014.
[8] R. Kotecha, R. Venkatramani, R.F. Jubran, A. Arkader, A.J. Olch, K. Wong, Clinical outcomes of radiation therapy in the management of Langerhans cell histiocytosis, Am. J. Clin. Oncol. 37 (6) (2014) 592–596.
[9] R. Haupt, M. Minkov, I. Astigarraga, et al., Langerhans cell histiocytosis (LCH): guidelines for diagnosis, clinical work-up, and treatment for patients till the age of 18 years, Pediatr. Blood Cancer 60 (2) (2012) 175–184.
[10] M.E. Lahey, Histiocytosis X: comparison of three treatment regimens, J. Pediatr. 87 (2) (1975) 179–183.
[11] R. Haupt, T.R. Fears, P. Rosso, et al., Increased risk of secondary leukemia after single-agent treatment with etoposide for Langerhans' cell histiocytosis, Pediatr. Hematol. Oncol. 11 (5) (1994) 499–507.
[12] K.A. Al-Anazi, A. Alshehri, H.A. Al-Zahrani, F.I. Al-Mohareb, I. Maghfoor, D. Ajarim, Successful outcome of Langerhans cell histiocytosis complicated by therapy-related myelodysplasia and acute myeloid leukemia: a case report, Cases J. 1 (1) (2008) 101.
[13] J. Donadieu, F. Bernard, M. van Noesel, et al., Cladribine and cytarabine in refractory multisystem Langerhans cell histiocytosis: results of an international phase 2 study, Blood 126 (12) (2015) 1415–1423.
[14] S.R. Vaiselbuh, Y.T. Bryceson, C.E. Allen, J.A. Whitlock, O. Abla, Updates on histiocytic disorders, Pediatr. Blood Cancer 61 (7) (2014) 1329–1335.
[15] M.L. Berres, K.P.H. Lim, T. Peters, et al., *BRAF-V600E* expression in precursor versus differentiated dendritic cells defines clinically distinct LCH risk groups, J. Exp. Med. 211 (4) (2014) 669–683.
[16] F. Sahm, D. Capper, M. Preusser, et al., BRAFV600E mutant protein is expressed in cells of variable maturation in Langerhans cell histiocytosis, Blood 120 (12) (2012) e28–e34.
[17] G. Badalian-Very, J.-A. Vergilio, B.A. Degar, C. Rodriguez-Galindo, B.J. Rollins, Recent advances in the understanding of Langerhans cell histiocytosis, Br. J. Haematol. 156 (2) (2011) 163–172.
[18] C. Nezelof, F. Basset, M.F. Rousseau, Histiocytosis X histogenetic arguments for a Langerhans cell origin, Biomedicine 18 (5) (1973) 365–371.
[19] C.L. Willman, Detection of clonal histiocytes in Langerhans cell histiocytosis: biology and clinical significance, Br. J. Cancer Suppl. 23 (1994) S29–S33.
[20] R.C. Yu, C. Chu, L. Buluwela, A.C. Chu, Clonal proliferation of Langerhans cells in Langerhans cell histiocytosis, Lancet 343 (8900) (1994) 767–768.
[21] N. Agarwal, R.H. Nussenzveig, S.I. Swierczek, C.J. Parker, J.T. Prchal, Does HUMARA assay for assessment of clonal hematopoiesis have shortcomings? Blood 114 (11) (2009) (2357–8– author reply 2358–9).
[22] W. Liu, X. Sun, Skewed X chromosome inactivation in diploid and triploid female human embryonic stem cells, Hum. Reprod. 24 (8) (2009) 1834–1843.
[23] R.M. Egeler, B.E. Favara, M. van Meurs, J.D. Laman, E. Claassen, Differential in situ cytokine profiles of Langerhans-like cells and T cells in Langerhans cell histiocytosis: abundant expression of cytokines relevant to disease and treatment, Blood 94 (12) (1999) 4195–4201.
[24] B. Senechal, G. Elain, E. Jeziorski, et al., Expansion of regulatory T cells in patients with Langerhans cell histiocytosis, PLoS Med. 4 (8) (2007) e253.
[25] R.M. Egeler, A.G.S. van Halteren, P.C.W. Hogendoorn, J.D. Laman, P.J.M. Leenen, Langerhans cell histiocytosis: fascinating dynamics of the dendritic cell-macrophage lineage, Immunol. Rev. 234 (1) (2010) 213–232.
[26] T. Satoh, A. Smith, A. Sarde, et al., B-RAF mutant alleles associated with Langerhans cell histiocytosis, a granulomatous pediatric disease, PLoS One 7 (4) (2012) e33891.
[27] G. Badalian-Very, J.-A. Vergilio, B.A. Degar, et al., Recurrent *BRAF* mutations in Langerhans cell histiocytosis, Blood 116 (11) (2010) 1919–1923.
[28] S.A. Yousem, S. Dacic, Y.E. Nikiforov, M. Nikiforova, Pulmonary Langerhans cell histiocytosis: profiling of multifocal tumors using next-generation sequencing identifies concordant occurrence of *BRAF* V600E mutations, Chest 143 (6) (2013) 1679–1684.
[29] D.S. Nelson, A. van Halteren, W.T. Quispel, et al., *MAP2K1* and *MAP3K1* mutations in Langerhans cell histiocytosis, Genes Chromosom. Cancer 54 (6) (2015) 361–368.
[30] R. Chakraborty, O.A. Hampton, X. Shen, et al., Mutually exclusive recurrent somatic mutations in *MAP2K1* and *BRAF* support a central role for ERK activation in

LCH pathogenesis, Blood 124 (19) (2014) 3007–3015.
[31] N.A. Brown, L.V. Furtado, B.L. Betz, et al., High prevalence of somatic *MAP2K1* mutations in *BRAF V600E*-negative Langerhans cell histiocytosis, Blood 124 (10) (2014) 1655–1658.
[32] C.E. Allen, L. Li, T.L. Peters, et al., Cell-specific gene expression in Langerhans cell histiocytosis lesions reveals a distinct profile compared with epidermal Langerhans cells, J. Immunol. 184 (8) (2010) 4557–4567.
[33] M.-L. Berres, C.E. Allen, M. Merad, Pathological consequence of misguided dendritic cell differentiation in histiocytic diseases, Adv. Immunol. 120 (2013) 127–161.
[34] D.J. Zinn, R. Chakraborty, C.E. Allen, Langerhans cell histiocytosis: emerging insights and clinical implications, Oncology 30 (2) (2016) (Williston Park, NY).
[35] W.T. Quispel, J.A. Stegehuis-Kamp, L. Blijleven, et al., The presence of CXCR4$^+$ CD1a$^-$ cells at onset of Langerhans cell histiocytosis is associated with a less favorable outcome, Oncoimmunology 5 (3) (2016) e1084463.
[36] M.-L. Berres, M. Merad, C.E. Allen, Progress in understanding the pathogenesis of Langerhans cell histiocytosis: back to Histiocytosis X? Br. J. Haematol. 169 (1) (2015) 3–13.
[37] M. Collin, V. Bigley, K.L. Mcclain, C.E. Allen, Cell(s) of origin of Langerhans cell histiocytosis, Hematol. Oncol. Clin. North Am. 29 (5) (2015) 825–838.
[38] J. Braier, Is Langerhans cell histiocytosis a neoplasia? Pediatr Blood Cancer, September 2016.
[39] N. Dhomen, R. Marais, New insight into *BRAF* mutations in cancer, Curr. Opin. Genet. Dev. 17 (1) (2007) 31–39.
[40] A.-M. Bubolz, S.E. Weissinger, A. Stenzinger, et al., Potential clinical implications of *BRAF* mutations in histiocytic proliferations, Oncotarget 5 (12) (2014) 4060–4070.
[41] E.R. Cantwell-Dorris, J.J. O'Leary, O.M. Sheils, BRAF^{V600E}: implications for carcinogenesis and molecular therapy, Mol. Cancer Ther. 10 (3) (2011) 385–394.
[42] J. Haroche, F. Cohen-Aubart, J.F. Emile, et al., Dramatic efficacy of vemurafenib in both multisystemic and refractory Erdheim-Chester disease and Langerhans cell histiocytosis harboring the *BRAF* V600E mutation, Blood 121 (9) (2013) 1495–1500.
[43] M. Arico, Langerhans cell histiocytosis in children: from the bench to bedside for an updated therapy, Br. J. Haematol. 173 (5) (February 2016) 663–670.
[44] R. Kansal, L. Quintanilla-Martinez, V. Datta, J. Lopategui, G. Garshfield, B.N. Nathwani, Identification of the V600D mutation in Exon 15 of the *BRAF* oncogene in congenital, benign Langerhans cell histiocytosis, Genes Chromosom. Cancer 52 (1) (2013) 99–106.
[45] R. Chakraborty, T.M. Burke, O.A. Hampton, et al., Alternative genetic mechanisms of BRAF activation in Langerhans cell histiocytosis, Blood 128 (21) (October 2016) 2533–2537.
[46] S. Mourah, A. How-Kit, V. Meignin, et al., Recurrent *NRAS* mutations in pulmonary Langerhans cell histiocytosis, Eur. Respir. J. 47 (6) (April 2016) 1785–1796.
[47] M. Hamdorf, A. Berger, S. Schüle, J. Reinhardt, E. Flory, PKCδ-induced PU.1 phosphorylation promotes hematopoietic stem cell differentiation to dendritic cells, Stem Cells 29 (2) (2011) 297–306.
[48] A. Puig-Kröger, M. Relloso, O. Fernández-Capetillo, et al., Extracellular signal-regulated protein kinase signaling pathway negatively regulates the phenotypic and functional maturation of monocyte-derived human dendritic cells, Blood 98 (7) (2001) 2175–2182.
[49] F. Geissmann, Y. Lepelletier, S. Fraitag, et al., Differentiation of Langerhans cells in Langerhans cell histiocytosis, Blood 97 (5) (2001) 1241–1248.
[50] L. Redd, M. Schmelz, W.R. Burack, J.R. Cook, A.W. Day, L. Rimsza, Langerhans cell histiocytosis shows distinct cytoplasmic expression of major histocompatibility class II antigens, J. Hematop. 9 (3) (2016) 107–112.
[51] R. Chen, P.-C. Peng, B. Wen, et al., Anti-programmed cell death (PD)-1 immunotherapy for malignant tumor: a systematic review and meta-analysis, Transl. Oncol. 9 (1) (2016) 32–40.
[52] S. Farkona, E.P. Diamandis, I.M. Blasutig, Cancer immunotherapy: the beginning of the end of cancer? BMC Med. 14 (1) (2016) 73.
[53] S. Yun, N.D. Vincelette, M.R. Green, A.E. Wahner Hendrickson, I. Abraham, Targeting immune checkpoints in unresectable metastatic cutaneous melanoma: a systematic review and meta-analysis of anti-CTLA-4 and anti-PD-1 agents trials, Cancer Med. 5 (7) (2016) 1481–1491.
[54] F.E. Craig, K.A. Foon, Flow cytometric immunophenotyping for hematologic neoplasms, Blood 111 (8) (2008) 3941–3967.
[55] W.Y. Kim, H. Kim, Y.K. Jeon, C.-W. Kim, Follicular dendritic cell sarcoma with immature T-cell proliferation, Hum. Pathol. 41 (1) (2010) 129–133.
[56] S. Mcclory, T. Hughes, A.G. Freud, et al., Evidence for a stepwise program of extrathymic T cell development within the human tonsil, J. Clin. Invest. 122 (4) (2012) 1403–1415.
[57] C. Tong, X. Jia, Y. Jia, Y. He, Langerhans cell histiocytosis in Chinese adults: absence of BRAF mutations and increased FOXP3$^+$ regulatory T cells, Int. J. Clin. Exp. Pathol. 7 (6) (2014) 3166–3173.
[58] M. Battistella, S. Fraitag, D.H. Teillac, N. Brousse, Y. de Prost, C. Bodemer, Neonatal and early infantile cutaneous Langerhans cell histiocytosis: comparison of self-regressive and non-self-regressive forms, Arch. Dermatol. 146 (2) (2010) 149–156.
[59] W.T. Quispel, J.A. Stegehuis-Kamp, S.J. Santos, R.M. Egeler, A.G.S. van Halteren, Activated conventional T-cells are present in Langerhans cell histiocytosis lesions despite the presence of immune suppressive cytokines, J. Interf. Cytokine Res. 35 (10) (2015) 831–839.
[60] M. Alaibac, A.C. Chu, T-lymphocytes bearing the gamma delta T-cell receptor in cutaneous lesions of Langerhans' cell histiocytosis, Med. Pediatr. Oncol. 21 (5) (1993) 347–349.
[61] B.M. Hall, T cells: soldiers and spies-the surveillance and control of effector T cells by regulatory T cells, Clin. J. Am. Soc. Nephrol. 10 (11) (2015) 2050–2064.
[62] W. Hua, A. Yuan, W. Zheng, et al., Accumulation of FoxP3$^+$ T regulatory cells in the tumour microenvironment of human colorectal adenomas, Pathol. Res. Pract. 212 (2) (2016) 106–112.
[63] X. Zhou, J. Tang, H. Cao, H. Fan, B. Li, Tissue resident regulatory T cells: novel therapeutic targets for human disease, Cell. Mol. Immunol. (April 2015) 1–10.
[64] C.I. Kingsley, M. Karim, A.R. Bushell, K.J. Wood, CD25$^+$CD4$^+$ regulatory T cells prevent graft rejection: CTLA-4- and IL-10-dependent immunoregulation of alloresponses, J. Immunol. 168 (3) (2002) 1080–1086.
[65] K. Nakamura, A. Kitani, W. Strober, Cell contact-dependent immunosuppression by CD4$^+$CD25$^+$ regulatory T cells is mediated by cell surface-bound transforming growth factor beta, J. Exp. Med. 194 (5) (2001) 629–644.
[66] R.M. Steinman, D. Hawiger, M.C. Nussenzweig, Tolerogenic dendritic cells, Annu. Rev. Immunol. 21 (2003) 685–711.
[67] M.V. Dhodapkar, M.D. Geller, D.H. Chang, et al., A reversible defect in natural killer T cell function characterizes the progression of premalignant to malignant multiple myeloma, J. Exp. Med. 197 (12) (2003) 1667–1676.
[68] J.W. Molling, M. Moreno, H.J.J. van der Vliet, et al., Generation and sustained expansion of mouse spleen invariant NKT cell lines with preserved cytokine releasing capacity, J. Immunol. Methods 322 (1–2) (2007) 70–81.
[69] N. Neparidze, M.V. Dhodapkar, Harnessing CD1d-restricted T cells toward antitumor immunity in humans, Ann. N. Y. Acad. Sci. 1174 (2009) 61–67.
[70] P. Sundström, F. Ahlmanner, P. Akéus, et al., Human mucosa-associated invariant T cells accumulate in colon adenocarcinomas but produce reduced amounts of IFN-γ, J. Immunol. 195 (7) (2015) 3472–3481.
[71] L. Ling, Y. Lin, W. Zheng, et al., Circulating and tumor-infiltrating mucosal associated invariant T (MAIT) cells in colorectal cancer patients, Sci. Rep. 6 (2016).
[72] D.I. Godfrey, A.P. Uldrich, J. Mccluskey, J. Rossjohn, D.B. Moody, The burgeoning family of unconventional T cells, Nat. Immunol. 16 (11) (2015) 1114–1123.
[73] J. Rossjohn, S. Gras, J.J. Miles, S.J. Turner, D.I. Godfrey, J. Mccluskey, T cell antigen receptor recognition of antigen-presenting molecules, Annu. Rev. Immunol. 33 (2015) 169–200.
[74] N. Shijubo, K. Shigehara, S. Tsutahara, S. Abe, Increased level of circulating gamma/delta T cells in a patient with eosinophilic granuloma, Chest 105 (3) (1994) 967–968.
[75] M. Rei, D.J. Pennington, B. Silva-Santos, The emerging Protumor role of γδ T lymphocytes: implications for cancer immunotherapy, Cancer Res. 75 (5) (2015) 798–802.
[76] E. Presti Lo, F. Dieli, S. Meraviglia, Tumor-infiltrating gamma delta T lymphocytes: pathogenic role, clinical significance, and differential programing in the tumor microenvironment, Front. Immunol. 5 (2014) 975.
[77] S.P. Berzins, M.J. Smyth, A.G. Baxter, Presumed guilty: natural killer T cell defects and human disease, Nat. Publ. Group 11 (2) (2011) 131–142.
[78] K. Held, L. Bhonsle-Deeng, K. Siewert, et al., αβ T-cell receptors from multiple sclerosis brain lesions show MAIT cell-related features, *Neurol. Neuroimmunol. Neuroinflamm.* 2 (4) (2015) e107.
[79] M.E. Wallace, M.B. Alcantara, Y. Minoda, G. Kannourakis, S.P. Berzins, An emerging role for immune regulatory subsets in chronic lymphocytic leukaemia, Int. Immunopharmacol. 28 (2) (2015) 897–900.
[80] H.J. van der Vliet, Blomberg von BM, N. Nishi, et al., Circulating Valpha24$^+$ Vbeta11$^+$ NKT cell numbers are decreased in a wide variety of diseases that are characterized by autoreactive tissue damage, Clin. Immunol. 100 (2) (2001) 144–148.
[81] C. Iwamura, T. Nakayama, Role of alpha-galactosylceramide-activated Valpha14 natural killer T cells in the regulation of allergic diseases, Allergol. Int. 56 (1) (2007) 1–6.
[82] R.M. McEwen-Smith, M. Salio, V. Cerundolo, The regulatory role of invariant NKT cells in tumor immunity, Cancer Immunol. Res 3 (5) (2015) 425–435.
[83] N.A. Gherardin, D.S. Ritchie, D.I. Godfrey, P.J. Neeson, OMIP-021: simultaneous quantification of human conventional and innate-like T-cell subsets, Cytometry 85 (7) (2014) 573–575.
[84] B. Anderson, L. Teyton, A. Bendelac, P. Savage, Stimulation of natural killer T cells by glycolipids, Molecules 18 (12) (2013) 15662–15688.
[85] A.C. Chan, E. Leeansyah, A. Cochrane, et al., Ex-vivoanalysis of human natural killer T cells demonstrates heterogeneity between tissues and within established CD4$^+$ and CD4$^-$ subsets, Clin. Exp. Immunol. 172 (1) (2012) 129–137.
[86] D.G. Pellicci, A.P. Uldrich, All work and no Id2 makes a dull NKT cell, Immunol. Cell Biol. 94 (7) (May 2016) 619–620.
[87] M.J. Smyth, K.Y. Thia, S.E. Street, et al., Differential tumor surveillance by natural killer (NK) and NKT cells, J. Exp. Med. 191 (4) (2000) 661–668.
[88] N.Y. Crowe, M.J. Smyth, D.I. Godfrey, A critical role for natural killer T cells in immunosurveillance of methylcholanthrene-induced sarcomas, J. Exp. Med. 196 (1) (2002) 119–127.
[89] T. Tachibana, H. Onodera, T. Tsuruyama, et al., Increased intratumor Valpha24-positive natural killer T cells: a prognostic factor for primary colorectal carcinomas, Clin. Cancer Res. 11 (20) (2005) 7322–7327.
[90] A.E. Najera Chuc, L.A.M. Cervantes, F.P. Retiguin, J.V. Ojeda, E.R. Maldonado, Low number of invariant NKT cells is associated with poor survival in acute myeloid leukemia, J. Cancer Res. Clin. Oncol. 138 (8) (2012) 1427–1432.
[91] A. Shaulov, S. Yue, R. Wang, et al., Peripheral blood progenitor cell product contains Th1-biased noninvariant CD1d-reactive natural killer T cells: implications for posttransplant survival, Exp. Hematol. 36 (4) (2008) 464–472.
[92] M. Salio, J.D. Silk, E.Y. Jones, V. Cerundolo, Biology of CD1- and MR1-restricted T cells, Annu. Rev. Immunol. 32 (1) (2014) 323–366.

