The role of CD73 in the pathogenesis of Juvenile Idiopathic Arthritis

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Declaration

I, Sophie Botta Gordon-Smith, confirm that the work presented in this thesis is my own. Where information or data has been derived from other sources or produced by others, I confirm that this has been indicated.

Abstract

Juvenile idiopathic arthritis (JIA) manifests as a persistent arthropathy, thought to be immune-driven, that when untreated leads to progressive joint destruction. This group of diseases represents an excellent model to investigate immunoregulation because of the possibility to sample cells aspirated from the site of inflammation. This PhD investigated the contribution of defects in purinergic pathways to the pathogenesis of JIA by examining the distribution and enzymatic activity of the ecto-nucleotidase CD73, together with some investigation of expression of CD39 and CD26.

The data presented here demonstrate the significantly decreased proportion of $CD73^+$ T and B synovial lymphocytes from JIA patients compared to peripheral blood lymphocytes of both patients and healthy subjects. This reduction increased with higher disease severity (worse in extended compared to persistent oligoarticular JIA patients) and correlated with patient's cumulative joint count, but not with disease duration. No genetic association for NT5E (encoding CD73) was found that could explain the different levels of CD73 observed within different subtypes of JIA. Treatment with methotrexate, the first line DMARD to control arthritis, did not affect the proportion of $CD73^+$ peripheral blood lymphocytes, nor did this proportion predict response to methotrexate.

The reduction of $CD73^+$ synovial lymphocytes and of CD73 protein expression per $CD73^+$ cell was associated with a reduced ability to generate immunoregulatory adenosine *in vitro*, suggesting low levels of adenosine in the synovium. An incapacity of $CD39^+$ and $CD73^+$ cells to act cooperatively to metabolize ATP to adenosine, further contributes to the impression of low adenosine generation in the JIA joint, and of defective attenuation of inflammation.

In vitro, downregulation of $CD73^+$ PBMC and purified $CD8^+CD73^+$ T cells was demonstrated upon cell activation. The loss of $CD73^+$ PBMC was associated with a diminished potential to generate adenosine. The loss of $CD73^+$ PBMC appeared to be restricted to proliferating cells. I propose that the CD73 downregulation is associated with defective adenosine levels within the joint, which could contribute to the locally destructive inflammation seen in JIA.

3

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Abbreviations

Ab	Antibody
ACR	American college of rheumatology
ACR-Ped70	70% improvement in joint count
ACPA	Anti-citrullinated protein/peptide antibody
ADA	Adenosine deaminase
ADP	Adenosine diphosphate
ADK	Adenylate kinase
AICAR	5-aminoimidazole-4-carboxamide ribonucleotide
AK	Adenosine kinase
AMP	Adenosine monophosphate
ANA	Antinuclear antibody
AP	Alkaline phosphatase
APC	Antigen presenting cell
APS	Ammonium persulfate
APC (dye)	Allophycocyanin
ATIC	AICAR transformylase
ATP	Adenosine triphosphate
BCR	B cell receptor
BSA	Bovine serum albumin
cAMP	Cyclic AMP
CD	Cluster of differentiation
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CHO	Chinese hamster ovary
CIA	Collagen induced arthritis
CNT	Concentrative nucleoside transporter
CRE	cAMP responsive element
CREB	cAMP-dependent co-activator CRE-binding protein
CRP	C reactive protein
CTL	Cytotoxic lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen -4

DC	Dendritic cells
DCK	Deoxycytidine kinase
DMARD	Disease-modifying anti-rheumatic drugs
DMSO	Dimethyl sulphoxide
DP	Double positive
DPP4	Dipeptidyl-peptidase 4
ENT	Equilibrative nucleoside transporter
ESR	Erythrocyte sedimentation rate
ERK	Extracellular signal-related kinase
FACS	Fluorescent activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
fMLP	N-formyl-methionyl-leucyl-phenylalanine
Foxp3	Forkhead box protein P3
FPGS	Folylpolyglutamate synthase
g	Gravity
GITR	Glucocorticoid-induced TNF-receptor-related protein
GPI	Glycosyl phosphatidlyinositol
HLA	Human leukocyte antigen
HPLC	High performance liquid chromatography
IFN	Interferon
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
ILAR	International league of associations for rheumatology
JIA	Juvenile idiopathic arthritis
LPS	Lipopolysaccharide(s)
MAPK	Mitogen-activated protein kinase
MFI	Median fluorescence intensity
MHC	Major histocompatibility locus
mRNA	Messenger ribonucleic acid
MTX	Methotrexate

NK	Natural killer
NSAID	Non-steroidal anti-inflammatory drugs
P	Probability
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	R-phycoerythrin
PeCy5	R-phycoerythrin-cyanine 5
PerCP	Peridinin chlorophyll protein
PBMC	Peripheral blood mononuclear cells
PKA	Protein kinase A
PRRs	Pattern recognition receptors
SAH	S-adenosyl homocysteine
SCID	Severe combined immunodeficiency
SF	Synovial fluid
SFMC	Synovial fluid mononuclear cells
SNP	Single nucleotide polymorphism
spMHC	Self-peptide presented by MHC
RA	Rheumatoid arthritis
RAG	Recombination-activating gene
RF	Rheumatoid factor
RT	Room temperature
TCR	T cell receptor
TdT	Terminal deoxynucleotidyl transferase
TEMED	N, N, N', N'-tetramethylethylenediamine
TGF-β	Transforming growth factor-Beta
TNF	Tumour necrosis factor
Treg	regulatory T cell
TSDR	T-reg specific demethylated region
TYMS	Thymylidate synthase
VEGF	Vascular endothelial growth factor
WTCCC2	Wellcome Trust case control consortium -2

Contents

List of Figures

15

List of Tables

Chapter 1 Introduction

1.1 From an effective adaptive immune response to autoimmunity: break of self-tolerance

Adequate protection against pathogens is ensured by continuous cross-talk between cells of the innate and the adaptive immune systems. This constant dialogue gives rise to a robust defence network against foreign antigens, while at the same time preventing responses to self. Innate immunity relies on the function of both leukocytes (dendritic cells, monocytes, granulocytes, etc.) and non-leukocytes (epithelia), and provides the first line of defence against infections. Innate responses are initiated by the recognition of microbial motifs, which are generally absent in mammals, by pattern recognition receptors (PRRs). Adaptive immunity, on the other hand, depends on lymphocytes that are able to recognize antigens via their uniquely specific cell surface receptors. Due to this specificity, only a small proportion of cells can recognise a given antigen. To generate a sufficient number of antigen-specific lymphocytes for a response, antigen recognition brings about division of lymphocytes. During this clonal expansion, lymphocytes proliferate typically for several days, differentiate into effector cells, and give rise to a "clone" of daughter cells of the same antigen specificity. For this reason, initiation of adaptive immune responses takes longer than innate responses, in particular upon first recognition of a specific antigen when naïve cells are stimulated. The specificity of each lymphocyte receptor arises by a genetic mechanism, involving gene rearrangement, which occurs during lymphocyte development, and is described below.

1.1.1 T cell development, generation of T cell receptor (TCR) diversity and recognition of antigen

Precursors of T cells, which arise in the bone marrow, migrate via blood to the outer cortex of the thymus to mature. At this initial stage, these progenitor T cells, called double negative (DN) cells, do not express the T cell receptor (TCR) or the coreceptors CD4 and CD8. Developing T cells, known as thymocytes, give rise to either the more common αβ T cell population or the minor γδ T cell population. Those thymocytes that develop down the αβ pathway first express an invariant pre TCR α chain (pre TCR α) and then rearrange at the TCR β locus to produce and express the TCR β chain (Saint-Ruf et al. 1994). These two chains associate together with a CD3 group to give rise to the pre-TCR complex and provide a signal that the DN can continue its maturation.

There are opposing theories on whether a functional interaction between the pre-TCR and an intra-thymic ligand is required for signalling or whether the mere assembly of the pre-TCR is sufficient to permit maturation of the thymocyte (Irving et al. 1998). At this stage, thymocytes upregulate the expression of the co-receptors CD4 and CD8 to become CD4$^+$ CD8$^+$ double positive (DP) cells and start to proliferate. Simultaneously, rearrangement of the TCR β chain by the recombination-activating gene (RAG) 1 and 2 proteins (Oettinger et al. 1990) is interrupted, provided that a successful version of the TCR β chain has been produced. At the end of the proliferative burst, RAG-1/2 genes are reactivated, allowing somatic recombination of the V, J, C regions of the TCR α-chain. During the recombination process the removal and addition of nucleotides known as P-nucleotides (for Palindrome) and N-nucleotides (non-template encoded) (Meier et al. 1993; Nadel et al. 1997) further contribute to the generation of TCR diversity. N region nucleotides are added through the activity of the enzyme terminal deoxynucleotidyl transferase (TdT), expressed in the thymus before birth (Deibel et al. 1983).

Once a DP thymocyte has acquired a functional TCR α chain, it is ready to undergo selection for its ability to recognise self-peptide presented by MHC (spMHC) proteins expressed on thymic stromal cells (Hinterberger et al. 2010). This process of positive selection that occurs in the thymic cortex by cortical thymic epithelial cells expressing MHC class I and II proteins allows the survival of only those T cells that express a TCR that can recognise self-peptide MHC molecules. If a cell succeeds in rearranging its TCR α chain to produce a TCR which binds MHC, it will be spared, otherwise it will die by apoptosis (Surh et al. 1994). The subsequent stage of clonal deletion eliminates those thymocytes bearing receptors for self-MHC or spMHC above an affinity threshold (Von Boehmer 1992). Class I/II molecules are expressed by antigen-presenting cells (APC): dendritic cells found at the cortico-medullary junction and macrophages scattered in both the cortex and thymic medulla. Depending on whether the thymocytes bind to MHC-class I or class II complexes, the selection process results in lineage-specific differentiation to either CD8$^+$ or CD4$^+$ T cells respectively. The ratio of these two T cell subsets depends on asymmetric thymocyte death (Sinclair et al. 2013). Fewer than 5% of T cells survive thymic selection and are able to leave the thymic medulla (Surh et al. 1994).

After exiting the thymus and entering the periphery, T cells circulate around the body via lymphatics and blood, while continually entering peripheral lymphoid tissues to receive

signals for homeostasis and survival, and to encounter foreign antigen. The two best defined signals required for naive T cell homeostasis are interactions of the IL-7 receptor with IL-7 and of the TCR with spMHC (Kieper et al. 1999). The cytokine IL-7 has a dominant non-redundant role in supporting survival (Schluns et al. 2000) and homeostatic expansion (Tan et al. 2001) of T cells. Both naive CD4^{+} and CD8^{+} T cells require interaction with MHC proteins, class II in the case of CD4^{+} cells (Takeda et al. 1996) and class I in the case of CD8^{+} cells (Tanchot et al. 1997), for maintenance. It is believed that competition for these signals is what allows the number of naive T cells in the periphery to remain constant. In addition, cells of the same clonality compete for a spMHC interaction (intraclonal competition), whilst those of different specificity are unaffected, sustaining TCR diversity (Troy et al. 2003).

The naive T lymphocyte that has recognised its specific antigen, displayed by an APC, and has received a second 'co-stimulation' signal such as binding of CD28 on the T cell by CD80 or CD86 molecule on the APC, gets activated. It then leaves the lymph node as an effector cell, typically trafficking to the site of infection to combat the pathogen. Among APC, dendritic cells (DC) are the most efficient at initiating immune responses. DC are located in most tissues (in an immature form); and they express PRRs able to recognise microbes as well as co-stimulatory molecules required for functional T cell stimulation. DC also undergo maturation while migrating to the lymphoid organs. During this process they upregulate CCR7 for homing (Cyster 1999) (also expressed by naive T cells), and prepare to prime T cells.

Since DC are the key drivers of the adaptive immune response, they are considered to be key cells to determine whether a peptide, presented in the context of MHC molecules, will trigger a T cell immune response or not. Many theories have been developed to explain how this "choice" between immune activation and lack of response is made. Briefly, Burnet and Medawar (in 1969) introduced the self-nonself discrimination model, whereby an immune response is triggered by all foreign "nonself" entities. There were clear limitations to this model, such as its inability to explain reactivity towards self-antigens during autoimmunity. Therefore, Matzinger introduced the Danger model (Matzinger 1994). This model proposes that resting APC are activated by alarm signals released by cells exposed to pathogens and mechanical injury, resulting in cell death, and include substances such as adenosine triphosphate (ATP), complement components and the S100 protein family (Tveita 2010). This model provides the alternative viewpoint that "self-ness" is not a guarantee of tolerance,

so damaged cells of the host can trigger an immune response and therefore potentially result in autoimmunity (Matzinger 2002). The implications of this theory in autoimmunity are further discussed in section 1.1.3.1.

Following stimulation by DC, T cells also interact with other cells, including B cells to help with antibody formation. Activated T cells in turn reinforce DC maturation through signals mediated by surface CD40L and release of IFN-γ (Frleta et al. 2003). The cytokine environment present at the start of the T cell-DC interaction influences T cell differentiation. For example, elevated IL-12 production by DC promotes a Th1 response (Koch et al. 1996).

1.1.2 B cell development and immunoglobulin gene rearrangement

Production of B cells as common lymphoid stem cells occurs in the bone marrow, after which they mature to progenitor B cells. These cells proliferate and differentiate into precursor B cells if they receive the supporting developmental cytokine IL-7 from bone marrow stromal cells (Funk et al. 1995). The antigen specific receptor of B cells is known as Immunoglobulin or B cell receptor (BCR), and is first expressed as a membrane immunoglobulin composed of light and μ heavy chains. During development, the B cell receptor (BCR) genes undergo gene rearrangement (V (D) J recombination) through the action of RAG-1/2, in a process that parallels that of TCR genes. During the pro-B cell stage, the enzyme TdT, responsible for insertion of N-nucleotides at the coding joints of the D-J and V-D-J regions is also active for a short period. This allows for a higher number of possible combinations of BCR (Tonegawa et al. 1981).

Negative selection also occurs for immature B cells in the bone marrow, where after encounter with self-antigen, strongly reactive cells are eliminated. B-cells are able to break away from this process of negative selection by "receptor editing", with additional productive rearrangements of their immunoglobulin gene segments, until they are no longer self-reactive (Nemazee et al. 1989). The degree of antigen-receptor crosslinking is a factor which determines whether self-reactive immature B cells will either induce apoptosis or anergy, a state of specific functional unresponsiveness (Hartley et al. 1991).

After leaving the bone marrow, B cells circulate in the blood surveying for antigen, and unless they encounter both soluble protein antigen and activated T helper cells, they die by apoptosis within two days, since they need tonic BCR signalling for survival (Lam et al. 1997). Many B cell responses, including B cell activation, isotype class switching and affinity

maturation require help from T cells and such responses are therefore known as T-dependent responses. T cell help is typically provided through T cell derived cytokines. After antigen binding, B cells can also themselves process antigen for presentation to T cells, and upregulate expression of MHC class II and co-stimulatory molecules CD80/CD86 to enhance their APC function. During a T-B cell interaction, T cells recognise the antigen presented by the MHC class II molecule on B cells, resulting in its activation and upregulation of CD40L. The CD40 molecule present on B cells binds to this CD40L, helping the B cell to enter the cell cycle. The interaction between CD80/CD86 and CD28 on B and T cells respectively leads to co-stimulation of T cells, which results in release of cytokines that bind B cell receptors, ultimately resulting in B cell differentiation. Because of the need of T helper cells to mediate B cell activation for T dependent responses, B cells typically do not respond to self-antigens if these cells are lacking. If abnormal B cell self-recognition occurs, as may be the case in autoimmune disease, B cells contribute to disease pathogenesis by autoantibody production as well as abnormal regulation of T cell function and activation.