[93] J.W. Molling, J.A.E. Langius, J.A. Langendijk, et al., Low levels of circulating invariant natural killer T cells predict poor clinical outcome in patients with head and neck squamous cell carcinoma, J. Clin. Oncol. 25 (7) (2007) 862–868.
[94] L. Lynch, M. Nowak, B. Varghese, et al., Adipose tissue invariant NKT cells protect against diet-induced obesity and metabolic disorder through regulatory cytokine production, Immunity 37 (3) (2012) 574–587.
[95] M.C. Gold, S. Cerri, S. Smyk-Pearson, et al., Human mucosal associated invariant T cells detect bacterially infected cells, PLoS Biol. 8 (6) (2010) e1000407.
[96] L. Loh, Z. Wang, S. Sant, et al., Human mucosal-associated invariant T cells contribute to antiviral influenza immunity via IL-18-dependent activation, Proc. Natl. Acad. Sci. U. S. A. 113 (36) (2016) 10133–10138.
[97] M. Salio, O. Gasser, C. Gonzalez-Lopez, et al., Activation of human mucosal-associated invariant T Cells Induces CD40L-dependent maturation of monocyte-derived and primary dendritic cells, J. Immunol. 199 (8) (September 2017) 2631–2638.
[98] T.S.C. Hinks, Mucosal-associated invariant T cells in autoimmunity, immune-mediated diseases and airways disease, Immunology 148 (1) (2016) 1–12.
[99] A. Mekinian, T. Mahevas, M. Mohty, et al., Mucosal-associated invariant (MAIT) cells are deficient in systemic sclerosis, Scand. J. Immunol. 86 (4) (July 2017) 216–220.
[100] S. Siddiqui, L. Visvabharathy, C.-R. Wang, Role of group 1 CD1-restricted T cells in infectious disease, Front. Immunol. 6 (2015) 337.
[101] G. De Panfilis, G.C. Manara, C. Ferrari, C. Torresani, P. Sansoni, Hairy cell leukemia cells express CD1a antigen, Cancer 61 (1) (1988) 52–57.
[102] S. Cortelazzo, M. Ponzoni, A.J.M. Ferreri, D. Hoelzer, Lymphoblastic lymphoma, Crit. Rev. Oncol. Hematol. 79 (3) (2011) 330–343.
[103] M.R. Hussein, Evaluation of Langerhans' cells in normal and eczematous dermatitis skin by CD1a protein immunohistochemistry: preliminary findings, J. Cutan. Pathol. 35 (6) (2008) 554–558.
[104] V. Oji, N. Seller, A. Sandilands, et al., Ichthyosis vulgaris: novel *FLG* mutations in the German population and high presence of CD1a+ cells in the epidermis of the atopic subgroup, Br. J. Dermatol. 160 (4) (2009) 771–781.
[105] H.A. Alshenawy, E.A. Hasby, Immunophenotyping of dendritic cells in lesional, perilesional and distant skin of chronic plaque psoriasis, Cell. Immunol. 269 (2) (2011) 115–119.
[106] A. de Jong, V. Peña-Cruz, T.-Y. Cheng, R.A. Clark, I. Van Rhijn, D.B. Moody, CD1a-autoreactive T cells are a normal component of the human αβ T cell repertoire, Nat. Immunol. 11 (12) (2010) 1102–1109.
[107] C. de Lalla, M. Lepore, F.M. Piccolo, et al., High-frequency and adaptive-like dynamics of human CD1 self-reactive T cells, Eur. J. Immunol. 41 (3) (2011) 602–610.
[108] A.G. Kasmar, I. Van Rhijn, K.G. Magalhaes, et al., Cutting edge: CD1a tetramers and dextramers identify human lipopeptide-specific T cells ex vivo, J. Immunol. 191 (9) (2013) 4499–4503.

ARTICLE TITLE

Foxp3^{+} regulatory T cells from Langerhans cell histiocytosis lesions co-express CD56 and produce transforming growth factor beta

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ABSTRACT
Langerhans cell histiocytosis (LCH) is defined by lesions containing $CD1a^+/CD207^+$ myeloid lineage 'LCH' cells. Other immune cells are present within LCH lesions and this includes T cells and specifically an enrichment of $Foxp3^+$ regulatory T cells (Tregs), which are suspected to be immunosuppressive in function within the lesions. Transforming growth factor beta (TGF-β) is also present in lesions, and LCH cells are suggested to produce this inhibitory cytokine, but it is also typically produced by $Foxp3^+$ Tregs under normal conditions. Due to the need for transcription factor staining and thus cell permeabilization, $Foxp3^+$ Tregs have been difficult to conduct functional studies on in the past. Newer methods to test the function of Tregs are now available that allow for functional assays with this population. Using flow cytometry our study comprehensively analyzed the relative frequency of Tregs in patients with LCH by using a suite of identifiers including markers some other studies have omitted. We identified the unusual expression of CD56 by a considerable proportion of Tregs from LCH lesions and also tested the production of TGF-β by Tregs from LCH lesions. We confirmed that Tregs from LCH lesions are able to produce TGF-β and we further propose that Tregs within LCH lesions and their subsequent production of TGF-β is an important component in LCH pathogenesis.

INTRODUCTION
Langerhans cell histiocytosis (LCH) is a rare disease involving inflammatory lesions, which most commonly affect children, but also occur in adults. Lesions can occur in essentially any organ of the body and common sites affected are the skin and bone, although involvement of the liver, spleen or hematological system puts patients at an increased mortality risk[1]. LCH lesions are defined by the presence of $CD1a^+/CD207^+$ myeloid lineage cells (LCH cells), which often have mutations within the RAS/RAF/MEK/ERK pathway[2-8] and constitutive activation of ERK[4]. Given the ability of CD1a to bind variety of lipids, some of which can prime T cells for specific immune responses[9-16], CD1a expression by LCH cells within lesions may be important for LCH cell-T cell interactions, and T cells are amongst the range of other immune cells present within LCH lesions (reviewed in Bechan et al[17]).

In particular there is an enrichment of $Foxp3^+$ regulatory T cells (Tregs) in the peripheral blood and lesions from patients with LCH[18-22]. $Foxp3^+$ Tregs can maintain tolerance in immune environments by competing with other T cells for available interleukin 2 (IL-2), or by competing for co-stimulation signals, for example, via the expression of cytotoxic lymphocyte-associated antigen 4 (CTLA-4). Tregs can also produce cytolytic molecules such as granzymes, and secrete the inhibitory cytokines transforming growth factor beta (TGF-β) and/or IL-10 (Reviewed in Shevach[23]). It is by such mechanisms that $Foxp3^+$ Tregs can inhibit anti-tumor immune responses and promote the development of tumors (reviewed in Sakaguchi et al[24] and Chaudhary & Elkord[25]). $Foxp3^+$ Tregs in LCH lesions express inducible costimulatory factor (ICOS) and are in close proximity to the LCH cells, which correspondingly express ICOS ligand[19,21]. These observations suggest that there might be specific $Foxp3^+$ Treg-LCH cell interactions within the lesional environment. Given the enrichment of $Foxp3^+$ Tregs within lesions there is potential for involvement of Tregs in the pathogenesis of LCH. Additionally, Allen et al[22] identified an upregulation of *CTLA4* in T cells from

LCH lesions compared with LCH donor peripheral blood T cell expression, indicating that Tregs may be suppressing other immune cells within lesions. Furthermore, $Foxp3^+$ Tregs were reported to be increased in the blood from patients with active LCH compared to controls[19], strengthening their potential importance in LCH. Expression of the forkhead box P3 (Foxp3) transcription factor is synonymous with low CD127 expression and typically defines $Foxp3^+$ Tregs along with their expression of CD3, CD4 and the IL-2 receptor CD25[26,27]. Not all studies investigating $Foxp3^+$ Tregs in LCH have used the stringent definition of $CD3^+CD4^+CD25^+Foxp3^+$ or $CD127^{low}$ lymphocytes in concert.

The immune suppressive cytokines TGF-β and IL-10, which are commonly produced by $Foxp3^+$ Tregs, have been detected within LCH lesions[18,19,21,28-30] and blood[31,32] from patients with active LCH. Groups have suggested that LCH cells are a source of TGF-β[22,29] and that TGF-β is one of the drivers of the LCH cell phenotype[31,33,34], whilst IL-10 was described to be produced largely by macrophages[35] and LCH cells[18] within lesions. Given that $Foxp3^+$ Tregs are enriched within LCH lesions, and the cytokines they are known to produce under normal conditions are also present, it is conceivable that lesional $Foxp3^+$ Tregs too are a source of TGF-β and/or IL-10 production. Historically the identification of $Foxp3^+$ Tregs has not allowed for functional studies to be conducted because staining for the Foxp3 transcription factor requires cell permeabilization. Consequently, the function of Tregs in LCH lesions is not currently established, but a newer surrogate gating strategy to detect $CD3^+CD4^+CD25^+CD127^{low}$ lymphocytes (Tregs)[26,27] allows for downstream functional assays on the Treg population.

Our study set out to comprehensively characterize and quantitate the relative frequency of Tregs in patients with LCH by using a stringent identification method, and also to test the functional ability of this population in order to better define the role of Tregs in LCH pathogenesis.

RESULTS

Clinical details of patients with LCH.

The cohort for this study included 10 male and 7 female patients with LCH ranging from age 2 months to 68 years (**Table 1**). Bone, skin and lung were the most commonly affected tissues. Other than a single patient, to the best of our knowledge no patients with active LCH had any treatment with steroids or chemotherapy prior to the collection of specimens. Most patients with non-active disease had received prior treatment.

$Foxp3^+$ Tregs are enriched in patients with active LCH compared to healthy donors.

A surrogate identification method[26,27] was employed to gate on $CD3^+CD4^+CD25^+CD127^{low}$ lymphocytes in the peripheral blood from healthy donors and patients with LCH and in lesions from patients with LCH (**Figure 1.A**). Using this gating strategy, we identified similarly to previous studies that LCH lesion Tregs were significantly increased in the $CD4^+$ ($p < 0.0001$) and the total T cell population ($p < 0.0001$) when compared to healthy donors (**Figure 1.B** and **C**). Furthermore, the proportion of Tregs in the total T cell population was significantly increased in the

peripheral blood from patients with active LCH compared to those in the blood from healthy donors (*p = 0.0232*). By staining for the transcription factor, we confirmed Foxp3 expression by Tregs in lesions from three LCH patients, and we confirmed that other LCH lesion $CD4^+$ T cells were mostly negative for Foxp3 (**Figure 1.D**).

LCH lesion $Foxp3^+$ Tregs unexpectedly display cell surface expression of CD56 and lack HLA-DR expression.

Due to our interest in unconventional T cells we analyzed specimens for CD56 expression in conjunction with investigating Tregs and we unexpectedly found CD56 expression on a reasonable proportion of the Treg population from LCH lesions (**Figure 2.A**). CD56 is also known as neural cell adhesion molecule (NCAM) although it is not typically foreign to immune cells. CD56 is one of the defining markers used to identify natural killer (NK) cells, however it is also expressed on the surface of unconventional T cells such as natural killer T (NKT) cells and mucosal associated invariant T (MAIT) cells[36-38]. The proportion of Tregs that displayed CD56 on their cell surface was significantly higher in the lesions from patients with LCH compared to the peripheral blood from healthy donors (*p = 0.0092*) and patients with active LCH (*p < 0.0001*) (**Figure 2.B**). Additionally, we found that the proportion of $CD56^+$ Tregs in total T cells from LCH lesions was directly correlated to the proportion of total Tregs in LCH lesion T cells (*r = 1, p = 0.0028*) (**Figure 2.C**) and importantly, we confirmed Foxp3 expression by both the $CD56^+$ and $CD56^-$ Treg populations in lesions from three LCH patients (**Figure 2.D**). High HLA-DR expression by Tregs has previously been associated with the population having a superior contact-dependent suppressive capacity[39]. We therefore investigated cell surface HLA-DR expression by Tregs in lesions from three independent LCH donors but found that HLA-DR expression was low and similar to that seen in healthy donors (**Figure 2.E**).

Investigating expression of CD56 by lymphocytes in patients with LCH.

Because of the unexpected expression of CD56 by Tregs in lesions from patients with LCH, we took a broader look at CD56 expression in the lymphocyte population by assessing CD3 expression versus CD56 expression (**Figure 3.A. i**). We then assessed the expression of CD4 and CD8 by $CD56^+$ T cells (**Figure 3.A. ii**). We found that the ratio of $CD3^-CD56^+$ lymphocytes, which are most likely to be NK cells, was not strikingly different in patients with LCH compared to healthy donors (**Figure 3.B. i**). In contrast, the ratio of $CD3^-CD56^+$ lymphocytes to $CD56^+$ T cells was significantly decreased within LCH lesions compared to blood from patients with active disease (*p = 0.0048*) (**Figure 3.B. ii**). The difference in ratios we observed was likely due to the significant increase in the proportion of $CD56^+$ T cells in LCH lesions compared to the peripheral blood from patients with active LCH (*p = 0.0136*) (**Figure 3.C**). Blood from patients with non-active LCH also had a significantly higher proportion of T cells expressing cell surface CD56 when compared to patients with active disease (*p = 0.0490*) (**Figure 3.C**), which suggests that there are abnormalities in the overall $CD56^+$ T cell population in patients with LCH compared with healthy donors. We did not observe any CD56 expression by $CD19^+$ (B) cells in the two LCH lesion specimens we analyzed (data not shown).

$CD56^+$ T cell subsets in LCH patients.

$CD8^+CD56^+$ T cells have potent cytolytic abilities[40] and patients with advanced cancers have responded to injections of $CD8^+CD56^+$ T cells into tumours[41]. We therefore continued our investigation into $CD56^+$ expression by CD4 and CD8 T cell subsets. We found that $CD8^+CD56^+$ T cells were significantly reduced in proportion in the peripheral blood (*p = 0.0080*) and lesions (*p =0.0296*) from patients with active LCH when compared to healthy donors (**Figure 3.D. i**). Additionally, we investigated whether there was a correlation between the proportion of Tregs and the proportion of $CD8^+CD56^+$ T cells in total T cells from patients with LCH and we identified a significant negative correlation between the two cell populations (*r = 0.56, p = 0.032*) (**Figure 3.D. ii**).

$CD4^-CD8^-CD56^+$ T cells were not considerably different in relative frequency in patients with LCH compared to healthy donors (**Figure 3.E. i**) but within LCH lesions the proportion of $CD4^+CD56^+$ cells in total T cells was significantly higher when compared with the peripheral blood from healthy donors (*p = 0.0218*) and from patients with active LCH (*p = 0.0094*) (**Figure 3.E. ii**).

The proportion of Tregs in the $CD4^+CD56^+$ T cell population was significantly higher within LCH lesions when compared to the peripheral blood from healthy donors (*p = 0.003*), and it also trended substantially higher when compared to the blood from patents with active and non-active LCH (**Figure 3.F**). The $CD4^+CD56^+$ T cell population within LCH lesions mostly comprised Tregs (*median = 84 % of $CD4^+CD56^+$ T cells*) and Tregs also accounted for a reasonable proportion of the total $CD56^+$ T cell population within lesions (*median = 67.08 %*) (**Figure 3.G**).

CD56 expression on other immune cells in patients with LCH.
Surprisingly, we also identified one pulmonary LCH lesion with expression of CD56 on viable $CD3^-CD19^-CD11c^+CD1a^+CD14^-$ cells within the lesion (**Figure 3.H**). We did not observe this in any other lesions (data not shown).

***In situ* expression of Foxp3 and TGF-β in LCH lesions.**
It is already established that LCH cells produce TGF-β[22,29], however to better understand the role of $Foxp3^+$ Tregs in LCH, we investigated the *in situ* localization of CD3, Foxp3 and TGF-β in archival formalin fixed paraffin embedded LCH lesion tissue from three individual donors (**Figure 4A** and **B**). We found that TGF-β intensity in $CD3^+Foxp3^+$ cells within lesions was low compared to larger (presumably LCH) cells within the environment (**Figure 4C**).

LCH lesion Tregs can produce active TGF-β.
Because the identification of Tregs can sometimes be confounded by activated T cells transiently expressing Foxp3[42,43], we further set out to confirm that the cytokine output from LCH lesion Tregs matched that of a natural $Foxp3^+$ Treg phenotype rather than a helper T cell phenotype. We purified and then stimulated Treg and $CD4^+$ responder T cell populations from healthy donors (**Supplementary Figure 1A**), and $CD56^+$ and $CD56^-$ Treg, and $CD4^+$ responder T cell populations from LCH lesions (**Supplementary Figure 1B**). Using cell culture supernatants from the assay we found that neither $CD56^+$ or $CD56^-$ Tregs from lesions from three LCH patients secreted any

detectable IFN-γ or TNF (**Figure 5.A** and **B**). We also confirmed by intracellular staining that the stimulated $CD56^+$ and $CD56^-$ Treg populations from LCH lesions were not producing TNF, but importantly maintained their CD25 expression (**Figure 5.B**). We measured Treg supernatants for the secretion of total TGF-β and found that Tregs from both healthy donors and lesions from patients with LCH were producing total TGF-β (**Figure 5.D**). Because TGF-β detection in cell supernatants can be confounded by serum supplements we included a 100 % fetal bovine serum (FBS) control when testing for the presence of active TGF-β and found that LCH lesion derived $CD56^+$ and $CD56^-$ Treg populations produced active TGF-β when challenged (**Figure 5.E**). We did not conduct any statistical analysis on the levels of cytokines produced by these purified populations because the number of cells was not the same for each purified population (**Table 2** and **3**). Nevertheless, we have confirmed that both $CD56^+$ and $CD56^-$ Treg populations from the lesions from patients with LCH are able to produce TGF-β.

CD1a-restricted T cells are present within the Treg population in healthy donors and patients with LCH.

We conducted a preliminary study to identify whether T cells from LCH lesions could recognize endogenous lipid-loaded CD1a using a rigorous gating strategy (**Figure 6A** and **Supplementary Figures 2A** and **B**). We found that CD1a-restricted T cells were present in the peripheral blood and lesions from patients with LCH (**Figure 6B**) and LCH patients displayed similar relative frequencies to those seen in healthy donors (data not shown), although the study requires further investigation for conclusive findings. Of note, however, was the identification of a CD1a-restricted T cell population within the Treg ($CD3^+CD4^+CD25^+CD127^{low}$) population in both healthy donors and patients with LCH, including within the $CD56^+$ Treg population from LCH lesions (**Figure 6C**). It is important to note that this population was not tested for Foxp3 expression, although our analysis of Foxp3 in the $CD56^+$ Treg population already demonstrated that almost all cells within the $CD56^+$ Treg population expressed Foxp3 (**Figure 2D**).

DISCUSSION

Functional studies on $Foxp3^+$ Tregs in LCH lesions have previously been difficult because staining for the Foxp3 transcription factor requires cell permeabilization. In this study, we have established that the surrogate Treg identification method that substitutes Foxp3 for CD127 is sufficient for detecting Foxp3 transcription factor expression in LCH patients. Furthermore our results using this identification method are consistent with the previous studies that report an enrichment of Tregs in patients with LCH[18-22].

Additionally, our study is the first to identify CD56 expression by a considerable proportion of Tregs within LCH lesions. Regarding CD56 expression by Tregs, the literature is sparse, with only two reports to the best of our knowledge. The first study to report CD56 expression by $Foxp3^+$ Tregs identified this population in hepatocellular carcinoma[44], while a more recent study demonstrated that $CD56^+Foxp3^+$ Tregs were enriched at the site of *Leishmania donovani* (*L.donovani*) infection[45]. Both studies demonstrated that these cells were suppressive in nature,

with the first demonstrating that $CD56^+$ Tregs were able to inhibit the proliferation of $CD4^+CD25^-$ T cells following anti-CD3 stimulation[44], while the latter confirmed that $CD56^+$ Tregs produced TGF-β[45].

Investigating other $CD56^+$ cell populations in patients with LCH we found that infiltrating $CD3^-CD56^+$ lymphocytes, likely to be NK cells, were at consistent levels relative to T cells in healthy donors and LCH patients, which suggests that NK cells are not enriched in lesions like $CD56^+$ Tregs. An interesting and unforeseen finding by this study was the expression of CD56 by approximately 50 % of lesional LCH cells from a single patient with pulmonary LCH. CD56 expression has previously been reported on myeloid cells in chronic myeloid leukemia (CML) [46] and is associated with a poor prognosis in CML[47]. Furthermore, aberrant CD56 expression by myeloid lineage monocytes and granulocytes has been described in essential thrombocythemia, primary myelofibrosis, and polycythemia vera[48].

Our study confirmed that neither $CD56^+$ nor $CD56^-$ Tregs derived from LCH donor lesions produced the inflammatory cytokines TNF or IFN-γ when challenged, although they did produce the immune suppressive cytokine TGF-β. Given their cytokine potential we have confirmed that Tregs derived from LCH lesions are biased towards a suppressive function and may be responsible for a portion of the TGF-β production found within lesions[21,29,30].