The presence of antibodies in individuals with T-cell deficiencies demonstrated that some antigens are able to stimulate naive B cells, even in the absence of T cell help. These antigens, known as thymus-independent (TI), are mostly bacterial polysaccharides and lipopolysaccharides (LPS). TI antigens are less efficient at inducing memory B cells and are not efficient at promoting isotype-switching (Coutinho et al. 1973).

1.1.3 Immune regulation and tolerance

Upon encounter with an antigen, the immune system must decide whether a lymphocyte should mount an immune response or should undergo anergy, deletion, and/or active regulation (suppression), to maintain tolerance. T cell anergy is a state of unresponsiveness, due to lack of costimulation, where potentially autoreactive T lymphocytes are kept in a dormant state. In addition, repeated stimulation of previously activated and expanded T cells by self-antigen can result in activation-induced cell death.

Active regulation can be mediated by a small subset of $CD4^+$ T lymphocytes that prevent excessive immune reactivity (Baecher-Allan et al. 2001), known as regulatory T cells (Treg). These cells are characterised by the expression of the transcription factor forkhead box P3 (Foxp3), which directs their suppressive function (Hori et al. 2003). Treg are able to inhibit the activation and expansion of self-reactive lymphocytes through a variety of mechanisms

(further details in section 1.2.2), one of which is the generation of anti-inflammatory adenosine. The role of this purine nucleoside is discussed below and further in section 1.3.2. Peripheral tolerance can also be mediated by DC when they are in a quiescent non-activated state. Recognition of self-antigens presented by DC to T cells in the absence of inflammation is likely to induce activation-induced cell death or anergy, and not activation (Chappert et al. 2008).

1.1.3.1 Break of self-tolerance and role of purines

Since the mechanisms of tolerance are complex, there can be a breakdown in the system, resulting in lymphocytes becoming activated by self-antigens. An example of a situation in which this can occur is when a naive T cell with low affinity for a self-antigen encounters and gets activated by an activated dendritic cell expressing high levels of co-stimulatory ligands. This dendritic cell has "decided" to initiate an immune response since it has detected a dysfunction or danger signal in the local environment. As described by the Danger model as above, the immune system is "alarmed" and set in motion by both exogenous and endogenous danger signals. External signals derive from recognition of bacterial/viral motifs by PRR, whilst tissue-derived messages are generated by activated cells (e.g. cytokines such as IL-1 (Sims et al. 2010)) or by damaged cells.

The nucleotide purine ATP is an example of a molecule normally almost absent in the extracellular space, but present at high intracellular concentrations, which is released following cell activation and damage. For these reasons, ATP can serve as a danger signal. Its immuno-activating functions include impairment of the suppressive function of Treg (Schenk et al. 2011) and stimulation of T cells (Baricordi et al. 1996). In contrast to ATP, adenosine, originating from the metabolism of ATP by the enzymes discussed in section 1.3.1., is mostly cytoprotective. Adenosine attenuates tissue damage (Ohta et al. 2001), promotes the expansion of Treg with increased immunoregulatory activity (Ohta et al. 2012) and induces T cell anergy, even in the presence of costimulation (Zarek et al. 2008).

Due to the described roles of ATP and adenosine in inflammation, an imbalance between these purines could represent a possible reason for or contribute to the pathogenesis of autoreactivity, potentially resulting in autoimmunity. Altered purinergic pathways have indeed been seen in several autoimmune diseases, such as Crohn's disease and ulcerative colitis (Friedman et al. 2009; Frick et al. 2009), diabetes (Chia et al. 2012) and childhood

arthritis known as juvenile idiopathic arthritis (JIA) (Moncrieffe et al. 2010b). With this background, it was hypothesized that alterations in adenosine generation may be part of the abnormalities seen during the chronic inflammation in JIA.

JIA is the focal point of this thesis: it represents a model to study defects in the purinergic pathway, since immune cells from the inflamed joints are readily accessible and can be used in both *ex vivo* and *in vitro* experiments. At the same time, peripheral blood cells from the same patient can be studied in parallel to identify changes in the purine pathway that are specific to the site of inflammation.

1.2 Juvenile Idiopathic Arthritis (JIA): definition, epidemiology and classification

Juvenile idiopathic arthritis (JIA) encompasses several heterogeneous forms of chronic arthritis of unknown etiology, beginning before 16 years of age. The disease is defined as JIA if it persists for at least 6 weeks affecting one or more joints, leading to arthropathy. In developed countries, the prevalence of JIA, the most common rheumatological disease in children (Manners et al. 2002) ranges between 16 and 150 per 100 000 (Ravelli et al. 2007). The International League of Associations for Rheumatology (ILAR) introduced the most recent classification with 7 mutually exclusive categories (Petty et al. 2004), with the oligoarthritis group further divided into 2 subtypes: persistent and extended. This categorization (depicted in Table 1.1) is based on clinical and laboratory characteristics, such as the number of joints affected in the first 6 months of illness.

In contrast to adult-onset rheumatoid arthritis (RA), where small joints are most commonly involved at onset, in JIA, large joints, such as the knee and ankle, are frequently affected at presentation. Some JIA categories such as psoriatic and rheumatoid factor (RF)-positive polyarticular JIA have a parallel form of disease in adults (psoriatic arthritis and RF-positive RA, respectively) (Szer et al. 2006; Firestein et al. 2008). Despite however both JIA and RA being autoimmune diseases characterized by destructive arthropathy, they are distinct diseases (as demonstrated by differences in clinical phenotype and outcome, subtypes and genetics) and may well have different underlying pathogenic mechanisms (Prahalad et al. 2002).

As shown in Table 1.1, oligoarthritis is the most common form of JIA and is also the subtype with the best outcome, with rates of remission ranging from 23 to 47% of patients after 6-10 years from disease onset (Ravelli et al. 2007). Oligoarthritis is characterised by high female prevalence (female: male, 3:1), young age of onset (<6 years), asymmetric arthritis, positive antinuclear antibodies (ANA) and a high risk of iridocyclitis (Ravelli et al. 2007). This subtype has a wide spectrum of outcomes and is relatively benign, particularly when fewer than five joints are affected during the first 6 months of disease. If the disease continues on a milder course, it is known as persistent oligoarthritis. When more than four joints become affected after 6 months, the disease is as such defined as extended oligoarthritis. Extended oligoarticular JIA can be a highly damaging and erosive disease, and bears more similarity to polyarticular JIA; usually requiring disease-modifying anti-rheumatic drugs (DMARDs).

JIA subtype	Definition	Extra-articular features	% of all JIA	Laboratory features	HLA associations
Oligoarticular JIA	affects 1-4 joints during first 6 months, 2 subcategories are recognised:	uveitis in 30% of cases	up to 50% of cases	mild ESR, 70% ANA+, RF-	HLA-A*0201, HLA-DRB1*0801,1101,1301
	1) Persistent Oligoarthritis affects $\leq$ 4 joints throughtout disease course	as above	50% of Oligo	as above	HLA-DRB1*1301
	2) Extended Oligoarthritis affects > 4 joints after 6 months of disease	as above	50% of Oligo	as above	HLA-DRB1*0101
Systemic arthritis	affects ≥1 joints with or preceded by fever, and at least either rash, serositis, splenomegaly	erythematous rash, serositis	<10%	high ESR and CRP	HLA-DRB1*11, -DQA1*05,-DQB1*03 (weak)
Polyarthritis (RF positive)	affects ≥ 5 joints during first 6 months of disease	rheumatoid nodules	< 5%	RF+, ACPA, high ESR	HLA-DRB1*0401, HLA-DR4
Polyarthritis (RF negative)	affects ≥ 5 joints during first 6 months of disease	uveitis in 10% of cases	20%	40% ANA+, mild anaemia	HLA-DRB1*0801
Psoriatic arthritis	asymmetric arthritis and psoriasis, 40% of patients have affected relative	dactylitis, nail pitting, uveitis 10% of cases	5-10%	50% ANA+, RF-mild/high ESR	HLA-DRB1*01, -DQA1*0101
Enthesitis-related arthritis	arthritis and enthesitis, with at least either HLA-B27+, acute anterior uveitis, history of spondylitis, lumbosacral pain or onset in a male >6 yr old	acute anterior uveitis, sacroiliac joint tenderness	10%	RF-, can be ANA+	HLA-B27+
Undifferentiated arthritis	fulfills criteria in no category or ≥ 2 categories	variable	10-20%	-	-

Table 1.1 JIA subtypes defined by ILAR criteria

ACPA anti-citrullinated protein/peptide antibody, ANA antinuclear antibody, ESR erythrocyte sedimentation rate, CRP C reactive protein, RF rheumatoid factor. Information in table derived from (Szer et al. 2006; Ravelli et al. 2007; Firestein et al. 2008; Macaubas et al. 2009; Hahn et al. 2010; Prakken et al. 2011; Martini 2012).

1.2.1 Aetiology of JIA

Despite the significant strides that have been made in the understanding of JIA pathology, its causes remain unknown and its exact trigger has still not been defined. It is possible that JIA is initiated and perpetuated by an immunological response to an infectious trigger and that it is the genetically programmed immune response of the host that establishes whether synovitis will be suppressed or perpetuated. Some evidence that the initial insult may be infectious derives from studies determining the presence of virus-specific antibodies in the serum of JIA patients (Hokynar et al. 2000; Gonzalez et al. 2007), together with similarities observed between the early clinical picture of JIA patients and that of patients infected with microbes and viruses (Pugh et al. 1993). If JIA truly is antigen-driven, the initiating antigens, instead of being auto-antigens, could be from infectious agents.

Antigen mimicry is also considered a potential cause for autoimmunity. Indeed oligoarticular JIA patients T cells were found to respond to heat-shock proteins (hsp), particularly to hsp60 (Prakken et al. 1997). Hsp are proteins expressed by both human and microbial cells after stress. Those T cells, derived from oligoarticular JIA patients, that did respond to hsp60 *in vitro* secreted high levels of anti-inflammatory IL-10 and showed an upregulation of CD30 surface expression (de Kleer et al. 2003). This phenotype, mostly associated with Th2 cells, or some forms of Treg cells, is suggestive of a regulatory function and an ability to counteract a Th1-driven immune response.

1.2.2 Immunopathology of JIA

All subtypes of JIA are characterized by chronic inflammation affecting the lining of the joints, the synovia. The synovium presents an infiltration in the sub-lining layer of both innate and adaptive immune cells: T cells, macrophages, B cells, natural killer (NK) cells and neutrophils (Bywaters 1977; Murray et al. 1996; Gregorio et al. 2007). The JIA hypertrophied synovial layer is vascular with the synovium presenting an abundant expression of the markers of activation human leukocyte antigen (HLA)-DR and intracellular adhesion molecule (ICAM)-1. A potential cause for this vascularisation could be the presence of elevated levels of the proangiogenic mitogen vascular endothelial growth factor (VEGF) in the JIA synovial fluid, which also correlated with disease severity (Vignola et al. 2002). Synovial angiogenesis, a critical determinant of JIA pathogenesis, could also be explained by

the raised levels of the proliferative protein osteopontin in JIA synovial fluid (Gattorno et al. 2004).

1.2.2.1 Alterations of synovial cytokines

A selective recruitment of activated T lymphocytes, of T helper-1 (Th-1) phenotype has previously been observed in the joint of children (Wedderburn et al. 2000). This finding, together with that of well documented associations with certain HLA haplotypes (Thomson et al. 2002) suggests that the pathogenesis of T cells is associated with T cell defects. However, it is clear that the immunopathology of JIA involves abnormalities of both innate and adaptive immunity. Thus, in the inflamed joints of children with JIA there are elevated levels of the cytokines TNF-α, IL-1 and IL-6 (de Jager et al. 2003) produced by monocytes and macrophages, of VEGF (Vignola et al. 2002) secreted by fibroblasts and synoviocytes, and of the phagocyte-specific S100 proteins (Prakken et al. 2011). In addition, cells of the innate system such as neutrophils, DC, and macrophages found in the synovial fluid (SF) have an activated phenotype (Varsani et al. 2003; Jarvis et al. 2006). This activation of APC could be in part driven by an interaction between APC and T cells through CD40L-CD40 signalling.

As mentioned previously, an array of cytokines released by infiltrating leukocytes are found elevated at the site of inflammation (de Jager et al. 2007). Among these, TNF-α, IL-1 and IL-6 are thought to be pivotal in the pathophysiology of synovial inflammation as they stimulate many cell types and activate several inflammatory pathways. Inflammatory cytokines, particularly TNF-α, IL-1 and IL-6, have been implicated in mediating the initiation and perpetuation of inflammation and joint destruction in JIA (Woo 2002). Since these cytokines have an important role in JIA pathogenesis and are targeted clinically for JIA treatment they are described in more detail here.

TNF-α is produced predominantly by activated macrophages and T lymphocytes and interacts with two receptors: TNFR1 and TNFR2, the soluble forms of which have been found to rise with increasing TNF-α levels in the synovium of children with JIA (Rooney et al. 2000). Production of IL-1 is an indicator of macrophage activation and is associated with induction of acute phase response, potentially linked to instances of high ESR observed in some oligoarticular JIA patients (Ravelli et al. 2007). The two forms of IL-1, IL-1α and IL-1β signal through the same receptor complex and have identical biological activities. However,

whereas IL-1β acts systemically and is produced mainly by monocytes and macrophages, IL-1α has a more localised effect and is highly expressed by keratinocytes and endothelial cells (Sims et al. 2010). The pro-inflammatory cytokine IL-6, known to enhance leukocyte transmigration to the site of inflammation (Clahsen et al. 2008) has been observed to inhibit Treg-mediated suppressive function (Fujimoto et al. 2011) and to stimulate NK cell activity (Malejczyk et al. 1992). The IL-6 receptor complex is composed of the gp130 subunit and the IL-6α chain, which may be present as soluble IL-6 receptor. A dysregulation in IL-6 signalling has been previously observed in autoimmune diseases, including RA, and in JIA a reduction of expression of gp130 was found on synovial monocytes, compared to peripheral blood monocytes, mediated by the stress-induced p38 mitogen-activated protein kinase (MAPK) (Honke et al. 2014). Downregulation of the IL-6R complex on JIA SF monocytes could serve to counterbalance the widespread inflammation present in the joint.

Another cytokine which is abundant in the synovial fluid of patients with oligoarticular arthritis (de Jager et al. 2007) is IL-10. This anti-inflammatory cytokine can inhibit the functions of activated macrophages/monocytes (Moore et al. 2001) and is produced by many cells of both the innate and adaptive immune system. The broadly anti-inflammatory cytokine transforming growth factor (TGF)-β is instead found in low levels in the synovial fluid of patients affected by JIA (Nistala et al. 2010). TGF-β is a pleiotropic cytokine implicated with the inhibition of effector functions of T cells, and of cytolytic and Th-1 differentiation (Rubtsov et al. 2007). It affects almost every type of immune cell, due to its broad distribution of receptors on immune cells, and mice lacking TGF-β receptor II were found to suffer from a fatal autoimmune disease (Marie et al. 2006). As this cytokine has an important role in regulating TCR-activation dependent effects, its absence in the JIA joint could be in part associated with the elevated expression of activation markers by synovial T cells (Black et al. 2002) discussed below.