Overall, the proportion of $CD56^+$ T cells in total T cells in the blood from patients with active LCH was reduced when compared to that seen in lesions, and also compared to the proportion in blood from patients with non-active disease. Conversely the $CD4^+CD56^+$ T cell population was increased in LCH lesions compared to the blood from patients with active LCH and healthy donor controls, and interestingly the enriched LCH lesion $CD4^+CD56^+$ and the total $CD56^+$ T cell populations comprised mostly Tregs. These results suggest that during active disease, $CD56^+$ T cells from the blood may be relocating to lesional tissue sites. $CD56^+$ T cells have previously been shown to assume Foxp3 and CD25 expression in the presence of TGF-β[44] and furthermore the correlation we identified between $CD56^+$ Tregs and total Tregs in LCH lesions suggests a direct relationship between these two cell populations. We hypothesize that $CD4^+$ T cells, including natural thymic $Foxp3^+$ Tregs and $CD4^+CD56^+$ T cells are recruited to the LCH lesional microenvironment and the production of TGF-β by LCH cells[22] and Tregs induces Foxp3 and CD25 expression along with TGF-β production by local $CD4^+$ T cells.

The recruitment of immune cells to the LCH lesion environment is likely led initially by LCH cells, for example $Foxp3^+$ Tregs may be recruited as a consequence of the immature phenotype (reviewed in Janikashvili[49]) of LCH cells, although other factors such as the CXCR3-CXCL11/I-TAC axis may be important[50]. For example, we know that CXCL11 is a chemoattractant for CXCR3[51]. Expression of CXCR3, which is commonly found on T cells with an activated memory phenotype, has been observed on T cells within LCH lesions, and CXCL11 is suggested to be expressed by LCH cells (unpublished data reported in study by Annels et al[50]).

Our study found a reduction in $CD8^+CD56^+$ T cells in both the blood and lesions from patients with active LCH compared to healthy donors, therefore we hypothesize similarly to Kumari et al[45] that the induction of Tregs in LCH patients is responsible for apoptosis or inhibited proliferation of the $CD8^+CD56^+$ T cell population. The negative correlation between Tregs and $CD8^+CD56^+$ T cells detected in our study supports this hypothesis.

An alternative mechanism to consider is that antigen specific $CD4^+$ T cells may be recruited to lesions and expanded in response to stimuli. Supporting this hypothesis is a very recent LCH study that showed proliferation of $Foxp3^+$ Tregs in response to LCH-like cells derived from notch-activated monocytes[52]. Although LCH cells have been reported to have mostly internalized major histocompatibility complex class II[35,53], which rules out the likelihood of peptide antigen presentation to $CD4^+$ T cells within lesions, LCH cells almost ubiquitously express CD1a on their cell surface, and CD1a-bound lipids can prime T cells for specific immune responses[9-16]. CD1a-restricted T cells can express the skin homing receptor CCR6[9] and there is also overexpression of the CCR6 ligand, CCL20/MIP-3α within lesions, including by LCH ($CD1a^+$) cells[50]. Thus, there is potential for CD1a-dependent stimulation of T cells and this is plausible given our preliminary results demonstrating that there is expression of CD25 and a lack of CD127 by CD1a-restricted T cells in LCH lesions.

As our understanding surrounding the basic biology of $Foxp3^+$ Tregs increases along with new technologies, we should in the future be able to assess whether Tregs directly suppress responder T cells through assays such as those that measure proliferation, however in agreeance with Quispel et al[21] this is currently beyond the scope of LCH studies. Furthermore, through co-culture studies we may be able to determine whether LCH cells specifically have the ability to stimulate Tregs to produce suppressive cytokines such as TGF-β and whether CD1a plays a role in Treg activation, although again this is currently technically challenging because attempts to establish LCH cell lines have resulted in phenotype alterations[54,55].

In summary, our study a has primarily established that the alternative $Foxp3^+$ Treg identification method that allows for downstream functional studies is appropriate in the context of assaying Tregs from LCH lesions. We have highlighted that $CD56^+$ T cells collectively may have an important role in the pathogenesis of LCH and that further studies are needed to determine the importance of CD1a-T cell interactions in LCH. Additional to our current understanding that Tregs and TGF-β are present within lesions, we have established that Tregs from LCH lesions are biased towards TGF-β production, which provides further evidence to suggest that Tregs are involved in LCH progression. This study takes us closer to elucidating the pathogenesis of LCH and encourages a path towards prospective immunotherapy treatments for patients with LCH.

MATERIALS AND METHODS

Human blood and tissue. This research project was approved by the Ballarat Health Services and Saint John of God Ballarat Hospital Human Research Ethics Committee (HREC) (HREC/15/BHSSJOG/5 and HREC/10/BHSSJOG/57) and Federation University Australia HREC (A08-100). Written, informed consent was provided by patients, and/or parents of children where appropriate. Buffy coats from healthy donors were obtained from the Australian Red Cross Blood Service as controls.

PBMCs were isolated from healthy donor buffy coats using Histopaque-1077 (Sigma-Aldrich). PBMCS were isolated from the peripheral blood from patients with LCH using Histopaque-1077 or Lymphoprep (Axis-Shield PoC AS, Oslo, Norway), otherwise white blood cells were isolated using an in house red cell lysis buffer. LCH lesion tissue was separated out into a single cell suspension using MACS tumor dissociation kit for humans (Miltenyi Biotec) where necessary as per manufacturer's instructions. LCH lesions and matched blood specimens were color coded for tracking in figures.

Cell surface antibody labeling for flow cytometry. We labeled cells with either viability dye 7-aminoactinomycin D (7-AAD; BD Pharmingen) or Fixable Viability Stain 510 (BD Horizon), Human Fc Block™ (BD Pharmingen) and anti-human cell surface antibodies as follows: CD1a-BV605 (SK9; BD Biosciences), CD3-BV650 (UCHT1; BD Horizon), CD3-PE-Cy7 (UCHT1; BD Pharmingen), CD4-PE (RPA-T4; BioLegend), CD4-PE-CF594 (RPA-T4; BD Horizon), CD4-BV650 or -BV711 (SK3; BD Horizon), CD8-APC-Cy7 (SK1; BD Pharmingen), CD8-BV510 (RPA-T8; BD Horizon), CD8-BV605 (SK1; BD Horizon), CD11c-PE-CF594 (B-ly6; BD Horizon), CD14-APC (MϕP9; BD Biosciences), CD14-AlexaFluor700 (TÜK4; AbDSerotec), CD19-BV510 (SJ25C1; BD Horizon), CD25-BV711 (2A3; BD Horizon), CD25-PE-Cy7 (M-A251; BD Pharmingen), CD56-BV786 (NCAM16.2; BD Horizon), CD127-BV421 (HIL-7R-M21; BD Horizon), and HLA-DR-BV605 (G46-6; BD Horizon).

Foxp3 transcription factor staining for flow cytometry. Following surface staining the eBioscience™ Foxp3/transcription factor staining buffer set (ThermoFisher Scientific) was used to fix and permeabilize cells and detect the transcription factor Foxp3, as per manufacturer's instructions.

Multiplex immunohistochemistry. Archival formalin fixed paraffin embedded tissue blocks from LCH patients were deposited as sections onto positively coated adhesive glass microscope slides by Australian Clinical Labs, Saint John of God Hospital, Ballarat.

Primary antibodies used for immunofluorescent labeling were polyclonal rabbit anti-human CD3 (Dako), monoclonal mouse anti-human FOXP3 [236A/E7] (Abcam) and polyclonal rabbit anti-human TGF β (Abcam).

Primary CD3 and FOXP3 stains were labeled with EnVision^{TM} FLEX HRP (Dako) conjugated secondary antibodies followed by tyramide signal amplification (TSA^{TM} - plus fluorescein system; Perkin-Elmer) as per manufacturer's instructions. Alexa

Fluor 568 goat anti-rabbit IgG (H + L) (Thermo Fisher Scientific) was used as a secondary antibody to identify TGF-β in tissue sections.

Both of the following multiplex combinations were used to investigate expression of TGF-β by $Foxp3^+$ Tregs: (1) CD3-Cy5 (TSA), FOXP3-fluorescein (TSA), TGF-β-Alexa Fluor 568 (standard immunofluorescent labeling), and DAPI nuclear stain (Thermo Fisher Scientific); (2) CD3-fluorescein (TSA), FOXP3-Cy5 (TSA), TGF-β-Alexa Fluor 568 (standard immunofluorescent labeling), DAPI nuclear stain.

For antigen retrieval, slides were boiled by microwaving in EnVision™ FLEX target retrieval solution, high pH (Dako) antigen retrieval buffer for 10 min. Prior to primary antibody labeling, slides were blocked in a PBS solution containing 0.25 % casein, 2 % goat serum, 0.1 % Triton X-100 (Sigma-Aldrich), 0.5 % TWEEN 20 (Sigma-Aldrich) and 0.05 % sodium azide (Sigma-Aldrich). Prior to HRP labeling, slides were blocked using EnVision™ FLEX peroxidase-blocking reagent (Dako). For washing, slides were rinsed with EnVision™ FLEX wash buffer (Dako) then immersed in wash buffer and agitated for 3 min, then placed into a second wash buffer and agitated for a further 3 min. Each incubation took place in an agitated humid chamber with protection from light where necessary.

Standard immunofluorescent labelling for TGF-β was completed following all TSA stains and antigen retrieval and blocking steps were as described previously. Following the completion of all antibody labeling, slides were incubated with DAPI for 5 min, then washed and coverslips were mounted using ProLong Gold™ antifade mountant (Thermo Fisher Scientific) and allowed to dry in the dark.

All immunohistochemistry images were captured using an Evos FL Auto 2 cell imaging system (Thermo Fisher Scientific) and analyses were completed using Image J software (National Institutes of Health).

Flow cytometry and cell purification. Flow cytometry analysis experiments were performed using either a BD FACS Aria II cell sorter or BD LSR Fortessa. Cell purification was performed using the BD FACS Aria II cell sorter. FlowJo software (Treestar) was used to analyze flow cytometric data. Unless otherwise stated, the gating strategy primarily identified viable lymphocytes with doublets excluded. All analyses were performed relative to the $CD3^+$ (T cell) population or sub-populations thereof.

Stimulation of Treg and T responder cell populations. $CD4^+CD25^+CD127^{low}$ T cells (Tregs) and other remaining $CD4^+$ T cells (not Tregs; T responder cells) were purified from blood from healthy donors. Similarly, these populations were purified from lesions from patients with LCH, except Tregs were further isolated into $CD56^+$ and $CD56^-$ populations. Cells were successfully isolated with purities of 95 % and above and then rested overnight at 37 °C in TexMACS medium (Miltenyi Biotec) with 10 % fetal bovine serum (Sigma-Aldrich) and 1x penicillin-streptomycin (Sigma-Aldrich). Treg cultures were supplemented with IL-2 (20 IU/mL). Following this, cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (10 ng/mL) and ionomycin (1

μg/mL) for a total of 16 hours and GolgiPlug (BD Biosciences) was added 4 hours prior to collecting the supernatant and harvesting the cells for analysis.

Bead based cytokine detection immunoassays. LEGENDplex (BioLegend) bead based cytokine detection immunoassays were performed to identify secreted cytokines following *in vitro* cell culture and stimulation. Cell culture supernatants were collected and stored at -80° C before use. Both the human Th cytokine panel (13-plex; BioLegend) and the human free active/total TGF-β1 assay (BioLegend) were used to detect secreted cytokines as per manufacturer's instructions. Analyses were performed in duplicate using LEGENDplex™ data analysis software (BioLegend) with cytokines quantified by comparing samples to a set of standard curves that were prepared in parallel with supernatant samples.

Intracellular cytokine staining. After the stimulation assay and subsequent cell surface labeling, the BD cytofix/cytoperm kit (BD Biosciences) was used to fix and permeabilize cells as per manufacturer's instructions. Cells were subsequently stained with antibodies directed towards the intracellular cytokine TNFα-APC-Cy7 (MAb11; BioLegend).

CD1a tetramer production for flow cytometry. Biotinylated human CD1a monomers loaded with endogenous lipids were kindly donated by the Godfrey laboratory (Dept. Microbiology and Immunology, Peter Doherty Institute, University of Melbourne, Parkville, Australia). For CD1a tetramer production, 20 μg PE streptavidin (BD Pharmingen) was added to endogenous lipid-loaded CD1a on ice by adding 2 μg every 15 min. Cells were labeled with tetramer as per standard cell surface antibody labeling.

Statistics. GraphPad Prism (GraphPad Software) was used to analyze data except where otherwise indicated. Four patient matched LCH blood and lesion specimens were available for the identification of Tregs. All other LCH lesions and blood specimens were from independent patients. To determine whether parametric or non-parametric statistical analyses were required, we conducted D'Agostino and Pearson omnibus normality tests. Statistical significance was based on an alpha level of 0.05.

REFERENCES

1. Gadner H, Minkov M, Grois N, et al. Therapy prolongation improves outcome in multisystem Langerhans cell histiocytosis. *Blood*. 2013;121(25):5006–5014.
2. Sahm F, Capper D, Preusser M, et al. BRAFV600E mutant protein is expressed in cells of variable maturation in Langerhans cell histiocytosis. *Blood*. 2012;120(12):e28–e34.
3. Satoh T, Smith A, Sarde A, et al. B-RAF mutant alleles associated with Langerhans cell histiocytosis, a granulomatous pediatric disease. *PLoS ONE*. 2012;7(4):e33891.
4. Badalian-Very G, Vergilio J-A, Degar BA, et al. Recurrent *BRAF* mutations in Langerhans cell histiocytosis. *Blood*. 2010;116(11):1919–1923.
5. Yousem SA, Dacic S, Nikiforov YE, Nikiforova M. Pulmonary Langerhans cell histiocytosis: profiling of multifocal tumors using next-generation sequencing identifies concordant occurrence of *BRAF* V600E mutations. *Chest*. 2013;143(6):1679–1684.
6. Nelson DS, van Halteren A, Quispel WT, et al. *MAP2K1* and *MAP3K1* mutations in Langerhans cell histiocytosis. *Genes Chromosomes Cancer*. 2015;54(6):361–368.
7. Chakraborty R, Hampton OA, Shen X, et al. Mutually exclusive recurrent somatic mutations in *MAP2K1* and *BRAF* support a central role for ERK activation in LCH pathogenesis. *Blood*. 2014;124(19):3007–3015.
8. Brown NA, Furtado LV, Betz BL, et al. High prevalence of somatic *MAP2K1* mutations in *BRAF V600E*-negative Langerhans cell histiocytosis. *Blood*. 2014;124(10):1655–1658.
9. de Jong A, Peña-Cruz V, Cheng T-Y, et al. CD1a-autoreactive T cells are a normal component of the human αβ T cell repertoire. *Nat. Immunol.* 2010;11(12):1102–1109.
10. de Jong A, Cheng T-Y, Huang S, et al. CD1a-autoreactive T cells recognize natural skin oils that function as headless antigens. *Nat. Immunol.* 2014;15(2):177–185.
11. Bourgeois EA, Subramaniam S, Cheng TY, et al. Bee venom processes human skin lipids for presentation by CD1a. *Journal of Experimental Medicine*. 2015;212(2):149–163.
12. Kim JH, Hu Y, Yongqing T, et al. CD1a on Langerhans cells controls inflammatory skin disease. *Nat. Immunol.* 2016;17(10):1159–1166.
13. Cheng J, Liu L, Pellicci D, et al. Total synthesis of the Mycobacterium tuberculosis dideoxymycobactin-838 and stereoisomers: Diverse CD1a-restricted T cells display a common hierarchy of lipopeptide recognition. *Chemistry*. 2017;23(7):1694–1701.
14. Birkinshaw RW, Pellicci DG, Cheng T-Y, et al. αβ T cell antigen receptor recognition of CD1a presenting self lipid ligands. *Nat. Immunol.* 2015;16(3):258–266.
15. de Lalla C, Lepore M, Piccolo FM, et al. High-frequency and adaptive-like dynamics of human CD1 self-reactive T cells. *Eur. J. Immunol.* 2011;41(3):602–610.
16. Kasmar AG, Van Rhijn I, Magalhaes KG, et al. Cutting edge: CD1a tetramers and dextramers identify human lipopeptide-specific T cells ex vivo. *The Journal*

of Immunology. 2013;191(9):4499–4503.
17. Bechan GI, Egeler RM, Arceci RJ. Biology of Langerhans cells and Langerhans cell histiocytosis. *Int. Rev. Cytol.* 2006;254:1–43.
18. Battistella M, Fraitag S, Teillac DH, et al. Neonatal and early infantile cutaneous Langerhans cell histiocytosis: Comparison of self-regressive and non-self-regressive forms. *Arch Dermatol*. 2010;146(2):149–156.
19. Senechal B, Elain G, Jeziorski E, et al. Expansion of regulatory T cells in patients with Langerhans cell histiocytosis. *PLoS Med.* 2007;4(8):e253.
20. Tong C, Jia X, Jia Y, He Y. Langerhans cell histiocytosis in Chinese adults: absence of BRAF mutations and increased $FOXP3^+$ regulatory T cells. *Int J Clin Exp Pathol*. 2014;7(6):3166–3173.
21. Quispel WT, Stegehuis-Kamp JA, Santos SJ, Egeler RM, van Halteren AGS. Activated conventional T-cells are present in Langerhans cell histiocytosis lesions despite the presence of immune suppressive cytokines. *Journal of Interferon & Cytokine Research*. 2015;35(10):831–839.
22. Allen CE, Li L, Peters TL, et al. Cell-specific gene expression in Langerhans cell histiocytosis lesions reveals a distinct profile compared with epidermal Langerhans cells. *The Journal of Immunology*. 2010;184(8):4557–4567.
23. Shevach EM. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity*. 2009;30(5):636–645.
24. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell*. 2008;133(5):775–787.
25. Chaudhary B, Elkord E. Regulatory T Cells in the tumor microenvironment and cancer progression: Role and therapeutic targeting. *Vaccines (Basel)*. 2016;4(3):.
26. Seddiki N, Santner-Nanan B, Martinson J, et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J. Exp. Med.* 2006;203(7):1693–1700.
27. Liu W, Putnam AL, Xu-Yu Z, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J. Exp. Med.* 2006;203(7):1701–1711.
28. Egeler RM, Favara BE, van Meurs M, Laman JD, Claassen E. Differential In situ cytokine profiles of Langerhans-like cells and T cells in Langerhans cell histiocytosis: Abundant expression of cytokines relevant to disease and treatment. *Blood*. 1999;94(12):4195–4201.
29. de Graaf JH, Tamminga RY, Dam-Meiring A, Kamps WA, Timens W. The presence of cytokines in Langerhans' cell histiocytosis. *J. Pathol.* 1996;180(4):400–406.
30. Kannourakis G, Abbas A. The role of cytokines in the pathogenesis of Langerhans cell histiocytosis. *Br. J. Cancer Suppl.* 1994;23:S37–40.
31. Carrera Silva EA, Nowak W, Tessone L, et al. $CD207^+CD1a^+$ cells circulate in pediatric patients with active Langerhans cell histiocytosis. *Blood*. 2017;130(17):1898–1902.
32. Morimoto A, Oh Y, Nakamura S, et al. Inflammatory serum cytokines and chemokines increase associated with the disease extent in pediatric Langerhans cell histiocytosis. *Cytokine*. 2017;97:73–79.
33. Geissmann F, Prost C, Monnet JP, et al. Transforming growth factor β1, in the

presence of granulocyte/macrophage colony-stimulating factor and interleukin 4, induces differentiation of human peripheral blood monocytes into dendritic Langerhans cells. *J. Exp. Med.* 1998;187(6):961–966.
34. Hutter C, Kauer M, Simonitsch-Klupp I, et al. Notch is active in Langerhans cell histiocytosis and confers pathognomonic features on dendritic cells. *Blood*. 2012;120(26):5199–5208.
35. Geissmann F, Lepelletier Y, Fraitag S, et al. Differentiation of Langerhans cells in Langerhans cell histiocytosis. *Blood*. 2001;97(5):1241–1248.
36. Van Acker HH, Capsomidis A, Smits EL, Van Tendeloo VF. CD56 in the Immune System: More Than a Marker for Cytotoxicity? *Front Immunol*. 2017;8:892.
37. Gherardin NA, Souter MNT, Koay H-F, et al. Human blood MAIT cell subsets defined using MR1 tetramers. *Immunol. Cell Biol.* 2018;96(5):507–525.
38. Berzins SP, Smyth MJ, Baxter AG. Presumed guilty: Natural killer T cell defects and human disease. *Nat. Rev. Immunol.* 2011;11(2):131–142.
39. Schmetterer KG, Neunkirchner A, Pickl WF. Naturally occurring regulatory T cells: markers, mechanisms, and manipulation. *The FASEB Journal*. 2012;26(6):2253–2276.
40. Pittet MJ, Speiser DE, Valmori D, Cerottini JC, Romero P. Cutting edge: cytolytic effector function in human circulating CD8+ T cells closely correlates with CD56 surface expression. *J Immunol*. 2000;164(3):1148–1152.
41. Hasumi K, Aoki Y, Wantanabe R, Mann DL. Clinical response of advanced cancer patients to cellular immunotherapy and intensity-modulated radiation therapy. *Oncoimmunology*. 2013;2(10):e26381.
42. Tran DQ, Ramsey H, Shevach EM. Induction of FOXP3 expression in naive human CD4+FOXP3 T cells by T-cell receptor stimulation is transforming growth factor-beta dependent but does not confer a regulatory phenotype. *Blood*. 2007;110(8):2983–2990.
43. Wang J, Ioan-Facsinay A, van der Voort EIH, Huizinga TWJ, Toes REM. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. *Eur. J. Immunol.* 2007;37(1):129–138.
44. Li X, Peng J, Pang Y, et al. Identification of a FOXP3(+)CD3(+)CD56(+) population with immunosuppressive function in cancer tissues of human hepatocellular carcinoma. *Sci. Rep.* 2015;5:14757.
45. Kumari S, Shivam P, Kumar S, et al. Leishmania donovani mediated higher expression of CCL4 induces differential accumulation of CD4+CD56+NKT and CD8+CD56+NKT cells at infection site. *Cytokine*. 2018.
46. Lanza F, Bi S, Castoldi G, Goldman JM. Abnormal expression of N-CAM (CD56) adhesion molecule on myeloid and progenitor cells from chronic myeloid leukemia. *Leukemia*. 1993;7(10):1570–1575.
47. Alegretti AP, Bittar CM, Bittencourt R, et al. The expression of CD56 antigen is associated with poor prognosis in patients with acute myeloid leukemia. *Rev Bras Hematol Hemoter*. 2011;33(3):202–206.
48. Gong P, Metrebian F, Dulau-Florea A, et al. Aberrant expression of CD56 on granulocytes and monocytes in myeloproliferative neoplasm. *J Hematopathol*. 2013;6(3):127–134.
49. Janikashvili N, Bonnotte B, Katsanis E, Larmonier N. The dendritic cell-regulatory T lymphocyte crosstalk contributes to tumor-induced tolerance.

Clin. Dev. Immunol. 2011;2011:430394.
50. Annels NE, da Costa CET, Prins FA, et al. Aberrant chemokine receptor expression and chemokine production by Langerhans cells underlies the pathogenesis of Langerhans cell histiocytosis. *J. Exp. Med.* 2003;197(10):1385–1390.
51. Hirota Y, Osuga Y, Koga K, et al. The expression and possible roles of chemokine CXCL11 and its receptor CXCR3 in the human endometrium. *J Immunol*. 2006;177(12):8813–8821.
52. Schwentner R, Jug G, Kauer MO, et al. JAG2 signaling induces differentiation of CD14+ monocytes into Langerhans cell histiocytosis-like cells. *J. Leukoc. Biol.* 2018.
53. Redd L, Schmelz M, Burack WR, et al. Langerhans cell histiocytosis shows distinct cytoplasmic expression of major histocompatibility class II antigens. *J Hematopathol*. 2016;9(3):107–112.
54. Murakami I, Gogusev J, Jaubert F, et al. Establishment of a Langerhans cell histiocytosis lesion cell line with dermal dendritic cell characteristics. *Oncol. Rep.* 2015;33(1):171–178.
55. Gogusev J, Telvi L, Murakami I, et al. DOR-1, A novel CD10+ stromal cell line derived from progressive Langerhans cell histiocytosis of bone. *Pediatr. Blood Cancer*. 2005;44(2):128–137.

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AUTHORSHIP
T.G., E.K., J-I.H. and G.K. recruited patients with LCH and provided tissue samples and clinical information. S.B. provided healthy donor control tissues. J.M., J.K., S.B. and G.K designed experiments. J.M. performed experiments, analyzed results, prepared figures and wrote the manuscript. S.B. and G.K. critically revised the manuscript. J.K., E.K., T.G., D.G.P. and J-I.H. reviewed the manuscript. S.B. and G.K led the investigation.

DISCLOSURE OF CONFLICTS OF INTEREST
The authors declare no competing interests.

FIGURE LEGENDS
Figure 1. Foxp3$^+$ Tregs are enriched in patients with LCH (**A**) Gating strategy to identify Foxp3$^+$ regulatory T cells (CD3$^+$CD4$^+$CD25$^+$CD127^{low} lymphocytes) in blood from healthy donors (left) and in blood (center) and lesions (right) from patients with LCH. (**B**) Proportion of Tregs in CD4$^+$ T cells from blood from healthy donors and from blood and lesions from patients with LCH. (**C**) Proportion of Tregs in total T cells from blood from healthy donors and from blood and lesions from patients with LCH. (**D**) Histograms confirm Foxp3 expression by CD3$^+$CD4$^+$CD25$^+$CD127^{low} T cells in lesions from 3 patients with LCH. Kruskal-Wallis tests with Dunn's multiple comparisons were conducted for (**B**) and (**C**), *$p < 0.05$, ****$p < 0.0001$, error bars indicate median + interquartile range. HD = healthy donor, PB = peripheral blood, NAD = non-active LCH, AD = active LCH.

Figure 2. Cell surface CD56 expression by LCH lesion Foxp3$^+$ Tregs. (**A**) Gating strategy to identify CD56 expression on Tregs from blood from healthy donors (left) and from blood (center) and lesions (right) from patients with LCH. (**B**) Proportion of CD56$^+$ T cells in Tregs from blood from healthy donors and from blood and lesions from patients with LCH. (**C**) Correlation between the proportion of total Tregs and CD56$^+$ Tregs in T cells from lesions from patients with LCH. (**D**) Plots demonstrate Foxp3 expression by CD56$^+$ and CD56$^-$ Tregs from lesions from 3 patients with LCH. (**E**) Histograms demonstrate that cell surface expression of HLA-DR is low by Tregs from lesions from 3 patients with LCH. A Kruskal-Wallis test with Dunn's multiple comparisons was conducted for (**B**), **p < 0.01, ****p < 0.0001, error bars indicate median + interquartile range. A Spearman's two-tailed non-parametric correlation test was conducted for (**J**) (95 % confidence interval). HD = healthy donor, PB = peripheral blood, NAD = non-active LCH, AD = active LCH.