1.2.2.2 Alterations of synovial lymphocytes

JIA synovial T cells express both the early and late activation markers: HLA-DR, CD69 and CD25 (Black et al. 2002). Synovial B cells are also highly activated and express the costimulatory molecules CD80 and CD86 (Corcione et al. 2009; Morbach et al. 2011). This evidence in concert with the restricted oligoclonal T cell expansion in the JIA joint (Wedderburn et al. 1999), would suggest the presence of autoantigen in the inflamed site

driving cell activation and proliferation. However, CD69 expression could also be upregulated after endothelial transmigration due to cell contact (Black et al. 2002).

The pathological JIA synovial T lymphocytes have a memory phenotype, as determined by expression of the memory marker CD45RO, and express high levels of the chemokine receptors CCR5 and CXCR3 receptors on both $CD4^+$ and $CD8^+$ T cell subsets (Wedderburn et al. 2000). As CCL5, CCL3 and CXCL10 are also released at high levels by synovial T cells (Pharoah et al. 2006), these chemokines could be driving the migration of these cells to the site of inflammation. CCR5 and CXCR3 are known to be associated with a Th1 profile and their expression on synovial T cells may be associated with the high IFNγ: IL-4 ratios observed in JIA synovial tissue (Scola et al. 2002).

The effector Th17 cells have also been associated with JIA pathogenesis. These cells produce the proinflammatory cytokine IL-17, elevated in the SF of both RA and JIA patients (Feldmann et al. 1996; de Jager et al. 2003), which is able to stimulate the production of IL-1 and TNF-α by macrophages. Th17 cells have a distinct phenotype from both Th1 and Th2 cell subsets and are elevated as a proportion of $CD4^+$ T cells among JIA synovial fluid compared to that in both patient and healthy control blood. The enrichment of these cells in the JIA joint is inversely related to the proportion of Treg (Nistala et al. 2008), which will be discussed later on.

No reliable predictor of extension has been identified yet, although the two subcategories of oligoarthritis are known to be strongly associated with different polymorphisms in the genes encoding for HLA: extended oligoarthritis with HLA-DQA1*01 and persistent oligoarthritis with HLA-DRB*1301 (Szer et al. 2006). Other differences have been observed prior to extension, between those patients who will remain mild (persistent) and those who will go on to have more severe disease (extended oligoarticular) JIA. These include: a lower ratio of CD4:CD8, due to $CD8^+$ T cell accumulation, higher levels of CCL5 and a series of genes expressed in synovial cells prior to extension (Hunter et al. 2010). In addition, there is a decreased proportion of $CD4^+CD25^{bright}$ cells (Treg) in the joints of patients with extended oligoarthritis compared to those with persistent oligoarthritis (de Kleer et al. 2004).

Due to its strong genetic link with HLA class II genes, and because the function of the HLA II molecule is to present antigen peptides to $CD4^+$ T cells via APC, the pathology of

oligoarthritis is considered to be at least in part CD4$^+$ T cell-mediated. As T lymphocytes may also drive the adaptive immune B cell response, they can also induce an autoimmune B cell response. The presence of autoantibodies in JIA and in many autoimmune diseases is likely to be due to an expansion of autoantigen specific B cells that bind and present self-antigen to T cells. Additional data suggesting a significant role of B cells in JIA pathology is the observation in some cases of lymphoid neogenesis in the joint (Gregorio et al. 2007). These structures are like germinal centres, the presence of which is related to the extent of infiltrating plasma cells and are more likely to be found in ANA-positive patients (Gregorio et al. 2007).

1.2.2.3 Mechanisms of immune regulation by regulatory T cells

As mentioned in section 1.1.3, one of the processes utilized by the immune system to achieve and maintain tolerance is active regulation, mediated for example by a cell subset called Treg. Treg can be either initiated in the thymus, which constitutively express CD25, Foxp3 and CTLA-4 (Sakaguchi 2004; Gavin et al. 2007) or adaptive/induced Treg (iTreg). iTreg are thought to be induced after chronic antigenic stimulation in the periphery. Induced Treg are more heterogeneous in terms of Foxp3 expression, phenotypically and functionally distinct from natural Treg, and can produce IL-10 or TGF-β. Due to the broad research on Treg, there is an abundance of terminology to describe them, which can lead to ambiguity. For this reason, Abbas et al. in (2013) recommended the following nomenclature for Treg: thymus-derived Treg instead of natural Treg, peripherally derived Treg instead of induced/adaptive, and *"in vitro"* induced Treg should be used to distinguish from those generated *in vivo*. The forkhead/winged transcription factor Foxp3 is known as the master regulator of Treg (Fontenot et al. 2003).

Because of their role in maintaining homeostasis, the ability of Treg to prevent autoimmunity or the associated inflammation has been the basis of much research. Treg transferred into nonobese diabetic (NOD) mice with recent onset diabetes were able to reverse the disease (Tang et al. 2004), while depletion of CD4$^+$CD25$^+$ cells in models of antigen-induced arthritis, enhanced symptoms of the disease, indicated by knee swelling and histological scores (Morgan et al. 2003; Frey et al. 2005).

Treg investigation in JIA led to some interesting and perhaps surprising results. A higher proportion of Treg among CD4$^+$ T cells, identified as either CD4$^+$Foxp3$^+$ or CD4$^+$CD25^{bright}

cells, was measured in the JIA joint, compared to that found among CD4$^+$ T cells from both patient and healthy control blood (de Kleer et al. 2004; Ruprecht et al. 2005; Nistala et al. 2008). As mentioned earlier, the proportion of Treg in the joint was inversely related to that of synovial Th17 cells (Nistala et al. 2008) and a higher proportion of Treg was observed in the joint of patients with persistent compared to those with extended oligoarticular JIA (de Kleer et al. 2004). This led to the question: why does the joint inflammation of JIA continue despite the high Treg frequency at the site of inflammation? Studies of the relationship between synovial Treg and effector cells have suggested that effector SF T cells are somehow resistant to suppression by SF Treg (Haufe et al. 2011), potentially linked to the observed PKB/AKT hyperactivation (Wehrens et al. 2011) found in these cells. These data suggest that one mechanism that contributes to continuing inflammation is resistance of SF T effector cells rather than a defect of function of Treg.

As shown in Figure 1.1, Treg are able to suppress self-reactive cells by a wide range of mechanisms. The specific mechanism used depends on either the environment or the type of immune response, whilst there is also the possibility that several mechanisms are acting synergistically to maintain tolerance. The ways in which Treg can suppress proliferation of antigen-stimulated naive T cells are many. These include: release of suppressive soluble factors (IL-10, IL-35 and TGF-β (Tran et al. 2007; Ring et al. 2011); cytokine consumption, particularly of the essential T cell growth factor IL-2, the absence of which can lead to deprivation-mediated apoptosis (Sakaguchi et al. 1995), and blockade of the binding of co-stimulator CD28 to CD80/CD86 by the CTLA-4 protein (Mayer et al. 2013). It has been shown that one mechanism by which CTLA4 on Treg mediates its suppressive effect is by removing the ligands of CD28, co-stimulatory molecules CD80 and CD86, from the surface of APC, by the process of trans-endocytosis (Qureshi et al. 2011).

Treg can also prevent interactions between T cells and antigen-presenting dendritic cells by physically out-competing T effector cells. This is achieved by Treg expression of neuropilin-1 and lymphocyte activation gene-3 which increased the affinity of Treg-dendritic cell interactions (Sarris et al. 2008). Treg can also induce apoptosis of effector T cells via release of cytolytic proteins perforin, granzyme B (Grossman et al. 2004) and by Fas/Fas ligand (FasL) and TNF/TNFR dependent pathways.

The second messenger cyclic adenosine monophosphate (cAMP), which is a potent inhibitor of IL-2 synthesis and T cell proliferation (Bodor et al. 2001), can suppress T cells through two mechanisms. These are: release through gap junctions of Treg (Bopp et al. 2007) and accumulation by the adenylate cyclase enzyme, following activation of the adenosine A_{2A} receptor ($A_{2A}R$) present on effector T cells (Lappas et al. 2005). The conversion of cAMP to AMP is mediated by phosphodiesterases, and subsequently by the ectonucleotidase enzyme CD73 to adenosine. It appears however, that in order to have a physiologically significant rise in adenosine, multiple cells would have to be releasing cAMP over a prolonged period (Colgan et al. 2006).

Degradation of extracellular ATP and the subsequent formation of adenosine by the action of the apyrase CD39 and CD73 is another suppressive pathway of Treg (Deaglio et al. 2007). This pathway is discussed in detail in section 1.3.
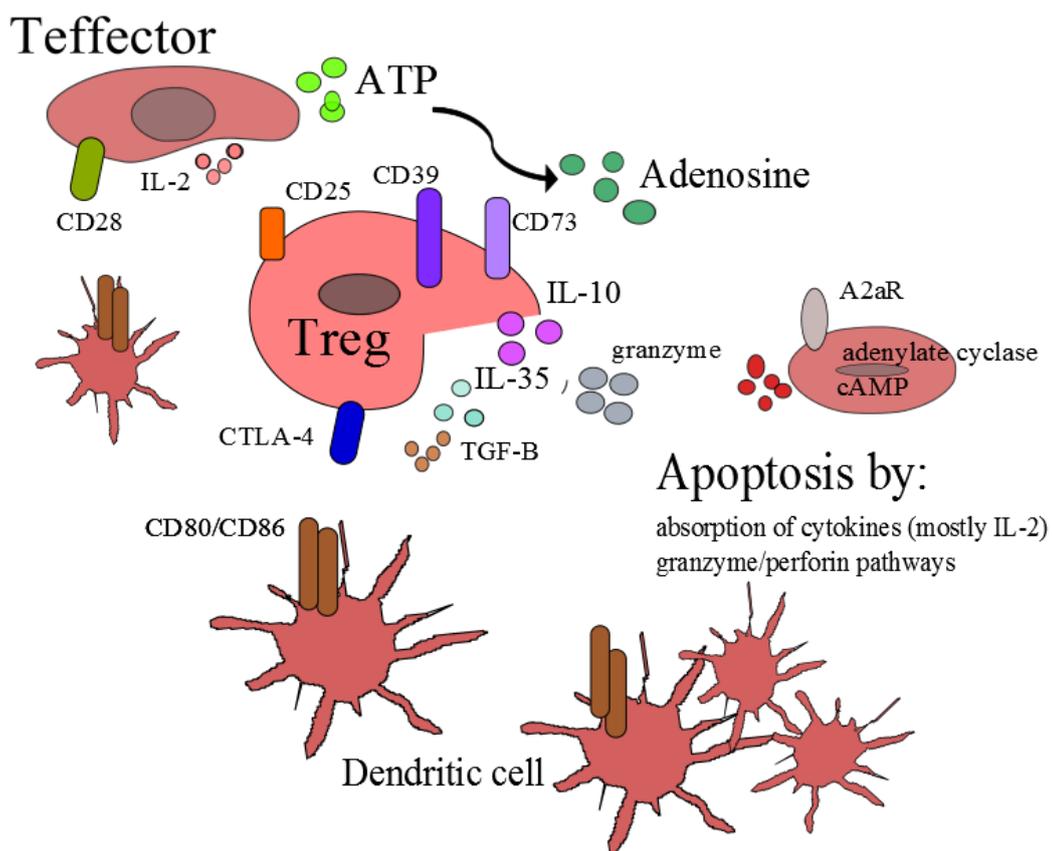
Figure 1-1 Mechanisms of suppression of regulatory T cells

Immunosuppressive pathways utilised by Treg to dampen inflammation include the release of soluble factors IL-10, IL-35, and TGF-β; generation of adenosine via CD39 and CD73 activity; prevention of interaction between CD80/CD86 and CD28 by CTLA-4 and apoptosis of effector cells mediated by granzyme and perforin, and absorption of IL-2 required for survival.

34

1.2.3 JIA treatment

Non-steroidal anti-inflammatory drugs (NSAIDs), intra-articular steroid injections, and oral steroids are used for the initial management of most JIA subtypes. These corticosteroids include prednisolone, methylprednisolone, betamethasone and triamcinolone hexacetonide/acetonide. If these are not sufficient, the established disease is treated with methotrexate (MTX) or other DMARDs such as leflunomide and sulfasalazine (Szer et al. 2006). Biologic agents such as the anti-TNF-α inhibitors, etanercept and inflixamab and the anti-IL-6R monoclonal antibody tocilizumab, are tested if the patient fails to respond to DMARDs, which happens in about 30% of cases (Becker et al. 2011). Combination therapies, such as the combination of anti-TNFα and MTX, are generally found to be more efficacious than DMARDs used alone (Martini 2012).

Despite the fact that its anti-inflammatory mechanism of action is still unknown, the DMARD MTX is the first choice of treatment for JIA. One of its proposed anti-inflammatory effects is mediated by adenosine, the production of which is dependent on the AMPase activity of CD73 (Montesinos et al. 2007). Further details of this mechanism and of how MTX inhibits purine and pyrimidine synthesis are in section 1.3.4.

1.3 Purines in health and disease

Several lines of evidence highlight the importance of ATP and adenosine in inflammation, as ubiquitous messengers with a wide range of immune functions. ATP is considered mostly a driver of inflammation, while adenosine counteracts its destructive effects. The level of availability of these two molecules is mainly regulated by the enzymes CD39 and CD73. CD39, also known as ectonucleoside triphosphate diphosphohydrolase-1 (ENTDP1), which converts ATP to ADP and then AMP has been previously reported in JIA to be upregulated on synovial T cells found to have an elevated capacity to breakdown ATP (Moncrieffe et al. 2010). In contrast, CD73, the driver of AMP conversion to adenosine, has not been yet fully investigated in JIA, and is the focus of this thesis.

1.3.1 Purine generation and catabolism
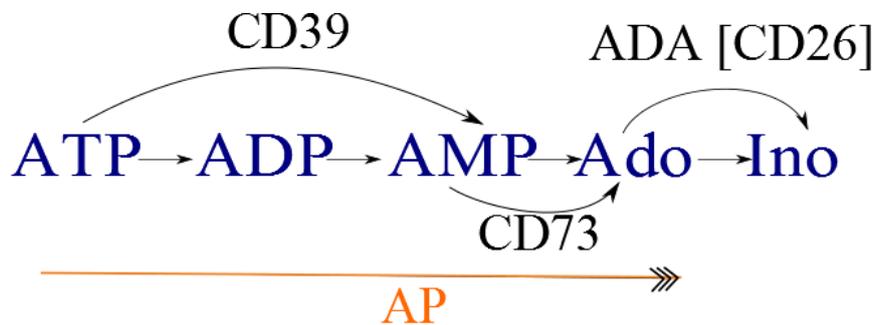
Figure 1-2 Main enzymes regulating extracellular levels of nucleotides and nucleosides

Ado adenosine, ADA adenosine deaminase, AP alkaline phosphatase, Ino inosine. Depiction of the nucleotides and nucleotides found upstream and downstream of adenosine and of the ectoenzymes that alter their extracellular levels.

The above figure depicts the key ectonucleotidases involved in determining the levels of available purines, while a more detailed scheme of the enzymes and transporters that affect both the extra- and intra-cellular concentrations of purine nucleotides is depicted in Figure 1.4 and described in detail below.

CD39 is the ectonucleotidase which hydrolyses ATP into ADP and ADP into AMP; with higher efficiency for ATP than for ADP (Dwyer et al. 2007). CD39 is an integral cell membrane 77kDa protein, which consists of two hydrophobic transmembrane regions and a large extracellular domain (Maliszewski et al. 1994) located within lipid rafts, and a small cytoplasmic domain containing the two N- and C-segments. A soluble catalytically active form of CD39 was found in both human and murine blood (Yegutkin et al. 2012), but its origin is unknown. CD39 protein is expressed by both T and B lymphocytes, on which it is upregulated after cell activation (Maliszewski et al. 1994), as well as endothelial cells, neutrophils, a subset of activated NK cells, Langerhans cells and monocytes/macrophages (Enjyoji et al. 1999). CD39 is consistently and abundantly expressed by murine Treg (Deaglio et al. 2007), for which it also considered a Treg marker. In contrast, in humans, the expression of CD39 is limited to a subset of Treg, and these have been shown to have an effector/ memory-like Treg phenotype (Borsellino et al. 2007).

CD73 or ecto-5'nucleotidase is the enzyme responsible for hydrolysing nucleotide monophosphates to their corresponding nucleosides. It is a surface-bound glycoprotein, composed of two identical subunits of 70kDa (Misumi et al. 1990) attached to the cell

membrane via a glycosyl-phosphatidylinositol (GPI) anchor at its C-terminus with no membrane embedded section (Sträter 2006). Its expression is mostly associated with glycolipid-rich domains, known as lipid rafts, characterised by high lateral mobility (Bianchi et al. 2003).

Both T and B lymphocytes, endothelial cells, granulocytes and monocytes derived from mice express CD73 (Flögel et al. 2012), while in humans CD73 expression is limited to lymphocytes and endothelial cells (Airas et al. 1993). On murine Treg, CD73 is highly co-expressed with CD39 (Deaglio et al. 2007), whilst in humans, expression of CD73 by Treg is negligible (Dwyer et al. 2010). Mandapathil et al. (2010) presented data showing that CD73 is present abundantly in the cytoplasm of human Treg.

Within this thesis, the expression of CD73 is characterized on leukocytes from both blood and synovial fluid of JIA patients, where a downregulation was observed (Botta Gordon-Smith et al. 2015). Detailed investigation of CD73 distribution is found in this thesis.

A soluble form of CD73 protein also exists, presumably derived from release of the GPI-anchored protein by the action of the phosphatidylinositol specific phospholipase C (PI-PLC) (Zimmermann 1992) or potentially secreted by the liver (Johnson et al. 1999). This soluble 5'-nucleotidase has been investigated less than the surface-bound protein, but from what is known, it appears to retain its AMPase catalytic activity, found to be particularly high in new-born blood plasma (Pettengill et al. 2013). The CD73 protein has complex cellular dynamics, with 50% of the enzyme residing intracellularly in a membrane-bound pool, as found in a rat hepatoma cell line (van den Bosch et al. 1988). There is evidence of a highly active membrane shuttle transporter for CD73 which allows a continuous cycle between the cell surface and cytoplasmic membranes (Widnell et al. 1982).

The CD73 enzyme has high affinity for AMP, for which it has a K_m ranging from 1-50 μM (Heuts et al. 2012). Competitive inhibitors of the enzyme include ATP, ADP and adenosine 5'-(α,β-methylene) diphosphate (APCP), a stable ADP analogue, which all bind to the catalytic site without being hydrolysed (Zimmermann et al. 2012). Uptake of adenosine from the extracellular milieu allows it to be reutilized for the generation of new RNA and DNA, preventing the need for *de novo* synthesis. CD73 has therefore an important function not only in adenosine signalling, but also in adenosine salvage.

Other seven forms of 5'nucleotidases with different subcellular localization which have been documented include: a PI-PLC resistant version (Klemens et al. 1990), five cytosolic 5'nucleotidases of which only cytosolic 5'-nucleotidase-I (cN-1A) dephosphorylates AMP, and a mitochondrial deoxyribonucleotidase (Hunsucker et al. 2005).

Adenosine deaminase (ADA) protein of which there are two distinct isoenzymes in humans, ADA1 and ADA2, deaminates adenosine and deoxy-adenosine to inosine and deoxy-inosine, both intracellularly and extracellularly. The two forms of this protein have differing distribution and structure, with ADA 1 (encoded by ADA), being a 35kDa Zn-binding protein, found in all cells with the highest levels in the lymphoid tissue. ADA 2 isoform (encoded by the cat eye syndrome critical region candidate 1 gene (CECR1) (Gakis 1996)), is instead the prevalent source of ADA activity in healthy human serum and plasma and is only expressed by monocytes and macrophages (Sakowicz-Burkiewicz et al. 2011).

As described in section 1.3.2, for the homeostasis of immune cells it is essential for deoxyadenosine to be kept low as despite lacking physiological functions, its accumulation inhibits DNA synthesis and triggers apoptosis by accumulation of the deoxy-ATP pool (Seto et al. 1986). ADA therefore, has an important cytoprotective role, highlighted by the fact that mutations of its gene, resulting in loss of enzymatic activity, are found in children who suffer from severe combined immunodeficiency (SCID) (Giblett et al. 1972). These ADA-deficient patients suffer from lymphophenia, absence of T and B cell-immunity and present elevated levels of intracellular and extracellular adenosine and derived compounds in body fluids (Franco et al. 1997). The severity of the disease depends on the concentration of accumulated adenosine. The potent inhibitory effect of adenosine on lymphocyte proliferation is considered to be part of the reason behind the lack of immune response in patients lacking ADA (Bessler et al. 1982).

ADA-1 is mostly a cytosolic protein and is brought to the cell surface by a range of anchoring proteins. Multiple ADA-1 binding proteins have been found and CD26 was the first to be discovered and is therefore considered a surrogate marker for ADA expression (Kameoka et al. 1993). Others include A_1R, $A_{2A}R$ and $A_{2B}R$ (Ciruela et al. 2001). **CD26** (encoded by gene dipeptidyl-peptidase IV (DPP4)]) is 110kDa glycoprotein with its own enzymatic activity as a peptidase (De Meester et al. 1999). Despite CD26 and ADA being co-localized on the cell surface, this is not the case in the cell cytoplasm, hinting that CD26 is not involved with the

transport of ADA to the cell membrane (Dong et al. 1996). CD26 is not expressed by NK or resting B cells, while it is present on T cells, particularly CD4$^+$ T cells (Morimoto et al. 1998), on which its expression is highly upregulated after T cell activation (Martín et al. 1995). This marked upregulation appears to be needed to sustain the activation (Franco et al. 1997). The expression of CD26 is upregulated in autoimmune diseases such as RA (Ellingsen et al. 2007), where its upregulation is correlated with disease activity. CD26 can also serve as a co-stimulatory molecule, enhancing T cell proliferation, following its crosslinking on the cell membrane (von Bonin et al. 1998).

Another enzyme that can metabolize nucleotides is **alkaline phosphatase** (AP). AP has a spectrum of substrates, including the 5'-nucleotides ATP/ADP/AMP and adenosine. This protein is mainly cytosolic, but like CD73 can be found at the cell surface anchored by a GPI-bond. AP has low affinity for AMP, with a K_m in the low millimolar range, is not specific for this substrate and functions best in an alkaline environment with pH between pH 8.2-10.7 (Millán 2006; Badgu et al. 2013). AP is expressed highly in the placenta, the intestines and in liver/bone/kidney where it is known as tissue non-specific AP (TNAP). The tissue non-specific form is encoded by the ALPL gene. Not much is known of lymphocyte AP expression in humans, whilst in mice it has been identified as a B cell activation marker (Burg et al. 1989). Soluble AP is derived mostly from bone and liver, and its measurement is a standard clinical test for detection of bone diseases, as it is a marker of osteoblastic activity. Total serum AP levels are increased in growth phases during childhood and puberty (Turan et al. 2011) and decrease with age gradually.

Following uptake into the intracellular compartment, adenosine has two potential fates. It can either be metabolised by ADA to inosine or can be rapidly rephosphorylated to AMP by the intracellular enzyme **adenosine kinase** (AK). AK uses ATP as a phosphate donor and has an optimum pH for activity above 9 and low affinity for adenosine (Fredholm 2007).

Subsequent to deamination of adenosine to inosine by ADA, inosine can follow only one pathway, conversion to hypoxanthine by the ubiquitously expressed **purine nucleoside phosphorylase** (PNP). As for ADA, mutations discovered in the PNP-encoding gene were found to result in severe immunodeficiency disorders, by causing defective T cell immunity (Thompson 2013). Another enzyme involved in purine salvage is **deoxycytidine kinase** (DCK), found in the nucleus, it is responsible for the phosphorylation of several

deoxyribonucleosides, such as deoxyadenosine, a derivative of adenosine (Simmonds et al. 1978). **SAH hydrolase** (*S*-adenosyl-homocysteine hydrolase) is an enzyme responsible for the reversible hydration of *S*-adenosyl-homocysteine (SAH) into adenosine and homocysteine. Leukocytes contain only low levels of SAH (Newby et al. 1981; Worku et al. 1983), therefore this enzyme cannot contribute significantly to adenosine synthesis.

The extracellular adenosine concentration also depends on the re-uptake by the bi-directional transport system. Adenosine is transported across the plasma membrane by two types of transporters. One of them is the subset of **equilibrative nucleoside transporters** (ENT) which move nucleosides down their concentration gradient by facilitated diffusion. The others are **concentrative nucleoside transporters** (CNT) which use the sodium ion gradient as a source of energy to mediate the intracellular flux of nucleosides against their concentration gradient (Antonioli et al. 2013). Almost all cells possess equilibrative adenosine transporters which allow adenosine to quickly enter the cell (J.-F. Chen et al. 2013). Other nonspecific nucleoside carriers exist, such as the family of ABC proteins.
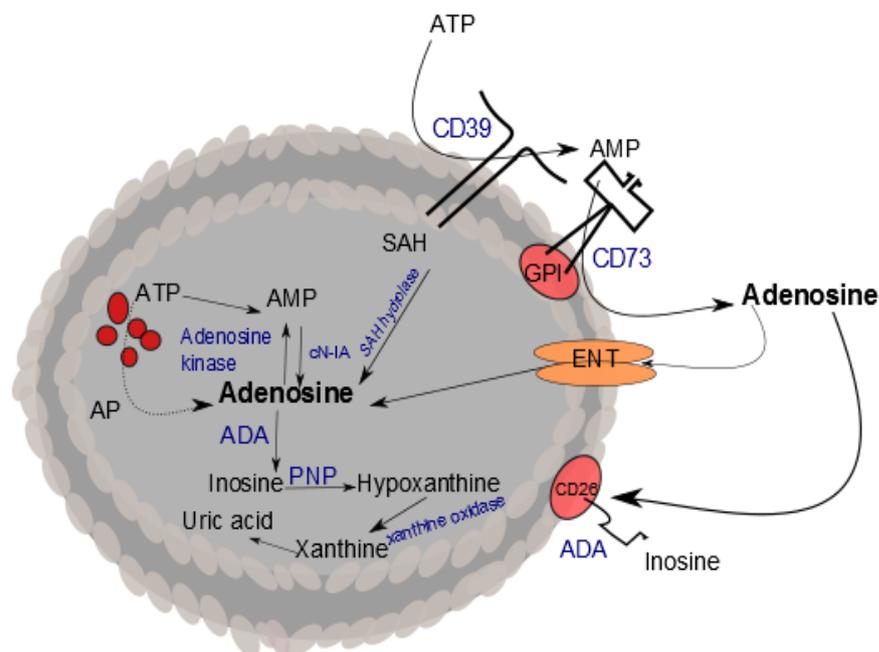
Figure 1-3 Metabolic pathways for adenosine generation and consumption

ADA adenosine deaminase, AP alkaline phosphatase, cN-1A cytosolic 5'-nucleotidase-I, ENT equilibrative nucleoside transporters, SAH *S*-adenosyl-homocysteine, PNP purine nucleoside phosphorylase. Complete depiction of all the enzymes and proteins that can alter both intra and extracellular levels of adenosine and of its metabolites.

1.3.2 Purines as immunomodulators in inflammation: focus on adenosine

ATP produced by mitochondria through the processes of glycolysis and oxidative phosphorylation is used as energy currency throughout the body to drive almost every cell function. It can for example maintain gradients for membrane potentials mediated by the sodium/potassium ATPase and ATP-driven proton pumps (Eguchi et al. 1997). A drop in mitochondrial membrane potential prevents ATP production, resulting in cell death (Ly et al. 2003).

Despite the fact that intracellular concentration of ATP is in the low millimolar range, under normal conditions it is almost not present in the extracellular space and cells are not exposed directly to its effects mediated by its surface receptors. Following mechanical injury, membrane deformation, and osmotic pressure leading to necrosis, there is leakage of the cytoplasmic content and therefore release of ATP to the extracellular space resulting in sudden high-fold increases in its extracellular levels. Release of intracellular ATP can also occur however, in an ordinate fashion, by opening of channel-like pathways or vesicular exocytosis, following shear stress of endothelial cells (Yegutkin et al. 2000) and degranulation of platelets in response to exogenous ADP.

In inflammatory conditions in response to activation of neutrophils and T cells, ATP is released through connexin 43 gap junctions (Eltzschig et al. 2006a) and pannexin-1 hemichannels (Schenk et al. 2008; Woehrle et al. 2010), respectively. This process occurs subsequently to increased mitochondrial activity, resulting in enlarged cytosolic Ca^{2+} entry and subsequent elevated ATP synthesis. The P2X subset of ATP receptors and pannexin-1 are translocated to the immune synapse following TCR stimulation (Woehrle et al. 2010), and since these surface receptors facilitate ATP liberation and their ligation by ATP induces elevated Ca^{2+} entry, the mechanism of ATP release can act as an autocrine feedback system. Immediate regulation of the extracellular levels of ATP, following its release, is considered particularly important as it has been found that minor changes in its levels translate to major (>100,000 times higher) increases in intracellular adenosine concentrations (J.-F. Chen et al. 2013).

The ATP nucleotide exerts a broad variety of effects on both hematopoietic and non-hematopoietic immune cells, such as stimulation of histamine and granule release from mast cells, neutrophils and monocytes (Dombrowski et al. 1998) and promotion of neutrophil

adhesion to epithelial cells (Pettengill et al. 2013). In contrast, the nucleoside adenosine found downstream of ATP, inhibits this adhesion (Cronstein et al. 1986). This is one of the many examples of the opposing effects of these two molecules, which are closely linked.