Figure 3. Cell surface CD56 expression by lymphocyte subsets in patients with LCH. (**A**) Gating strategy to identify cell surface CD3 and CD56 expression by lymphocytes (**i**), and CD4$^+$, CD8$^+$ and CD4$^-$CD8$^-$ T cells in CD56$^+$ T cells (**ii**) in blood from healthy donors (left) and blood (center) and lesions (right) from patients with LCH. (**B**) Ratio of CD56$^+$CD3$^-$ (NK) cells to T cells (**i**) and CD56$^+$CD3$^-$ (NK) cells to CD56$^+$ T cells (**ii**) in blood from healthy donors and in blood and lesions from patients with LCH. (**C**) Proportion of CD56$^+$ T cells in total T cells from blood from healthy donors and from blood and lesions from patients with LCH, including and excluding Tregs (CD4$^+$CD25$^+$CD127^{low} T cells) in the lesional population. (**D**) Proportion of CD8$^+$CD56$^+$ cells in T cells from blood from healthy donors and from blood and lesions from patients with LCH (**i**), and correlation between CD8$^+$CD56$^+$ cells and Tregs in T cells from blood from patients with active (triangles) and non-active (open squares) LCH, and from lesions from patients with LCH (circles) (**ii**). (**E**) Proportion of CD4$^-$CD8$^-$ CD56$^+$ (**i**) and CD4$^+$CD56$^+$ (**ii**) T cells in total T cells from blood from healthy donors and from blood and lesions from patients with LCH. (**F**) Proportion of Tregs (CD4$^+$CD25$^+$CD127^{low} T cells) in CD4$^+$CD56$^+$ T cells from blood from healthy donors and from blood and lesions from patients with LCH. (**G**) Pie chart demonstrates the median proportion of Tregs in CD56$^+$ T cells from lesions from patients with LCH. (**H**) Plots demonstrate a single pulmonary LCH lesion where approximately half of the CD3$^-$CD19$^-$CD11c$^+$CD1a$^+$ (LCH cells) express CD56. Kruskal-Wallis tests with Dunn's multiple comparisons were conducted for (**B**), (**C**), (**D. i**), (**E**) and (**F**), *p < 0.05, **p < 0.01, ***p < 0.001,****p < 0.0001, error bars indicate median + interquartile range. A Spearman's two-tailed non-parametric correlation test was conducted for (**D. ii**) (95 % confidence interval). HD = healthy donor, PB = peripheral blood, NAD = non-active LCH, AD = active LCH.

Figure 4. *In situ* Foxp3 TGF β localization in LCH lesions. (**A**) Merged image shows tissue section from osseous LCH lesion with DAPI, CD3, Foxp3 and TGF-β labeling. **(B)** Images show a magnified field from (**A**) (white square) with CD3 labeling in magenta (**i**), nuclear (DAPI) labeling in blue (**ii**), nuclear Foxp3 expression in green (**iii**) and TGF-β in red (**iv**). Green arrows indicate CD3$^+$Foxp3$^+$ cells. (**C**) (**i**) Image of the field in (**A**) with only nuclear (DAPI; blue) and TGF-β (red) labeling showing previously identified CD3$^+$Foxp3$^+$ cell (green arrow) with low intensity nuclear TGF-β compared

to larger cell (red arrow) with strong TGF-β expression, and the field from (**i**) (white square) is magnified in (**ii**). Images are representative lesions from 3 LCH patients.

Figure 5. *In vitro* cytokine production by Tregs from LCH lesions. Graphs demonstrate the supernatant levels of IFN-γ (**A**), and TNF (**B**) produced by purified and stimulated populations of T responder cells from blood from healthy donors and from lesions from patients with LCH and by $CD56^+$ and $CD56^-$ Tregs from lesions from patients with LCH. (**C**) Plots demonstrate intracellular TNF production by purified and stimulated T responder cells from lesions from patients with LCH (left) and maintenance of CD25 expression but a lack of TNF production by $CD56^+$ and $CD56^-$ Tregs. Plots are representative of cells from 3 independent patient lesions. Graphs demonstrate the supernatant levels of total (**D**) and active (**E**) TGF-β produced by purified and stimulated populations of Tregs from blood from healthy donors and by $CD56^+$ and $CD56^-$ Tregs from lesions from patients with LCH. Green line indicates minimum detectable concentration as determined by a standard curve. Dashed line indicates media background control. Blue line indicates detection level of 100 % fetal bovine serum. HD = healthy donor, Resp = T responder cells.

Figure 6. CD1a-restricted T cells are present within LCH lesions including within the Treg population. Representative plots show the identification of T cells that bind with endogenous lipid-loaded human CD1a tetramers (CD1a-restricted T cells) from (**A**) peripheral blood from healthy donors and (**B**) peripheral blood and lesions from patients with LCH and CD4 versus CD8 expression of this population. (**C**) Representative plots show the presence of CD1a-restricted T cells within the Treg population from blood from healthy donors and from blood and lesions from patients with LCH, and within the $CD56^+$ Treg population from lesions. Plots are representative of at least 2 individuals. PB = peripheral blood. HD = healthy donor, PB = peripheral blood.

Figure S1. Purification of Treg and T responder cell populations. (**A**) Gating strategy for identifying and purifying Tregs ($CD4^+CD25^+CD127^{low}$ T cells) and T responder (other $CD4^+$) cells (Resp) from blood from healthy donors and post-FACS analysis of these populations. (**B**) Gating strategy for identifying and purifying T responder cells (Resp) and $CD56^+$ and $CD56^-$ populations in Tregs ($CD4^+CD25^+CD127^{low}$ T cells) from lesions from patients with LCH and post- FACS analysis.

Figure S2. Identification of CD1a-restricted T cell. (**A**) Gating strategy for identifying T cells that bind with endogenous lipid-loaded human CD1a tetramers. (**B**) Fluorescence minus one (FMO) control demonstrating that detection in the PE channel is specific for tetramer labeling.