Engagement of ATP with one of its ligand-gated ion channel receptors P2X$_7$ allows entry of NLRP3 inflammasome agonists into the cell, leading to the assembly of the inflammasome, a multi-protein complex with a central role in innate immunity. This subsequently directs the clustering of caspase-1, resulting in caspase-1-dependent maturation and secretion of IL-1 and IL-18 cytokines (Mariathasan et al. 2006). In a zebrafish wound model, ATP release triggered activation of Dual oxidase (Duox)-1, a member of the multi-component, membrane-associated NADPH oxidase family, which triggers the formation of an H_2O_2 gradient, enhancing leukocyte recruitment to the site of injury. ATP together with H_2O_2, serves as a danger signal to activate *in vivo* the redox-sensitive transcription factor NF-κB (de Oliveira et al. 2014), the point of convergence of all inflammatory signalling pathways, which drives cytokine transcription and production.

Discharge of ATP into the extracellular space following T cell activation (Schenk et al. 2008; Yip et al. 2009) triggers mitogenic stimulation of T lymphocytes by signalling through its P2X and P2Y purinergic receptors (Baricordi et al. 1996), prolonging their activation and IL-2 secretion. ATP can therefore act as an autocrine positive feedback signal, resulting in signal amplification. In addition to T cells, Schena et al. (in 2013) reported that in human B cells, ATP is stored in late endosomal/lysosomal vesicular compartments released extracellularly after treatment of the cells with the stimuli CpG and anti-Ig.

ADP is stored together with ATP in secretory vesicles of blood platelets and released upon their activation, promoting platelet aggregation and activation (Robson et al. 1997). Therefore, in addition to acting on smooth muscle and the central nervous system, ADP has a role in thrombosis (Lüthje 1989). No effect has been reported for ADP on lymphocytes. ADP can induce changes in shape, calcium flux and inhibition of adenylate cyclase in platelets leading to their aggregation, together with further enhancing the secretion of ADP from activated platelets, amplifying its own effects (Foster et al. 2001). The cytoskeletal protein kindlin-2, a regulator of integrin function, can control platelet aggregation by changing the levels of available ADP/AMP in the bloodstream by reducing expression of CD39 and CD73 on the surface of endothelial cells via clathrin-dependent endocytosis (Pluskota et al. 2013).

The effects of **AMP** appear to be limited to the central and peripheral nervous system (Dunwiddie et al. 1997; Brundege et al. 1997), with no known immunomodulatory capacities. It is particularly difficult to account for the actions of AMP, because of its rapid conversion (in the order of milliseconds) to adenosine and because of the widespread distribution of CD73 AMPase on all tissues in the body.

The main source of extracellular **adenosine,** stored freely in the cytosol of all cells, is dephosphorylation of AMP (adenosine monophosphate) by the action of ecto-5'-nucleotidase CD73. The levels of available adenosine are closely linked that of extracellular ATP, so that in response to cell damage and inflammation in conditions of hypoxia, ischemia and trauma, both purines are elevated compared to healthy normal levels (Eltzschig et al. 2006b; Eltzschig et al. 2011).

Adenosine and its receptors are implicated in several biological functions, and its effects can be observed in almost every organ and tissue of the body (Drury et al. 1929). Physiological adenosine has widespread cardiovascular functions, such as inducing vasodilatation, increasing blood flow and therefore oxygen supply to tissues such as the brain and the heart (Adair 2005) and attenuating ischemia and reperfusion injuries (Ely et al. 1992). It is also implicated in neuroprotection following cerebral ischemia (Rudolphi et al. 1992), stimulation of nociceptive afferent neurons (Burnstock et al. 1996) and inhibition of neurotransmitter release by reducing rates of firing (Fredholm 2007). Rapid release of adenosine following tissue-disturbing stimuli mediates homeostasis by both acting as a pre-eminent alarm molecule and as a sensor of tissue damage. Adenosine can therefore prevent excessive damage to various organs following inflammation. The protective effect of adenosine during hypoxia is mostly due to its ability to increase blood flow and stimulate angiogenesis (Haskó et al. 2008).

Adenosine can inhibit the production of pro-inflammatory cytokines, while enhancing the production of anti-inflammatory cytokines by a broad variety of leukocytes. This mechanism, mediated by the A_{2A} receptor, is considered the dominant anti-inflammatory effect of adenosine. Cronstein et al. (in 1983) observed the ability of physiological adenosine to potently inhibit the release of oxygen radicals such as superoxide anions generated by neutrophils in response to the chemoattractant and cell activator n-formyl-methionyl-leucyl-phenylalanine (fMLP), while exerting little or no effect on their aggregation and

43

degranulation. Together with blockade of superoxide (0_2^-) anion generation by neutrophils, via A_{2A} receptors, adenosine also prevented neutrophil adhesion to endothelial cells (Cronstein et al. 1986) by decreasing the avidity and expression of their adhesion molecules and therefore reducing their damaging effects to the endothelium. Adenosine also prevented phagocytosis and the bactericidal function of neutrophils (Burnstock et al. 2014). In concert with the inhibition of Th1 cytokine release (Romio et al. 2011), engagement of the A_{2A} receptor of human LPS-stimulated neutrophils prevents the release of CCL3, CCL4 and CXCL2 involved in the onset and progress of inflammation (McColl et al. 2006). Inhibition of NF-κB and of p38 MAPK, a kinase involved with phosphorylation of nuclear factor kappa B (NF-κB) inhibitors and their signal transduction pathways, has been observed in human synoviocytes of patient with osteoarthritis after stimulation of their A_{2A} and A_3 receptors resulting in inhibition of IL-8 and TNF-α production (Varani et al. 2010a). In addition to inhibition of synthesis of inflammatory cytokines, adenosine can also trigger the production and release of regulatory, anti-inflammatory cytokines such as IL-10 (Haskó et al. 2008).

T cell activation induces a rapid upregulation of adenosine receptor expression, particularly of the A_{2A} receptor, the activation of which results in suppression of IFNγ release, a major T cell-driven inflammatory response (Lappas et al. 2005). This rapid induction of $A_{2A}R$ expression following TCR signalling has been suggested to be a mechanism that limits both the activation of T cells and the secondary activation of macrophages in inflamed tissues.

The activation of $A_{2A}R$ inhibits the upregulation of IL-2 receptor (Huang et al. 1997), normally observed after TCR-activation, preventing proliferation and expansion of lymphocytes, as IL-2 is required for their growth. Adenosine is also simultaneously able to inhibit IL-2 secretion *in vitro* (Erdmann et al. 2005) by naive CD4$^+$ T cells following TCR stimulation, further reducing their proliferation (Naganuma et al. 2006). In addition, the release of adenosine suppresses other TCR-triggered effector functions of T lymphocytes, such as the upregulation of the mRNA of the death receptor Fas (also known as CD95) and its ligand FasL in cytotoxic lymphocytes (Koshiba et al. 1997). Since the Fas/FasL system controls cell death by inducing apoptosis in those cells expressing Fas, this prevention of their upregulation by adenosine blocks activation-induced cell death (Himer et al. 2010). Together with the inhibition of cytotoxic activity of T lymphocytes, adenosine was found capable of inhibiting the ability of IL-2-activated NK cells from killing tumour cells

(Raskovalova et al. 2005). Adenosine can also dose-dependently suppress TCR signalling, contributing to the maintenance of a normal number of naive T cells (Cekic et al. 2013).

Adenosine therefore affects effector cell proliferation both directly and indirectly to limit the collateral damage associated with cell activation.

Apart from the anti-inflammatory responses that adenosine exerts on a variety of immune cells, adenosine can also regulate the adaptive responses to hypoxia. This is mediated by promoting vasodilatation, increasing blood flow (Manfredi et al. 1982) and mediating angiogenesis by ligation of the A_{2A} receptor, resulting in increased wound healing (Montesinos et al. 2002). The angiogenic effects of adenosine are executed both dependently and independently of the release of pro-angiogenic factors: basic fibroblast growth factor (bFGF) and VEGF. Unfortunately this pathway can contribute to cancer development and progression, particularly with the ability of adenosine to inhibit the cytotoxic activity of NK cells to clear the body of tumour cells (Raskovalova et al. 2005).

Despite its well-known cytoprotective role, adenosine can also have pro-inflammatory, detrimental effects, by inducing mast cell degranulation and activating pathways that promote tissue injury and fibrosis, particularly when high levels persist in acute injury (Fredholm 2007). Chronic elevated levels of adenosine are in fact considered toxic. This is particularly the case for a derivative of adenosine, deoxyadenosine as for SCID patients with defective adenosine deaminase (ADA), which breaksdown adenosine, and deoxyadenosine to inosine and deoxy-adenosine, there is no alternative catabolic route for degradation. Adenosine can instead be rephosphorylated to AMP by adenosine kinase as described later on. The major cause of the cytotoxic activity of deoxyadenosine appears to be mediated by its ability to block de novo deoxynucleotide synthesis, preventing the generation of new, undamaged DNA. This together with the effects of deoxyATP, formed by phosphorylation of accumulated deoxyadenosine, which causes high levels of single strand breaks in the DNA of resting lymphocytes, results in inhibition of DNA repair by these toxic metabolites (Seto et al. 1986).

The deleterious effects of long-term elevated adenosine in hypoxia are offset by an upregulation of active ADA, on both the cell membrane tethered by CD26 and in plasma, resulting in increased breakdown of adenosine to inosine (Eltzschig et al. 2006b).

Inosine was considered, for a long time, an inactive metabolite without any biological effects. This has however been found to be incorrect, as it was established to have both neuroprotective and potent immunomodulatory properties (Haskó et al. 2004). These are described in the next section that illustrates through which receptors these effects are mediated. After its generation from adenosine, inosine can be either returned to the pool of purines after catalysis by hypoxanthine-guanine phosphoribosyl-transferase or it is further degraded into hypoxanthine and uric acid (Sakowicz-Burkiewicz et al. 2011).

1.3.3 Purinergic signalling

ATP receptors were first known as P2 purinergic receptors. These were subdivided into seven ionotropic nucleotide-gated ion channels P2X receptors, which gate extracellular cations in response to ATP, and eight metabotropic G-protein coupled P2Y receptors based on their structure and signalling properties. These receptors are expressed by most immune cells (Bours et al. 2006). $P2Y_2$ receptor expression by monocytes allows for their recruitment to the location of apoptopic cells which release ATP as a "find-me" signal for these cells (Elliott et al. 2009) and it is through this receptor that ATP mediates adhesion of neutrophils to endothelial cells. Of the P2Y receptors, $P2Y_{11}$ is the only receptor which can activate adenylate cyclase causing intracellular accumulation of immunosuppressive cAMP (Abbracchio et al. 2006). It is through this cAMP elevation that activation of the $P2Y_{11}$ receptor by extracellular ATP serves to inhibit NK cell proliferation (Miller et al. 1999).

For the reasons stated above, ATP should not be solely considered as a "danger signal", but also as a negative feedback signal, able to limit harm associated with inflammation, particularly in situations where there is chronic exposure to low levels of extracellular nucleotides. This has been shown in part by microarray studies, in which exposure to ATP upregulated the expression of genes involved with immunosuppression. These included thrombospondin-1 which inhibits T cell proliferation and stimulates TGF-β secretion (Di Virgilio et al. 2009). Immune responses to ATP are also affected by receptor desensitisation, particularly of the $P2X_1$ receptor, whereby a significant fraction of the receptor pool enters into a long-lasting refractory state at a nanomolar concentration of ATP (Rettinger et al. 2003).

ADP does not appear to affect lymphocytes, whilst it has an important role in thrombosis by influencing platelets through three purinergic receptors, namely $P2Y_1$, $P2Y_{12,}$ and $P2X_1$.

Until recently, no receptor was known for **AMP**. Characterization of the receptor which mediates AMP effects was obtained recently by (Rittiner et al. 2012) who determined AMP to activate the G_i-coupled A_1R and no other receptor subtype. As the A1 receptor subclass is negatively coupled to adenylate cyclase, resulting in inhibition of generation of immunoregulatory cAMP, able to inhibit IL-2 production (Bopp et al. 2007), AMP is considered to exert mainly pro-inflammatory effects.

Adenosine exerts its multiple effects by engaging with its widespread cell surface G-protein coupled receptors, known as A_1, A_{2A}, A_{2B}, and A_3. These asparagine-linked glycoproteins are characterised by seven transmembrane domains and multiple intracellular and extracellular loops (Haskó et al. 2008). Adenosine receptors have different distributions throughout the body and variable affinities for their substrate adenosine. A_1 and A_{2A} are high affinity receptors sensitive to submicromolar concentrations of adenosine, while A_{2B} and A_3 are low affinity receptors, only responsive to micromolar concentrations (Fredholm et al. 2001). A_1 and A_3 are primarily coupled to the G_i protein, while A_{2A} and A_{2B} are coupled mostly to G_s proteins, causing the first two receptor types to inhibit adenylate cyclase and the latter to activate it, leading to accumulation of cAMP. An accumulation of cAMP can activate protein kinase A (PKA) that mediates phosphorylation of "cAMP- response element binding protein" (CREB). This phosphorylation causes an increase in its transactivating potential and therefore transcription of the CEBPβ gene. Transcription of this gene can result in transcription and subsequent release of IL-10. All adenosine receptors are coupled to MAPK pathways regulating phosphorylation of ERK, ERK2 kinases and p38 MAPK in a time and dose-dependent manner (Fredholm et al. 2001)

All four adenosine receptor subtypes are expressed by lymphocytes, but while A_1Rs are highly expressed in the brain, particularly at synapses (Augusto et al. 2013), the expression of this receptor is limited or even absent on lymphocytes (Franco et al. 1998). Among these cells, $A_{2A}R$ is the sole adenosine receptor expressed by B lymphocytes, but its expression is much higher on T cells compared to B cells (Koshiba et al. 1999).

The A_{2A} receptor of the P1 class, is the receptor most involved with the regulatory capacity of adenosine and dictating lymphocytes responses, inhibiting activation of T cells by prompting T-cell anergy, by preventing the TCR-triggered CD25 upregulation and induction of Treg (Huang et al. 1997; Zarek et al. 2008). Adenosine binding to $A_{2A}R$ on effector cells alters the

nuclear translocation of the transcription factor NF-κB after antigenic stimulation and subsequently diminishes the release of proinflammatory cytokines IL-2, TNF-α and IFNγ (Romio et al. 2011).

Inosine has recently been reported to also have some anti-inflammatory effects mediated by binding to A_1R, $A_{2A}R$ and A_3R (Fredholm et al. 2001; Haskó et al. 2004). This however, has been a matter of discussion, as there is inconsistent data regarding its binding capacity to these receptors. For example, a study which revealed the ability of inosine to stimulate mast cell degranulation via the A_3R (Jin et al. 1997), also reported the incapacity of inosine to bind to the A_1R and $A_{2A}R$. While the reported property of inosine to inhibit the production of proinflammatory cytokines (i.e. TNF-α and IFN-γ) and enhance production of IL-10 (Hasko et al. 2000) was through ligation to the A_1 and A_{2A} receptors. Not much is known of the intracellular pathways triggered by binding of inosine to these receptors, but consistent with adenosine binding, A_1R and A_3R occupancy results in prevention of cAMP accumulation.
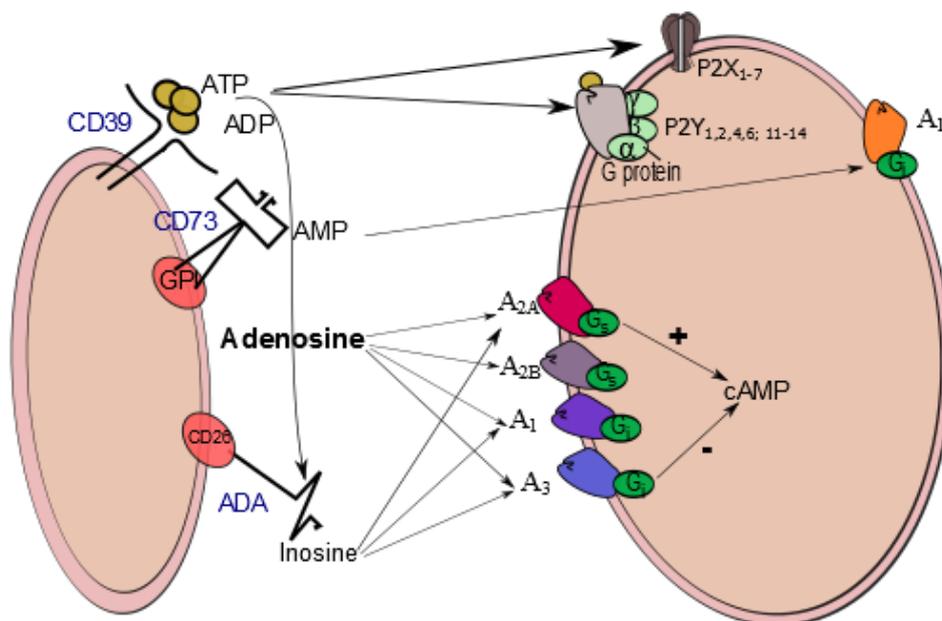
Figure 1-4 Nucleotide and nucleoside receptors

Depiction of the ectoenzymes responsible for the generation of extracellular adenosine and its metabolism, and of the receptors through which these nucleotides/nucleotides mediate their effects. The G proteins to which the GPCR receptors are coupled to are also represented, including the downstream effect of G protein activation for adenosine receptors.

1.3.4 Therapeutic approaches centred on modulating adenosine

Adenosine is a building block for nucleic acids; it belongs to the same pathway as ATP, the main biological energy currency used throughout the body, and mediates its effects through four receptors ubiquitously expressed. For these reasons and the non-redundant anti-inflammatory effects of adenosine described previously, much research has gone into developing drugs that can either target adenosine receptors directly or modulate adenosine levels by inhibition of its uptake and metabolism.

As discussed in the following section, MTX, the most widely used treatment for JIA, alters extracellular adenosine concentrations and signalling (Cronstein et al. 1993), as one of its mechanisms of immunomodulation. Other current treatments that prolong the actions of extracellular adenosine and enhance its signalling events are dipyridamole, an adenosine uptake inhibitor, currently under clinical trial for the treatment of RA in conjunction with the steroid prednisolone (Chen et al. 2013) and pentostatin, an ADA inhibitor which blocks the conversion of adenosine to inosine.

Unfortunately, because of the widespread distribution of adenosine receptors throughout the body, targeting of these ubiquitously expressed receptors generally leads to unwanted side effects. Because of this, and since it is practically unfeasible to correctly determine the concentrations of extracellular adenosine at a particular site as its concentration fluctuates so rapidly, it has been challenging for compounds altering adenosine biology to enter the clinical setting. Due to these complexities, the only A_{2A}-receptor specific agent to have been approved for use in humans is regadenoson, used for myocardial perfusion imaging in patients with suspected coronary artery disease (Ghimire et al. 2013). A novel pharmacological tool that could circumvent these issues, particularly the hypotensive effects of A_{2A} receptor agonists, has been developed. It consists of an A_{2A} receptor agonist prodrug, which is cleaved preferentially within tissues which over express CD73 and the A_{2A} receptor, such as found with synovial leukocytes from the knee of experimental mice with collagen-induced arthritis (Flögel et al. 2012).

Adenosine receptors are considered promising therapeutic targets in autoimmune diseases, as for example, both A_{2A} and A_3 receptors have been found upregulated on blood lymphocytes

of RA patients compared to healthy controls (Varani et al. 2011). In the Varani et al study, the activation of these receptors was found to inhibit the NF-κB pathway, blocking the release of pro-inflammatory cytokines, demonstrating that these receptors could be used as therapeutic targets. Two A_3 receptor agonists, CF101 and CF102, currently in phase II trial for rheumatoid arthritis and psoriasis, ameliorated arthritis symptoms in mouse models (Baharav et al. 2005; Fishman et al. 2006; Ochaion et al. 2008) and in the clinical trial in humans (J.-F. Chen et al. 2013). Upon treatment with these agents, an improvement in disease activity of RA was observed, with reduction of inflammation, pannus formation and cartilage destruction (Varani et al. 2010b). This suppression of pathological manifestations of RA was mediated by dose-dependent inhibition of the proliferation of fibroblast-like synoviocytes (Ochaion et al. 2008), via down-regulation of the NF-κB signal transduction pathway. The administration of adenosine attenuated adjuvant-induced knee arthritis in rats, while surprisingly also enhancing bradykinin-induced plasma extravasation in the rat knee joint, demonstrating that plasma extravasation protects against joint injury (Green et al. 1991).

Further evidence for the importance of adenosine in regulating pathophysiological conditions derives from studies with the nonspecific adenosine receptor agonist *N*-ethylcarboxamide adenosine (NECA), found to ameliorate the course of diabetes and prevent the β-cell mediated destruction of the pancreas in animal models of type 1 diabetes (Sakowicz-Burkiewicz et al. 2011).

1.3.4.1 Targeting adenosine with anti-inflammatory methotrexate

The anti-folate agent MTX is the first choice course of treatment for inflammatory diseases such as JIA and RA. This anti-proliferative agent, which disrupts the production of DNA precursors, was originally developed for the management of cancer. Despite its anti-inflammatory mechanism of action still not being fully understood, low dosages of the drug have been used to treat rheumatologic diseases for several years.

MTX is taken up by cells, including erythrocyte and synoviocytes, via the reduced folate carrier SLC19A1, where by the action of the enzyme folylpolyglutamate synthase (FPGS), glutamate moieties are added to its structure, which is converted to long-lived and metabolically active MTX polyglutamates (MTXGlu$_n$). The enzyme γ-glutamyl hydrolase (GGH) reverses the action of FPGS, removing the glutamate group from MTX, so that it is in

a form that can be transported to the outside of the cell by the ATP-binding cassette (ABC) family of transporters (de Rotte et al. 2012).

MTXGlu$_n$ polyglutamates are potent competitive inhibitors of the enzyme dihydrofolate reductase (DHFR) responsible for the conversion of folic acid to tetrahydrofolate, a methyl donor for the formation of methionine from homocysteine (Hillson et al. 1997). Whereas, *S*-adenosyl-methionine (SAM), a metabolite of methionine, is a methyl donor for the formation of the polyamines spermine and spermidine, which are found elevated in the urine, synovial fluid and lymphocytes of patients with RA and are considered important for IL-2 responsiveness in lymphocytes (Hillson et al. 1997).

Since the polyamines spermine and spermidine can be transformed into the lymphotoxins ammonia and hydrogen peroxide by the action of monocytes (Chan et al. 2010), inhibition of these toxic metabolites is considered to be one of the many protective effects of MTX. Pyrimidine synthesis is also blocked by low doses of MTX, as the drug inhibits thymylidate synthase (TYMS) the enzyme responsible for their formation. Inhibition of *de novo* purine and pyrimidine synthesis, together with the obstruction of the creation of lymphotoxins was considered the main immunomodulatory action of MTX. However, as supplementation with folic acid to RA patients treated with MTX did not compromise the efficacy of the drug, (Morgan et al. 1994), MTX was deemed to be acting via another metabolic pathway.

As MTX had been previously observed to increase the release of adenosine from fibroblasts and endothelial cells (Cronstein et al. 1991), this mechanism was further investigated, leading to the finding that MTX also inhibits AICAR transformylase (gene name ATIC) resulting in the intracellular accumulation of its metabolite AICAR (Cronstein et al. 1993) which affects adenine nucleotide metabolism. The build-up of AICAR results in release of adenosine into the circulation by direct inhibition of AMP deaminase and therefore accumulation of AMP, together with competitive inhibition of ADA by AICA ribonucleoside, a dephosphorylated metabolite of AICAR (Baggott et al. 1986). The addition of CD73 inhibitor APCP in a murine air-pouch model of inflammation (Morabito et al. 1998) abrogated entirely the release of adenosine, revealing that the promotion of adenosine release by MTX is dependent on the action of CD73 ecto-nucleotidase. Further evidence of this derives from a study using CD73-deficient mice which were found to be resistant to the anti-inflammatory action of methotrexate (Montesinos et al. 2007), as after treatment there was no MTX-associated

increase in adenosine exudates and diminished TNF-α and leukocyte accumulation at the site of inflammation as observed for wild-type mice.

Despite these reports suggesting that MTX mediates its anti-inflammatory action by release of adenosine and that its effects were reversed by either an $A_{2A}R$ antagonist, addition of adenosine deaminase or of the nonselective adenosine receptor antagonists theophylline and caffeine, or the use of knockout mouse models (Cronstein et al. 1993; Montesinos et al. 2000; Montesinos et al. 2003), this mechanism still remains a matter of discussion.

A study by Andersson et al. (2000) using a rat model of antigen-induced arthritis demonstrated that adenosine antagonists combined with MTX did not affect the drugs anti-arthritic effect. While another study by (Dolezalová et al. 2005) attempting to measure adenosine plasma concentrations of JIA patients under treatment with MTX and correlate it to erythrocyte $MTXGlu_n$ concentrations, which can be used to predict drug response, found no difference with healthy control adenosine levels, and no correlation with MTX. Since adenosine has an extremely short half-life, due to its rapid uptake inside cells (Dawicki et al. 1988), and as it is rapidly formed following release of ATP and ADP by platelet destruction during sampling, it is extremely challenging to accurately measure extracellular levels of adenosine. This could be the reason behind the failure of the (Dolezalová et al. 2005) study to find a difference between patient and control plasma concentrations of adenosine.

In an attempt to circumvent this issue, adenosine was quantified by determining its vasodilator effect in patients with active arthritis taking MTX (Riksen et al. 2006). This study found that MTX did indeed inhibit adenosine deaminase and potentiate adenosine-induced vasodilatation by activation of $A_{2A}R$. In addition, adenosine was also found to be elevated, as compared to healthy controls in the urine of patients treated with MTX (Baggott et al. 1999).

Not all JIA patients treated with MTX react successively to the drug, with about 30% of cases not responding to treatment (Becker et al. 2011). This percentage was found to not be improved by doubling the standard dosage of $10mg/m^2$/week as the plateau of efficacy of MTX was reached at the 15 mg/m^2/week parenteral dosage (Ruperto et al. 2004).

Figure 1-5 Diagram of the main enzymes involved in metabolism of methotrexate

ADA adenosine deaminase; ATIC AICAR transformylase; FPGS folylpolyglutamate synthase; GGH γ-glutamyl hydrolase; IMP inosine monophosphate, SAH S-adenosyl-L-homocysteine; SAM S-adenosyl methionine; polyG. Polyglutamates; TYMS thymylidate synthase. Red arrows depict the enzymes that MTX polyglutamates inhibit.

Due to the high percentage of non-responders, much investigation has gone into determining predictors of response to MTX. When JIA patients were tested for genetic differences that correlate with variability in response to the drug, they were found to have genetic variations in the ATIC, SLC16A7 (part of the solute carrier SLC10A1 family) and in the GGH genes associated with response to MTX (Moncrieffe et al. 2010a; Hinks et al. 2011; Yanagimachi et al. 2011). More recently a genome wide association study of response to MTX in JIA has identified 31 novel genetic loci which are different between responders and non-responders (Cobb et al. 2014).

SNPs within the ATIC and ADOR2A (A_{2A} receptor) were found to differentiate with clusters of intracellular MTX-Glu concentrations (Becker et al. 2011), suggesting that the ability of the patients cells to adequately produce or respond to adenosine, may determine the anti-inflammatory response to the drug and therefore its capacity to suppress the disease.

1.4 Project hypotheses and aims:

Many studies with CD73 knockout mice have highlighted the importance of this protein and its enzymatic activity in the control of autoimmune diseases. Examples of these include the study by (Blume et al. 2012), where lack of CD73 resulted in renal injury, with glomeruli inflammation and that of (Buchheiser et al. 2011) in which CD73 deletion caused atherogenesis, due to inhibition of resident macrophages and T cells, following changes in lipid metabolism and NF-κB signalling pathways. Termination of the effects of pro-inflammatory ATP by CD39 is also essential for disease control, as demonstrated by an induced model of colitis, whereby CD39 ablation was associated with increased disease severity (Friedman et al. 2009). In light of the studies suggesting the importance of the ectonucleotidases CD39 and CD73 and their ability to breakdown ATP and produce adenosine, in controlling autoimmune diseases the work described in the following chapters had the objective of further investigating the purine pathway in the context of juvenile idiopathic arthritis. Despite the fact that major strides have been made recently in the field of purine catabolism, there remains the open question as to how adenosine synthesis is modified at the site of inflammation of juvenile arthritis patients.

In this thesis, the following key questions/aims were addressed:

1) Characterisation of the expression of CD73 and other ectonucleotidases on blood and JIA synovial fluid leukocytes (Chapter 3);

2) Is there a correlation between CD73 expression on JIA synovial fluid lymphocytes and clinical characteristics of patients? (Chapter 4);

3) Does reduced synovial lymphocyte expression of CD73 result in reduced potential to generate adenosine? Is there a requirement for CD39 and CD73 coexpression for synthesis of adenosine from ATP? (Chapter 5);

4) Does the synovial inflammatory milieu drive the downregulation of CD73? (Chapter 6)

Chapter 2 Materials and Methods

2.1 Sample preparation

2.1.1 Sample collection

The samples analysed in this study were obtained from individuals who provided full informed consent in accordance with the local research ethics committee (Great Ormond Street Hospital/ Institute of Child Health Research Ethics committee) ref 95RU04. Samples were collected from children with full-informed parental consent. Children with JIA provided peripheral blood and synovial fluid samples taken at the time of required clinical blood tests and of therapeutic joint aspirations respectively. Each sample was allocated an anonymous code. Eighty patients with JIA who fulfilled the International League of Associations for Rheumatology (ILAR) criteria (Petty et al. 2004) were evaluated in this study. Clinical characteristics of these 80 patients are shown in Table 2.1. In addition, a large cohort of JIA patients in whom whole genome genotype was available, through the SPARKS-CHARM (Childhood arthritis response to medication) study, were analysed (see section 2.5).

Healthy adult volunteers were staff at the Institute of Child Health who provided peripheral blood samples, which were assigned an AW (adult well) code to maintain anonymity. Peripheral blood from healthy children (2 females and 4 males with mean age 6 ± 2.19 years) was used as age-matched controls, but as access to paediatric control samples was limited, the majority of control blood samples were from adult subjects. All samples were processed within 2h of collection, as described below.

A cohort of children was recruited from the SPARKS-CHARM study, which has the aim of developing understanding of the variability in response to treatment observed in children with JIA. All cases were about to start MTX treatment for active arthritis. For ten CHARMS patients, blood was collected just prior to starting MTX and again at 6 months of treatment with MTX. Response to MTX treatment was measured using the JIA definition of improvement (Giannini et al. 1997). In this definition, six core set variables are measured at start of treatment and at time of assessment of response. These variables are: 1) physician's global assessment of overall disease activity (measured on a 10-cm visual analogue scale (VAS)); 2) parent global assessment of overall well-being (measured on a VAS); 3) functional ability; 4) number of joints with active arthritis or, if no swelling is present, limitation of motion accompanied by heat, pain, or tenderness, 5) number of joints with limited range of motion; and 6) erythrocyte sedimentation rate (ESR).