FIGURES

Figure 1

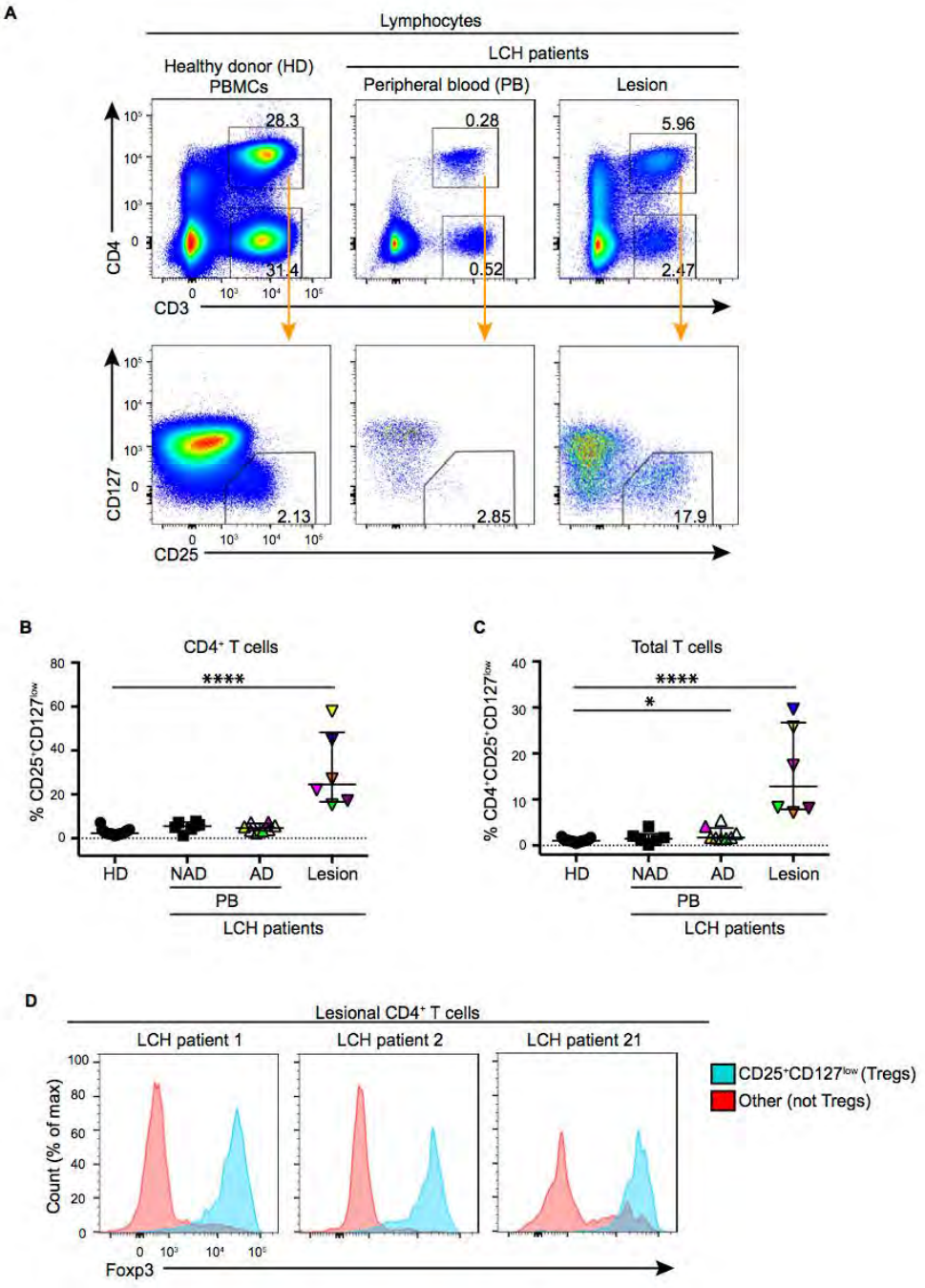


Figure 2

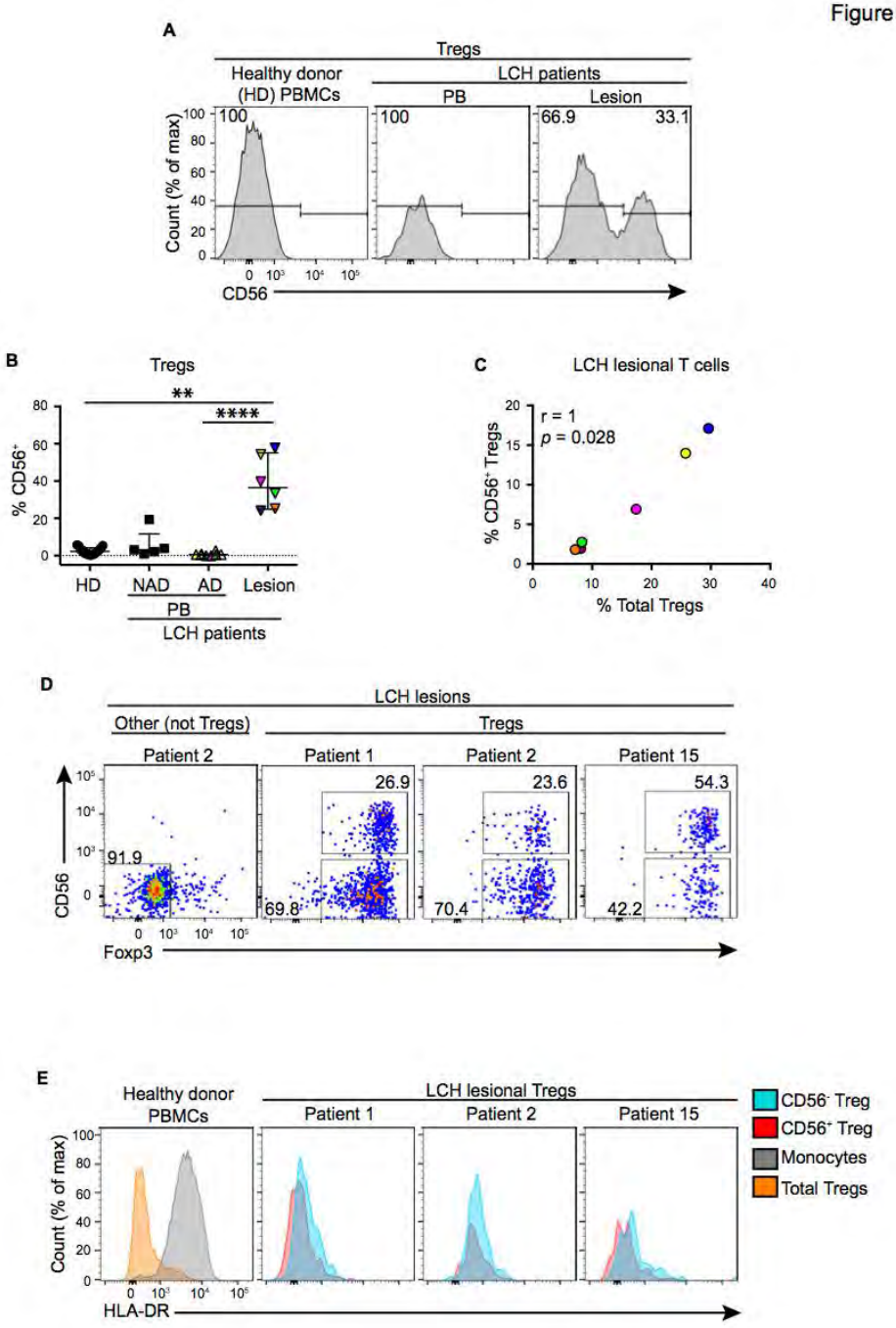
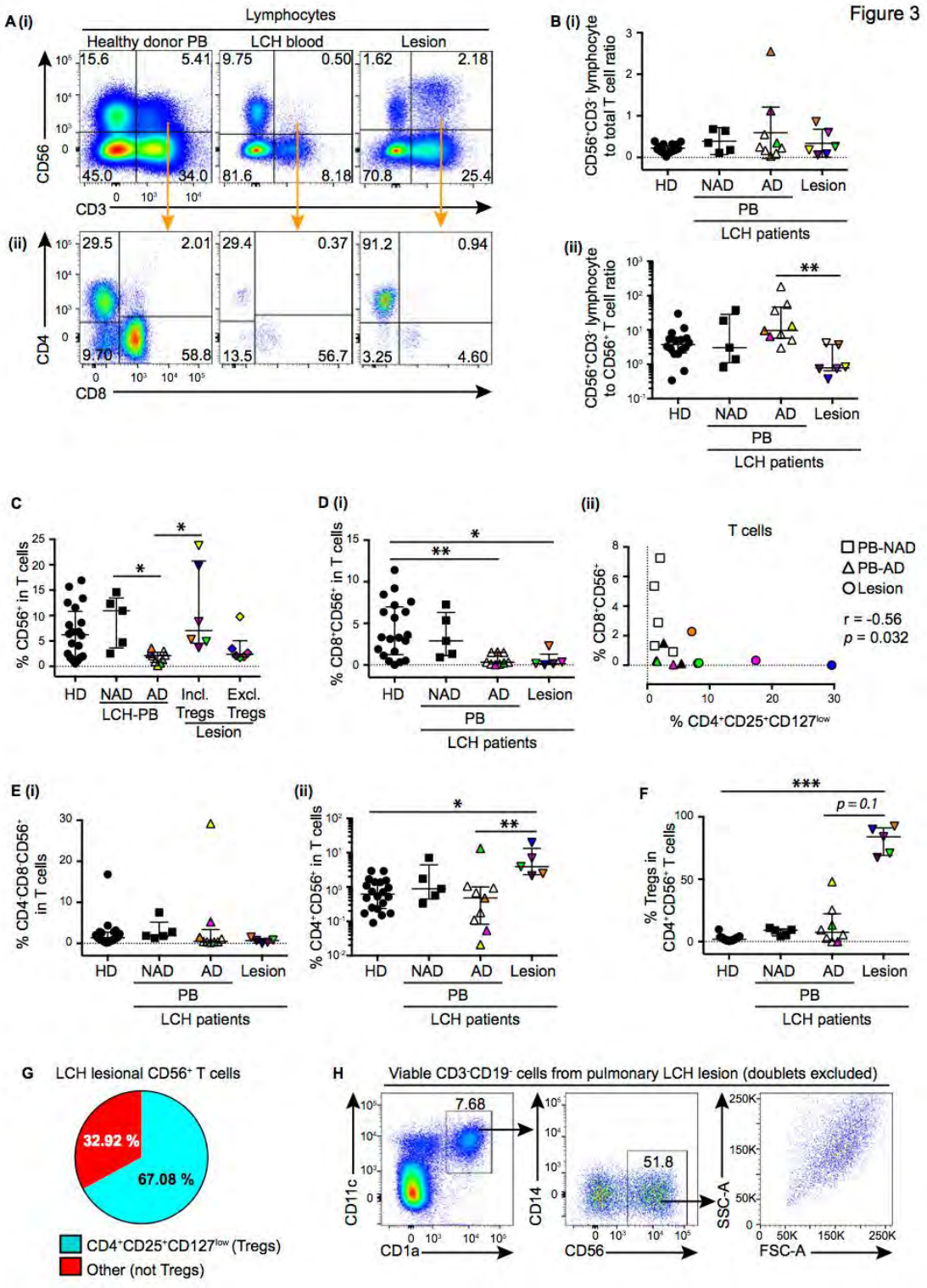
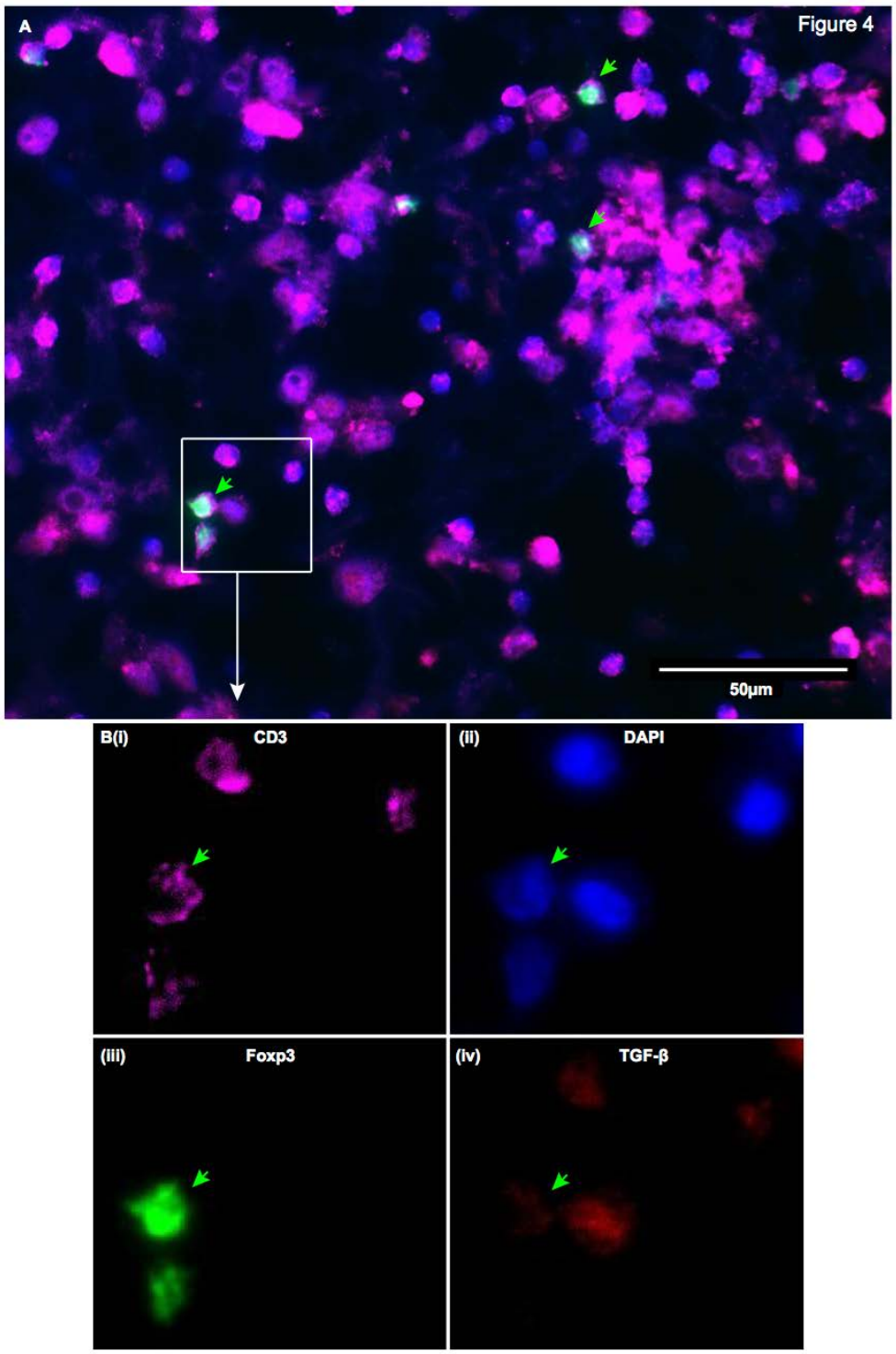

A
Tregs
Healthy donor (HD) PBMCs
LCH patients
PB
Lesion
100
100
66.9
33.1
Count (% of max)
CD56
B
Tregs
**
****
% CD56+
HD
NAD
AD
Lesion
PB
LCH patients
C
LCH lesional T cells
r = 1
p = 0.028
% CD56+ Tregs
% Total Tregs
D
LCH lesions
Other (not Tregs)
Tregs
Patient 2
Patient 1
Patient 2
Patient 15
91.9
26.9
69.8
23.6
70.4
54.3
42.2
CD56
Foxp3
E
Healthy donor PBMCs
LCH lesional Tregs
Patient 1
Patient 2
Patient 15
Count (% of max)
HLA-DR
CD56- Treg
CD56+ Treg
Monocytes
Total Tregs

Figure 3


A (i)
Lymphocytes
Healthy donor PB
LCH blood
Lesion
CD56
CD3
15.6
5.41
45.0
34.0
9.75
0.50
81.6
8.18
1.62
2.18
70.8
25.4
(ii)
CD4
CD8
29.5
2.01
9.70
58.8
29.4
0.37
13.5
56.7
91.2
0.94
3.25
4.60
B (i)
CD56+CD3- lymphocyte to total T cell ratio
HD
NAD
AD
Lesion
PB
LCH patients
(ii)
CD56+CD3- lymphocyte to CD56+ T cell ratio
**
C
% CD56+ in T cells
*
HD
NAD
AD
Incl. Tregs
Excl. Tregs
LCH-PB
Lesion
D (i)
% CD8+CD56+ in T cells
**
(ii)
T cells
PB-NAD
PB-AD
Lesion
r = -0.56
p = 0.032
% CD8+CD56+
% CD4+CD25+CD127low
E (i)
% CD4-CD8-CD56+ in T cells
(ii)
% CD4+CD56+ in T cells
F
***
p = 0.1
% Tregs in CD4+CD56+ T cells
G
LCH lesional CD56+ T cells
32.92 %
67.08 %
CD4+CD25+CD127low (Tregs)
Other (not Tregs)
H
Viable CD3-CD19- cells from pulmonary LCH lesion (doublets excluded)
7.68
51.8
CD11c
CD1a
CD14
CD56
SSC-A
FSC-A
250K
150K
50K
0
50K
150K
250K


A
Figure 4
50µm
B(i)
CD3
(ii)
DAPI
(iii)
Foxp3
(iv)
TGF-β

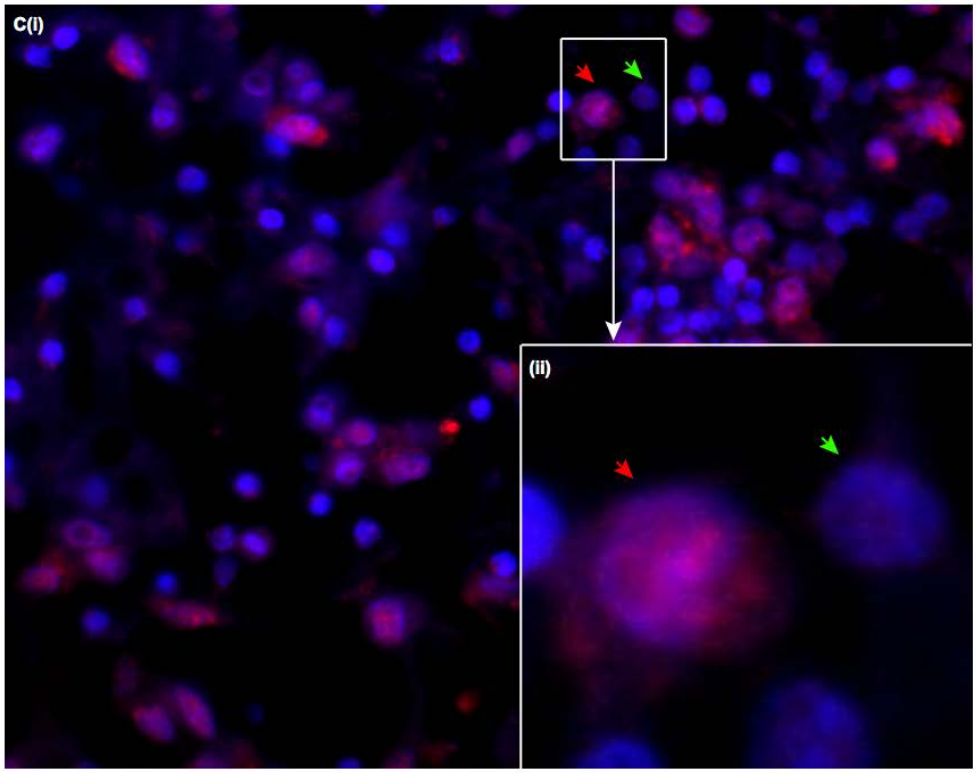

C(i)
(ii)