	Persistent Oligoarticular JIA patients (n=30)	Extended Oligoarticular JIA patients (n=34)	Polyarticular JIA patients (n=16)
No. male/female	9/21	8/26	4/12
Age at sampling, mean ± SD (years)	8.0 ± 3.6	9.5 ± 3.6	7.03 ± 3.1
Age at disease onset, mean ± SD (years)	5.4 ± 3.2	3.1 ± 2.1	4.5 ± 3.8
Disease duration, mean ± SD (years)	2.5 ± 2.8	6.3 ± 3.0	2.4 ± 2.1
Treatment received: MTX (%)	*3/29 (10)	20/34 (58.8)	14/16 (88)
Treatment received: steroids (%)	*2/29 (6.9)	9/34 (26.5)	3/16 (19)
No. of cumulative joints involved at up to sampling, mean ± SD	1.9 ± 0.85	5.6 ± 2.0	6.3 ± 2.3

* Treatment data not available for one patient. MTX=Methotrexate

Table 2.1 Characteristics of patient population used in this study

2.1.2 Isolation of peripheral blood mononuclear cells (PBMC)

Whole peripheral blood was collected into sterile tubes containing 35U of preservative free heparin (Wockhardt, UK) and processed in a sterile Holten LaminAir category 2 laminar flow hood with sterile and endotoxin-free reagents. The blood was diluted with an equal amount of RPMI 1640 (Life Technologies, UK) culture medium containing 2mM L-glutamine with added 100U/ml penicillin and 100µg/ml streptomycin antibiotic mix (Life Technologies, UK), subsequently called RPMI-PSG. The diluted blood was then layered onto half its volume of Lymphoprep ^{TM} (Axis-shield, Norway) and centrifuged at 800g for 20min in a Sorvall Legend (Germany) RT centrifuge. The interface of mononuclear cells was carefully pipetted into a new tube containing RPMI-PSG and centrifuged at 500g for 10min. The cell pellet was resuspended in RPMI-PSG with 10% v/v heat inactivated batch-tested FBS (Fetal Bovine serum) (Life Technologies, UK) in a volume of medium generally equal to the amount of blood initially taken and counted as described in section 2.1.4. Cells were then spun at 300g for 7min and prepared for cryopreservation as described in section 2.1.5.

2.1.3 Isolation of synovial fluid mononuclear cells (SFMC)

Synovial fluid samples were initially incubated at 37°C in a water bath for 30-40min with 10U/ml hyaluronidase (Sigma, UK). Samples were periodically shaken in order to break up hyaluronan. Following this treatment, SFMC were processed the same way as PBMC as described in the above section.

2.1.4 Enumeration of viable cells

Viable cell numbers were determined by placing 10µl of cell suspension onto a Neubauer haemocytometer chamber (Hawksley, UK) with 10µl of 0.4% dye trypan blue (Sigma, UK). Dead cells took up the dye and stained blue so could be excluded to obtain a measure of cell viability. The haemocytometer with a 25-box field and counting area of depth 0.1µl was placed under a light microscope (Euromex, Holland) for counting. The final concentration of viable cells per ml of sample was obtained by multiplying the counted number of cells by 2 (dilution factor) and by $1x10^4$ (as 1ml/ 0.1µl= 10^4). Cell preparations used had a cell viability > 90%.

2.1.5 Cryopreservation of cells

Cells were resuspended in freezing medium, composed of FBS supplemented with filtered 10% v/v dimethyl sulphoxide DMSO (Sigma, UK) and frozen in cryovials (NUNC, Thermo Fisher Scientific, Denmark) at a concentration of $1x10^7$cells/ml of freezing medium. Samples were initially stored at -80°C for 24-48hrs in an isopropanol "freezing pot" (Nalgene) and were then transferred to liquid nitrogen tanks at -196°C for long-term storage. This freezing process was carried out in stages to allow the cells to cool slowly (mean speed of 1°C per hour) to maximize cell viability upon recovery.

2.1.6 Thawing of frozen cells

Cryo-preserved cells were thawed by placing the cryovial in a 37°C water bath immediately after removal from the liquid nitrogen tank. Cells were then diluted in warm RPMI-PSG with 10% FBS and counted with a haemocytometer (as in section 2.1.4.) to check viability. The cell suspension was centrifuged at 300g for 7min with the final cell pellet resuspended in RPMI-PSG at 1-2x10^6 cells per ml, depending on the requirement of the experiment.

2.1.7 Extraction of synovial fluid or plasma

The fluid was obtained from peripheral blood or synovial fluid collected in a tube containing EDTA anti-coagulant. This was transferred to an eppendorf, first spun at 1000g for 10min, then 10.000g for 10min on a microfuge, before being stored at -80°C.

For the preparation of the pooled samples, 5 healthy control plasma samples and 10 synovial fluid samples were used. These had already been spun down and were stored at -80°C. After thawing of the samples, each type of fluid was pooled into a separate eppendorf for a final

volume of 3mls and treated with 3µl of hyaluronidase for 30 minutes at 37°C. Samples were then cooled, and at stored -80°C in 50µl aliquots.

2.2 Fluorescent activated cell sorting (FACS)

2.2.1 Buffers and Reagents

- Phosphate buffered saline (PBS): from tablets Dulbecco A (Oxoid, UK) dissolved in MilliQ H_2O

- FACS buffer: 1 x PBS, 1% FBS, 0.1% sodium azide

- Paraformaldehyde (PFA): 1xPBS, 4% PFA

- Foxp3 and intracellular staining with Foxp3 staining buffer set (eBioscience, UK), freshly prepared each time according to manufacturer's instructions):
  Foxp3 Fixation/permeabilization working solution: 1part concentrate, 3 parts diluents
  1x Foxp3 permeabilization buffer: 1 part permeabilization buffer, 9 parts filtered MilliQ H_2O

- Perm Buffer: 1 x PBS, 1% FBS, 0.1% sodium azide, 0.1% saponin

- Sort Buffer: 1x PBS, 2% FBS, 4µM EDTA

2.2.2 Antibodies

Table 2.2 contains a list of monoclonal antibodies (mAb) used in this thesis. Antibodies were diluted in FACS buffer at concentrations pre-determined by titration.

Monoclonal primary antibody (antihuman)	Conjugated fluorochrome	Species source	Clone	Isotype	Commercial source	Dilution
CD73	PE	mouse	AD2	IgG1,k chain	Becton Dickinson (BD)	1/10
CD73	BV421	mouse	AD2	IgG1,k chain	Biolegend	1/50
CD73	PE	mouse	AD2	IgG1,k chain	eBioscience	1/50
CD39	PeCy7	mouse	A1	IgG1	eBioscience	1/50
CD4	APC	mouse	OKT4	IgG2b	eBioscience	1/25
CD4	QDot605	mouse	S3.5	IgG2a	Invitrogen	1/200
CD4	B711	mouse	OKT4	IgG2b,k chain	Biolegend	1/50
CD3	FITC	mouse	UCHT1	IgG1,k chain	BD	1/25
CD3	V450	mouse	UCHT1	IgG1,k chain	BD	1/100
CD8	FITC	mouse	SK1	IgG1,k chain	BD	1/100
CD8	APC	mouse	SK1	IgG1,k chain	eBioscience	1/500
CD8	PE	mouse	DK25	IgG1,k chain	DAKO	1/100
CD25	Pac blue	mouse	BC96	IgG1,k chain	Biolegend	1/10
IFN-γ	Alexa488	mouse	4S.B3	IgG1,k chain	eBioscience	1/50
Perforin	FITC	mouse	dG9	IgG2b,k chain	Biolegend	1/50
CD26	APC	mouse	BA5b	IgG2a,k chain	Biolegend	1/50
Ki67	eFluor450	mouse	20Raj1	IgG1,k chain	eBioscience	1/50
CD45 RA	BV510	mouse	HI100	IgG2b,k chain	BD	1/200
CD45 RO	FITC	mouse	UCHL1	IgG2a	Biolegend	1/25
CD19	QDot655	mouse	SJ25-C1	IgG1	Invitrogen	1/300
CD19	FITC	mouse	HIB19	IgG1,k chain	BD	1/25
CD14	PerCP Cy5.5	mouse	61D3	IgG1,k chain	eBioscience	1/25

CD14	FITC	mouse	61D3	IG1,k chain	eBioscience	1/200
Foxp3	Alexa488	mouse	150D/E4	IgG1	eBioscience	1/20
Foxp3	APC	rat	PCH101	IgG2a,k chain	eBioscience	1/25
CD66B	FITC	mouse	G10F5	IgM, k chain	BD	1/25
CD66B	PE	mouse	G10F5	IgM, k chain	Biolegend	1/25

Table 2.2 Antibody reagents used in this thesis

2.2.3 Staining with Live/dead exclusion dye

A live/dead discriminant dye was used for flow cytometry staining in accordance with the manufacturer's instructions (Invitrogen, UK). Cells were washed twice with 200µl of PBS, centrifuged at 300g for 3min, and then flicked to remove supernatant. The LIVE/DEAD Fixable Blue dead cell stain kit was used with the dye diluted at 1/250 in PBS, with 40µl of this solution added per well and cells stained in the dark at 4°C for 25min. After staining, cells were washed with 160µl of PBS, centrifuged, and set for surface stain. The dye used was visualised through the DAPI channel.

2.2.4 Staining for surface markers

After treatment such as sorting, live/dead staining (section 2.2.3) or cell culture (section 2.10), cells were resuspended in cold FACS buffer in round-bottom 96 well plates (Greiner Bio-one) at a concentration of 1-2 x 10^6 cells per ml according to the experiment. Typically, 100,000 cells were stained per Ab combination. After centrifugation at 300g for 3min at 4°C, the plates were flicked and cell pellet resuspended in 25µl of FACS buffer with the required antibodies added at the concentration specified in Table 2.2. Cells were incubated in the antibody mixes at 4°C for 30min in the dark. After incubation, cells were washed with 100µl FACS buffer and centrifuged as above. The pellet was either resuspended in 250µl of FACS buffer, to be run on a flow cytometer (LSR II, BD) for acquisition after transfer in FACS tubes, or underwent fixation/permeabilization for staining of intracellular markers as described below.

2.2.5 Staining for intracellular markers or intra-nuclear transcription factors

Foxp3, intracellular CD73 and Ki67 staining was performed using the eBioscience 'Foxp3 staining buffer set' with specific buffers prepared freshly, prior to staining, according to manufacturer's instructions. After staining of surface markers, cells were resuspended in 100µl of fixation/permeabilization working solution and incubated for 45min at 4°C in the dark. Cells were then washed and resuspended in 25µl of 1x permeabilization buffer either alone or with the antibody at the appropriate dilution. Incubation then occurred with the intracellular staining antibodies at 4°C in the dark for 45min. After staining, cells were washed twice with 1x permeabilization buffer, then once in FACS buffer and resuspended in 250µl FACS buffer in FACS tubes in order to be analysed by flow cytometry on the LSR II (BD).

2.2.6 Preparation of cells for cytokine production assessment

To detect intracellular cytokine production by PBMC and SFMC, cells were incubated in RPMI-PSG with 10% FBS and stimulated with 0.05µg/ml Phorbol 12-myristate 13- acetate (PMA), 0.5 µg/ml Ionomycin in the presence of 5µg/ml Brefeldin A (all from Sigma). Pre-treatment was added to cells plated at 2M/ml in a 96well plate and incubated for 3h at 37°C, 5% CO_2. After the stimulation procedure, cells were washed with plain PBS and stained with live/dead dye and surface antibodies, before fixation in 100µl of 4% paraformaldehyde for 10min in the dark at room temperature (RT). Cells were washed in FACS buffer and PERM buffer and resuspended in 25µl PERM buffer containing the appropriate intracellular antibodies for 30min at 4°C in the dark. Staining was followed by a further wash in PERM and FACS buffer, with cells resuspended in the end in 250µl FACS buffer in FACS tubes, before being run on the LSRII. Perforin expression was detected using the same fix/perm protocol used for cytokines, without the PMA/Ionomycin stimulation step.

2.2.7 Preparation of whole blood and synovial fluid for surface staining

In order to have a broader investigation of CD73 expression on all leukocytes, some peripheral blood and synovial fluid samples underwent FACS staining in whole blood, after treatment with lysis reagent, to remove erythrocytes. Peripheral blood was drawn from control subjects and 100µl was added to each polystyrene tube to which the required antibodies were added and incubated at 4°C for 30min. Lysis buffer, containing ammonium chloride (BD, UK), was diluted 1:10 with MilliQ water, 2ml were added per tube, before gently vortexing. Synovial fluid was similarly treated without the lysis step. Tubes were left to stand at RT for a further 20min. Tubes were centrifuged at 300g for 5min in 2ml of FACS

buffer twice. Cell pellets were resuspended in 250µl of FACS buffer before analysis by flow cytometry.

2.2.8 CFSE labelling

Carboxyfluorescein diacetate succinimidyl ester (CFSE) labelling was used to analyse the proliferation of PBMC in culture. CFSE (Sigma, UK) was made in DMSO at 5mM, aliquoted and stored at -20°C. Cells were first washed in PBS and then stained in 1ml of a 1µM solution of CFSE in PBS for 10min at RT. The same volume of FBS was then added to cells and these were incubated at RT for a further 10min. Cells were then washed 3 times in RPMI-PSG with 10% FBS, counted and plated out.

2.2.9 Data acquisition and analysis

Flow cytometry data were acquired on a FACScan (BD, UK) or a LSR II FACS machine (BD, UK) using CellQuest Pro software version 5.2 (BD, UK). Data were saved uncompensated and cells were gated by their light scatter properties. For PBMC/SFMC, typically $30\text{-}50 \times 10^4$ events were acquired per sample. For experiments using whole blood, 50×10^4 events were acquired on the LSR II, with higher voltages for FSC (forward) and SSC (side) scatter, adjusted for the bigger cell size as compared to PBMC. Flow cytometric analysis and compensation was performed using Flowjo software (TreeStar, USA).