Figure 5

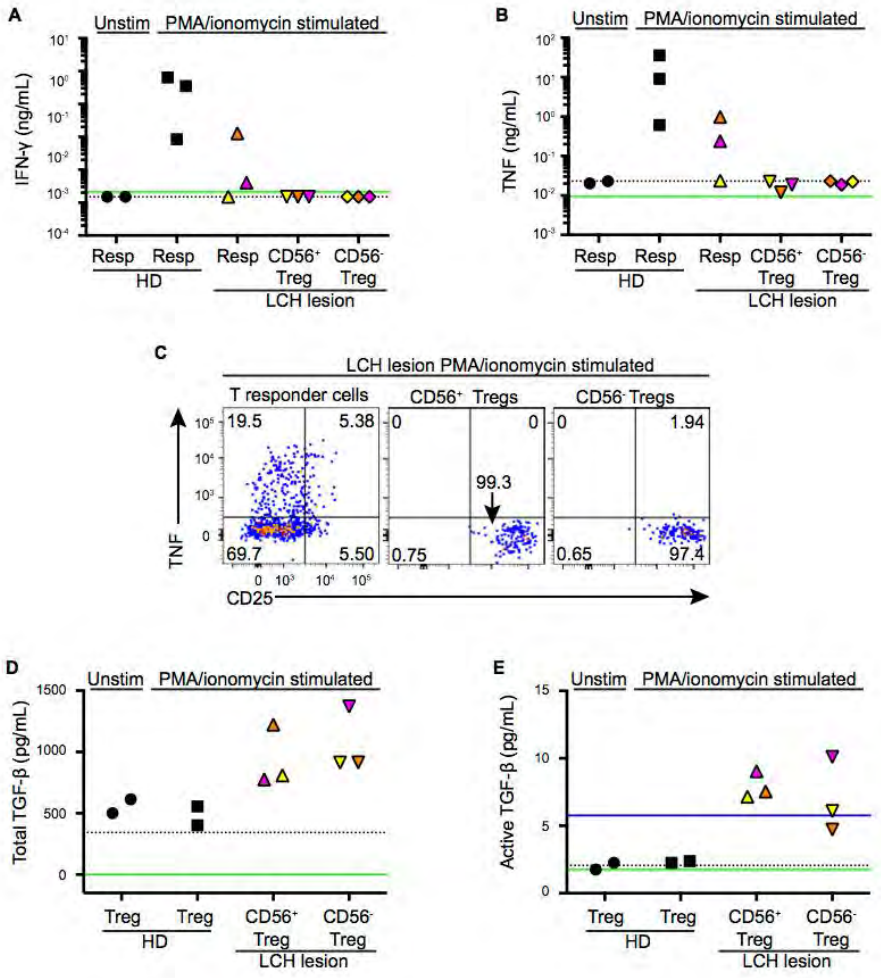

A
Unstim
PMA/ionomycin stimulated
IFN-γ (ng/mL)
Resp
Resp
Resp
CD56+ Treg
CD56- Treg
HD
LCH lesion
B
Unstim
PMA/ionomycin stimulated
TNF (ng/mL)
Resp
Resp
Resp
CD56+ Treg
CD56- Treg
HD
LCH lesion
C
LCH lesion PMA/ionomycin stimulated
T responder cells
CD56+ Tregs
CD56- Tregs
19.5
5.38
69.7
5.50
0
0
99.3
0.75
0
1.94
0.65
97.4
TNF
CD25
D
Unstim
PMA/ionomycin stimulated
Total TGF-β (pg/mL)
Treg
Treg
CD56+ Treg
CD56- Treg
HD
LCH lesion
E
Unstim
PMA/ionomycin stimulated
Active TGF-β (pg/mL)
Treg
Treg
CD56+ Treg
CD56- Treg
HD
LCH lesion

Figure 6

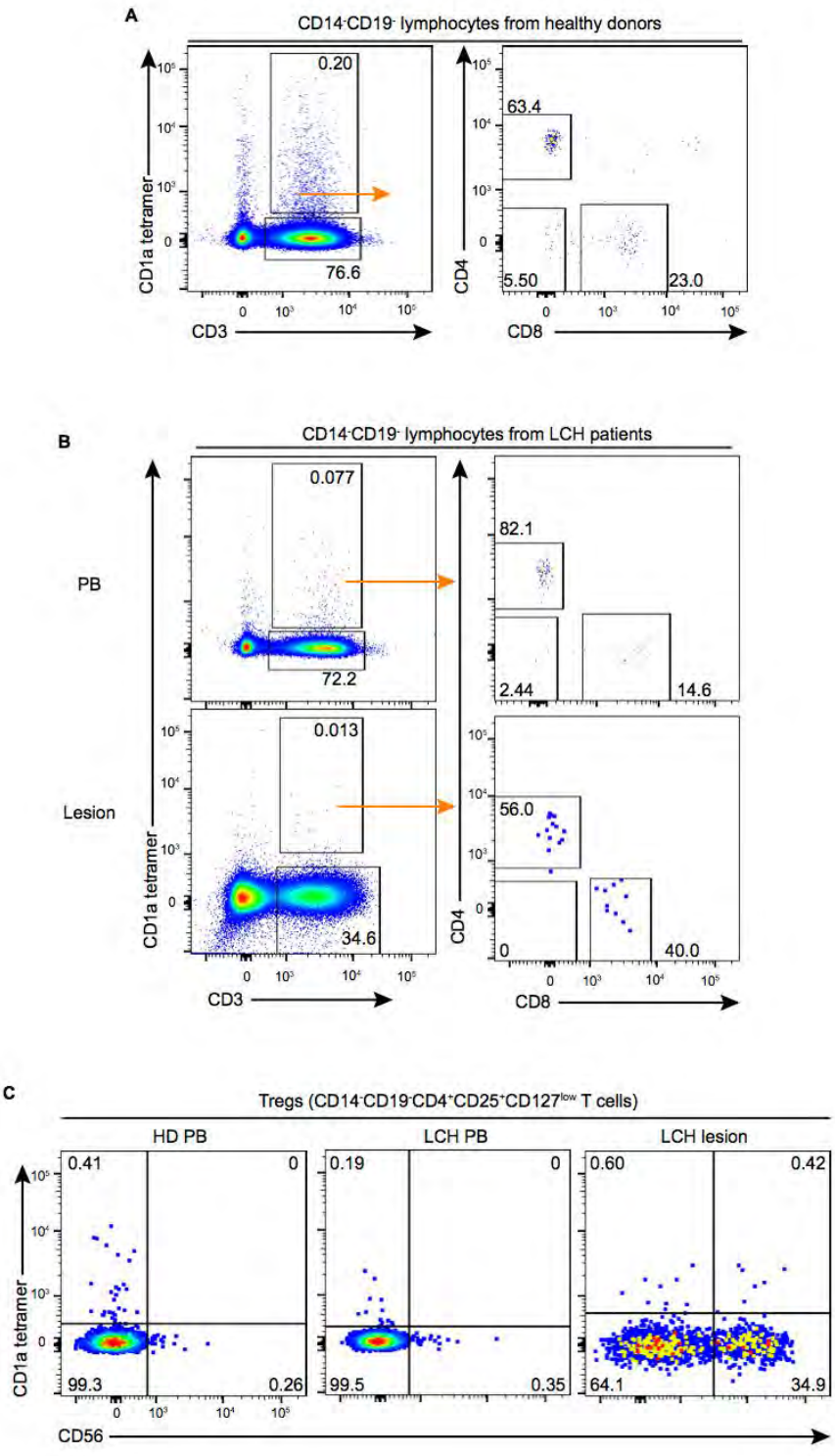

A
CD14-CD19- lymphocytes from healthy donors
0.20
76.6
63.4
5.50
23.0
CD1a tetramer
CD3
CD4
CD8
B
CD14-CD19- lymphocytes from LCH patients
PB
Lesion
0.077
72.2
82.1
2.44
14.6
0.013
34.6
56.0
0
40.0
CD1a tetramer
CD3
CD4
CD8
C
Tregs (CD14-CD19-CD4+CD25+CD127low T cells)
HD PB
LCH PB
LCH lesion
0.41
0
99.3
0.26
0.19
0
99.5
0.35
0.60
0.42
64.1
34.9
CD1a tetramer
CD56

Figure S1

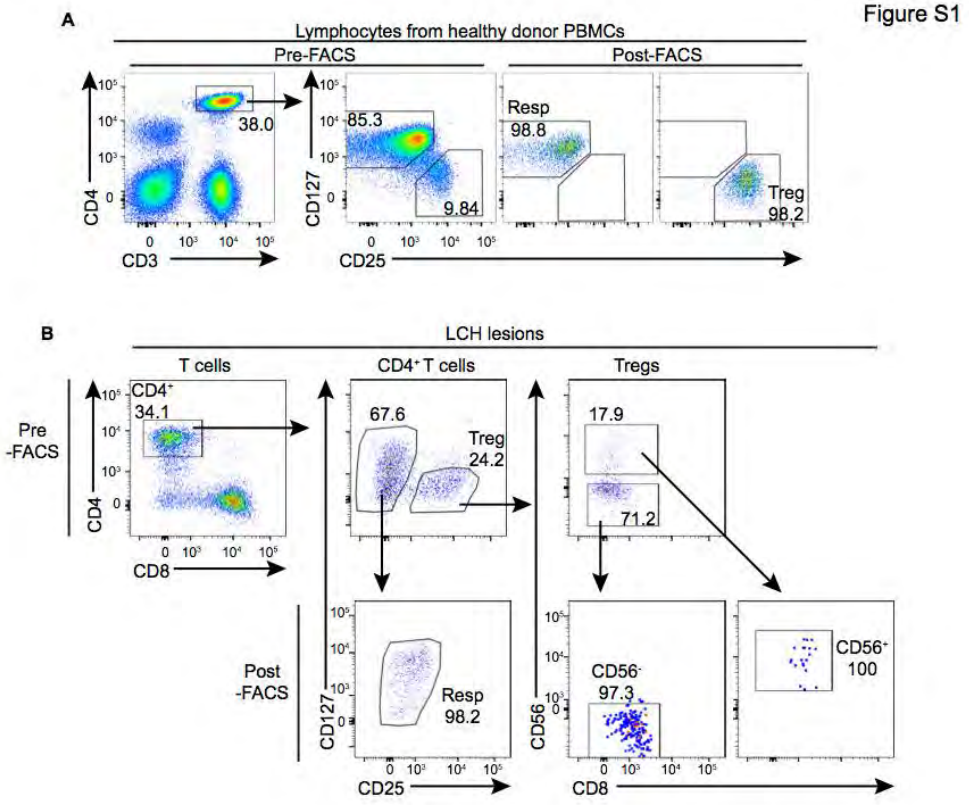
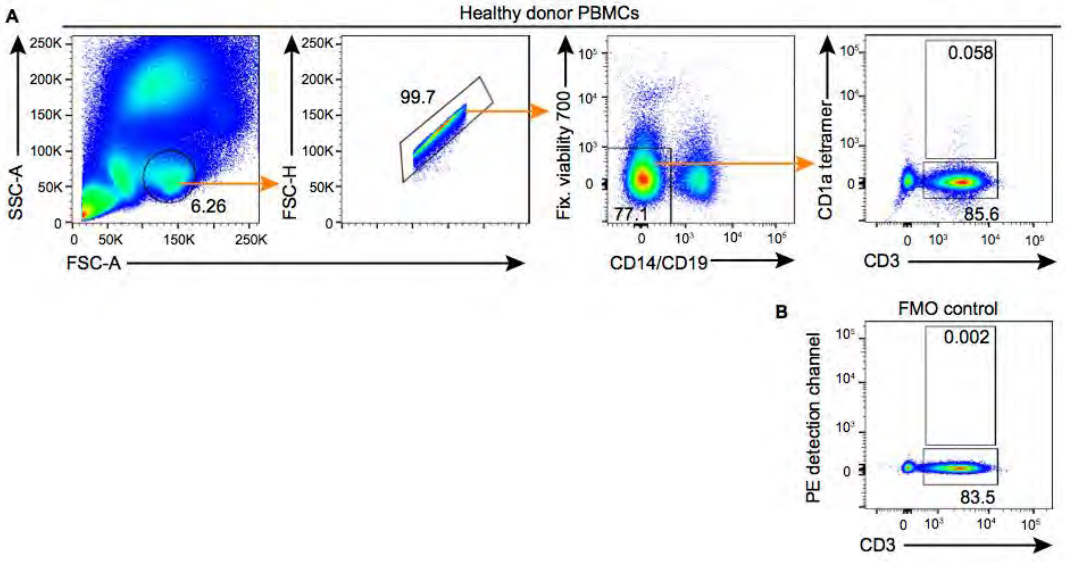


Figure S2



TABLES

Patient code	Specimen description	Sex	Age at diagnosis	Tissues affected	Age at specimen	Treatment prior to specimen	Disease status at specimen	Other
1	Bone lesion	M	68	Bone	68	Previous irradiation at site of and excision of hip lesion	Active LCH (AD)	Diabetes insipidus from age 55
1	Matched blood							
2	Pulmonary lesion	M	40	Lung	40	Nil	AD	Mild pulmonary fibrosis, smoker
3	Involved lymph node	M	9	Bone, lymph node	9	Nil	AD	Skull defect
4	Blood	M	18 months	Skin	13	Nil	Non-active LCH (NAD)	
5	Blood	M	53	Bone, lung, bone marrow	64	Vinblastine/prednisolone	NAD	Mild pulmonary fibrosis
6	Blood	M	39	Lung	52	Vinblastine/prednisolone	NAD	
7	Blood	F	60	Skin	64	Methotrexate/prednisolone	NAD	Leg scarring
8	Blood	F	36	Bone	37	Nil	NAD	
9	Blood	F	25	Bone	42	Nil	NAD	Ataxia at time of specimen
10	Blood	M	41	Skin	41	Biopsy and vinblastine/prednisolone	AD	
11	Blood	F	39	Bone, lung	39	Nil	AD	

12	Blood	F	34	Lung	54	Nil	NAD	Long-term pulmonary fibrosis
13	Bone lesion	M	2	Bone	2	Nil	AD	Mutation in BRAF V600, CNS-risk lesion
14	Bone lesion	M	7	Bone	7	Nil	AD	Mutation in BRAF V600
14	Matched blood							
15	Bone lesion	M	2	Bone, CNS	2	Nil	AD	Mutation in BRAF V600, unifocal disease at the time of specimen
15	Matched blood							
16	Blood	F	2	Bone	2	Nil	AD	
17	Blood	F	14	Bone	14	Nil	AD	

Table 1 – Clinical details of study participants.

LCH lesion patient number	No. of responder T cells (100 µl)	No. of $CD56^+$ Tregs (100 µl)	No. of $CD56^-$ Tregs (100 µl)
1	5000	1000	1000
2	5000	350	1000
15	300	200	250

Table 2 – Approximate number of cells cultured per well in purified T cell populations from lesions from patients with LCH.

No. of responder T cells (100 µl)	No. of Tregs (100 µl)
500	500
5000	1000
50,000	N/A

Table 3 – Approximate number of cells cultured in purified T cell populations from healthy donor blood.