Flow cytometry gating strategy
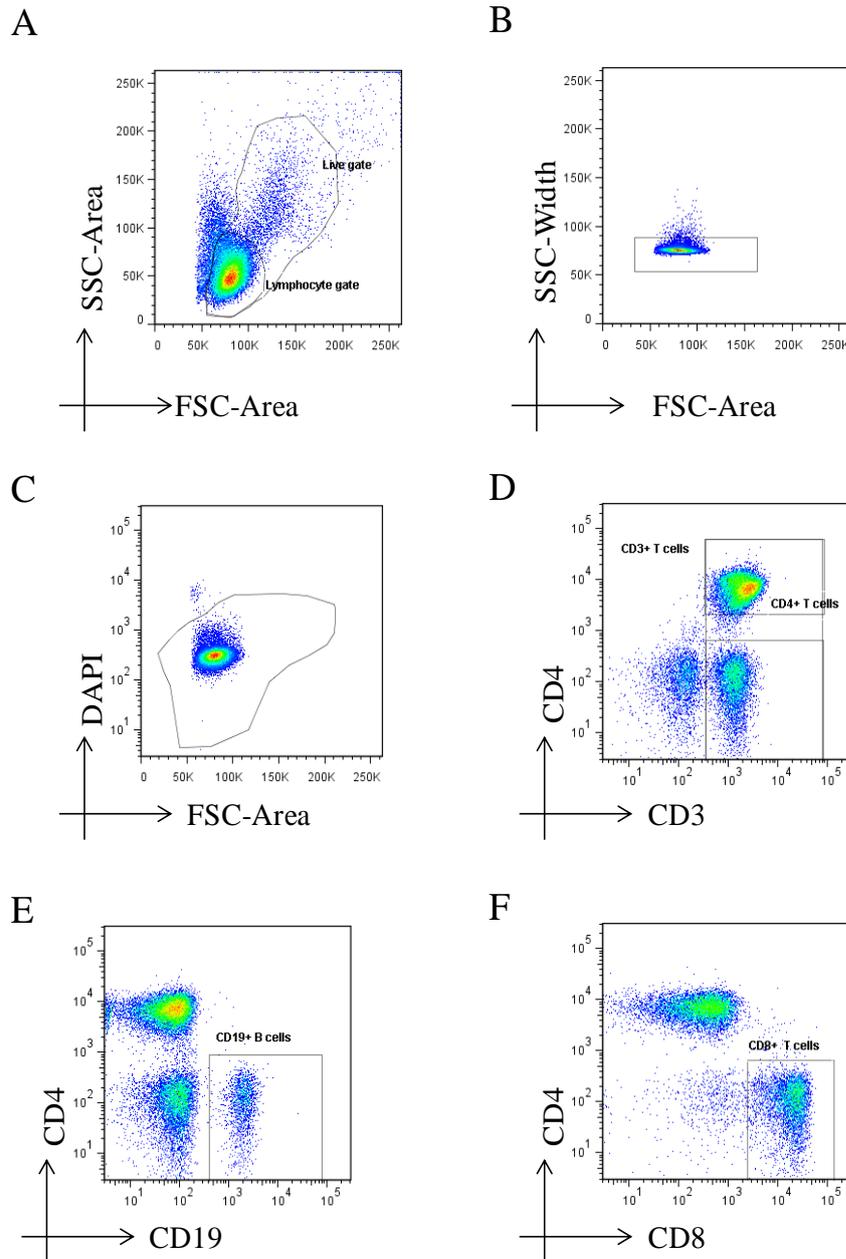
Figure 2-1 Flow cytometry gating strategy.

A) Forward (size) and side (granularity) scatter plot showing lymphocyte and live gate of healthy PBMC. B) To exclude doublets, forward scatter of lymphocytes was plotted against the side scatter width to discriminate those doublet cells for which there were disproportions between cell size and cell signal. C) Exclusion gate for those cells that stained positive for the Live/dead exclusion dye visualised through the DAPI channel, from lymphocyte gate. D) Characteristic distribution of CD3 and CD4 proteins on T lymphocytes, from lymphocyte gate. E) Lymphocyte gate showing strategy used to identify B cells. F) CD3 T cell gate showing strategy used to identify CD8^{+} T cells.

2.3 Cell sorting

2.3.1 Magnetic bead cell sorting- CD8$^+$ T cell enrichment

The human CD8$^+$ enrichment kit version 4.01 (EasySep, StemCell Technologies) was used according to the manufacturer's instructions. 10-30x10^6 cells were centrifuged to form a cell pellet and all culture medium was removed. Cells were then resuspended in sort buffer (amount adjusted to cell number used) composed of PBS, 2% FBS and 4mM EDTA (Sigma) and transferred to a 5ml polystyrene FACS tube (BD). 5-15μl of enrichment cocktail was added to the cell preparation and left to incubate for 10min at RT. 15-45μl of mixed magnetic particles were added and set aside for 5min at RT. The cell preparation volume was brought up to 2.5ml with sort buffer, the tube inserted into a magnet for 5min, after which negatively selected cells were poured into a new 15ml falcon, counted, and checked for purity by flow cytometry (section 2.2).

2.3.2 Cell sorting by flow cytometry

10-20x10^6 cells were thawed, spun, supernatant removed, resuspended in sort buffer (PBS, 2% FBS, 4mM EDTA) and stained for surface markers (section 2.2.3) for 30min at 4°C. Cells were spun down, resuspended in 1-1.5ml of sort buffer and passed through a 50μm filter (Partec, UK), to which 2μl of DAPI for dead exclusion was added, just prior to acquisition on the sorter. Either the MoFlo XDP cell sorter (Beckman coulter, UK) or the BD FACSAria III (BD Biosciences, UK) were used for cell sorting into 15ml falcons with collection medium (RPMI-PSG with 20% FBS). Sorted samples were each checked for purity, counted, and resuspended at the required concentration for cell culture in previously coated plates (section 2.10).

2.4 Western blotting (SDS-PAGE)

Cell lysates were prepared by lysing 1-2x10^6 total PBMC or CD8$^+$ enriched pelleted T cells (purified by bead sorting, see previous section 2.3) with 100-200μl total lysis buffer (2% 2-mercaptoethanol, 2% sodium dodecyl sulfate (SDS), 10mM Tris pH 7.5). The suspension was then centrifuged at 10000 rpm for 10min. The supernatant was then transferred to an eppendorf and stored at -80°C until use.

Thawed lysates were homogenised with Laemmli buffer (6x) (SDS, glycerol, Tris 0.5M pH 6.8, H$_2$O, Dithiothreitol (DTT), bromophenol blue, and 10% 2-mercaptoethanol) and heated

on a heat block at 95°C for 3min to ensure protein denaturation. Protein concentration was determined by addition of Bradford reagent (Sigma, UK) by using Bovine Serum Albumin (BSA) as a standard.

The gel cassette was assembled and filled with the resolving gel (12%), composed of acrylamide 30% solution (Sigma, USA), resolving buffer [H_2O, 30% acrylamide mix, 1.5M Tris pH 8.8, 10% SDS, 10% ammonium persulfate, TEMED], glycerol (Sigma, USA), ammonium persulfate (APS) (Fisher Scientific, USA), tetramethylethylenediamine (TEMED) (Sigma, USA), and H_2O. The stacking gel (4%) was then poured gently onto the resolving gel. The stacking gel was composed of acrylamide 30% solution, stacking buffer [H_2O, 30% acrylamide mix, 1M Tris pH 6.8, 10% SDS, 10% ammonium persulfate, TEMED], APS, TEMED, and H_2O. A 1mm comb was inserted and the gel left to set at RT for 40min.

After setting, the comb was removed, the electrode assembly assembled and the inner and outer buffer chambers filled with running buffer with a final concentration of 25mM Trizma base, 192mM Glycine and 0.1% SDS. Equal amounts of protein were loaded on a 4-12% (stacking- resolving) gel, together with the SeeBlue2 Plus2 (Invitrogen, UK) reagent used as protein ladder and the gel run at 120V for 1hr.

The gel was transferred to the wet blotting cassettes according to manufacturer's instructions (Biorad), in direct contact with a nitrocellulose Hybond C extra (Amersham Biosciences, UK) membrane. The transfer was run under a current of 200mAmp for 70min with a cooling block in a tank with transfer buffer at a final concentration of 25mM, 192mM Glycine, and 5% methanol.

After transfer, the membrane was then placed for 1hr in blocking solution (5% milk PBS, 0.1% Tween 20) on a rocker. Blots were probed with rabbit anti-human CD73 antibody, clone D7F9A (Cell Signalling, USA) at 1:1000 or mouse anti-human A_2AR antibody, clone 7FG-G5-A2 (Abcam, UK) at 1:200, made up in blocking solution and incubated overnight at 4°C. The membrane was then washed 3 times in 0.1% PBS, Tween and incubated with HRP-conjugated anti-rabbit or anti-mouse Ab (Santa Cruz Biotechnology, USA) at 1:2000 or 1:1000 for CD73 and A_2AR respectively, in blocking solution for 1.5hr. The membrane was placed on film in cassette and developed in the dark room with the ECL Western blotting Detection reagent (Amersham Bioscience).

After stripping the membrane with 0.2M NaOH for 15min, a positive control β-actin was done by staining the membrane with mouse anti-human β-actin 1:1000 in 5% milk PBS/Tween for 1hr at RT, followed by a further stain with HRP-conjugated anti-mouse Ab 1:2000 for 45min at RT. Development with ECL was then repeated.

2.5 CD39 Genotyping (done in collaboration with Dr Simona Ursu, ICH)

DNA was extracted from PBMC by phenol-chloroform method (Sigma-Aldrich, Dorset, UK). The genomic fragment containing rs10748643 SNP was amplified by PCR using primers for CD39: forward 5'GGTCACATCTGACTACATTTAG and reverse 5'CAGAGATTCTTCCTGGCTCTC. PCR products were sequenced at the UCL Wolfson Institute for Biomedical Research (London, UK) using the BigDye 3.1 chemistry method (Applied Biosystems). The PCR reactions were cleaned up using in-house produced sephadex filtration plates and run on an Applied Biosystems 3130XL Genetic Analyser.

2.6 Candidate gene investigation (performed by Dr Joanna Cobb, University of Manchester).

Methodology used was similar to that in (Hinks et al. 2013; Cobb et al. 2014) and is described briefly below. All genotyping was performed using DNA extracted from PBMC from venous blood.

Candidate genes were selected based on the adenosinergic pathway of interest. Data were then obtained from two previously published studies, one a JIA susceptibility analysis using the Illumina Immunochip and the other an analysis of MTX response in JIA using the Illumina HumanOmniExpress-12v1 GWAS chip (Hinks et al. 2013; Cobb et al. 2014). SNP genotyping was performed on the Immunochip array designed for dense mapping of susceptibility loci for multiple autoimmune diseases, according to manufacturer's instructions (Hinks et al. 2013). As this chip did not cover all the candidate gene regions, some analysis was also done from data generated by genome wide genotyping on the Illumina HumanOmniExpress-12v1 GWAS chip. SNPs were analysed by logistic regression adjusted for the top 5 principal components, calculated after quality control to minimise population stratification. SNPs were excluded from the analysis if they had a minor allele frequency <0.05 (GWAS chip) or <0.01 (Immunochip) and failed the Hardy-Weinberg equilibrium test (p≤ 0.001). Genotype frequencies were compared using the logistic regression in Plink.

Genome wide genotypes were available for a total of 1469 JIA cases, 374 CHARMS samples and 5195 WTCCC2 controls. Table 2.3 below illustrates the breakdown of JIA and CHARMS cases analysed by their subset.

	All JIA cases (n=1469)	CHARMS (n=374)			
	-	NR (117)	ACR30 (32)	ACR50 (54)	ACR70 (171)
Missing	85	9	4	5	17
Systemic	99	15	2	0	17
Persistent oligo	388	20	3	4	18
Extended oligo	229	21	6	16	34
RF-ve poly	384	31	11	22	62
RF+ve poly	81	4	0	2	11
ERA	88	7	6	4	4
Psoriactic	81	9	0	0	7
Undifferentiated	34	1	0	1	1

Table 2.3 Categories of JIA-CHARMS samples used in candidate gene investigation

ACR: American college of Rheumatology response level, where ACR30, ACR50, and ACR70 represent increasing levels of response to treatment after 6 months of methotrexate (MTX) treatment. NR: Non-responder, children who failed to reach even an ACR30 level of response.

2.7 High performance/pressure liquid chromatography (HPLC) for detection of etheno- and non-etheno Adenosine monophosphate breakdown products

2.7.1 Cell incubation with Etheno substrates

A total of 50×10^4 unsorted PBMC or SFMC, or $CD8^+$ bead sorted (see section 2.3) from PBMC or SFMC were plated in round-bottom 96 well plates (Greiner Bio-one, Germany) or 25µl of cell culture supernatant/ healthy blood plasma/ synovial fluid and resuspended in RPMI-PSG with 25µM of etheno-AMP (E-AMP) or etheno-ATP (E-ATP) substrate (Biolog, Germany) in the absence or presence of 10µM APCP (Adenosine 5′-(α,β-methylene)diphosphate), (Sigma-Aldrich, UK). Controls used were cells incubated in RPMI-PSG alone and RPMI-PSG in the absence of cells. Cells or fluids were incubated for different time points (0, 15, 30, 45, 60min) at 37°C in a humidified 5% CO_2 atmosphere (Galaxy S+, Biotech, UK).

2.7.2 Preparation of samples for HPLC detection

After incubation, the reaction was stopped by addition of 35μl of 15mM HCl (Sigma Aldrich, UK); samples were then transferred to 0.5ml eppendorfs and centrifuged at 12,000rcf at 4°C for 0.5min. The resultant supernatant was transferred to glass tubes containing 50μl of 0.2M KH_2PO_4 buffer (VWR Prolabo, UK) and frozen at -80°C until analysis by HPLC assay.

2.7.3 Preparation of buffer for HPLC and derivatization procedure for standards

The buffer used as eluent to run samples was a 0.2M KH_2PO_4 solution with pH of 5, prepared by dilution of KH_2PO_4 in MilliQ water. All adenine derivatives (ATP, ADP, AMP, adenosine, adenine), (Sigma Aldrich, UK) were diluted in MilliQ water at 1mM solution and stored at -20°C. In order for fluorescence detection to be carried out, these standards were derivatized to etheno forms. The use of etheno derivatives compared to the non-derivatized reagents monitored by UV absorbance, allows for a clearer separation of compounds peaks (Sonoki et al. 1989) and higher sensitivity and specificity.

For derivatization of samples, according to the method previously described by (Larson et al. 2001) 100μl of standard mix composed of 20μl of each adenine derivative, was reacted with 100μl of CAA reagent (0.5M chloroacetaldehyde, 0.15M citrate/citric acid pH 4.0) and heated to 80°C for 20 min. The samples were removed from the heater and placed in the autosampler tray of the HPLC to cool, prior to injection.

For the measurement of adenosine deaminase (ADA) activity, which metabolises adenosine to inosine, UV detection was used, since due to the lack of an adenine ring, inosine cannot be converted to an etheno form. A variety of buffers at different gradients was first tested to run the non-etheno ATP derivatives. These buffers included: 0.2M KH_2PO_4 with acetonitrile or 26mM KH_2PO_4 and 39mM K_2HPO_4 and 4mM of the ion pairing reagent tetrabutylammonium hydrogen sulphate (TBAHS), with 26mM KH_2PO_4 and 39mM K_2HPO_4 or 0.2M KH_2PO_4 with 5mM of the ion pairing reagent tetrabutylammonium bromide (TBAB) run with an acetonitrile gradient.

2.7.4 HPLC apparatus and columns

The HPLC apparatus was a JASCO AS-1555 autosampler connected to two JASCO PU-1580 pumps set up in binary fashion, to allow mixture of the two eluting solvents. Chromatographic peaks were recorded via a JASCO MD-1510 multi-wavelength detector, while fluorescent spectra were detected by a JASCO FP-1520 fluorescence detector. Columns

used were 15cm x 4.6mm 3micron ODS Hypersil from Thermo Scientific. Peak areas, used as a measurement of fluorescence intensities, were integrated via EZChrom Elite Chromatography software and chemical nature of the separated compound was determined by comparison with retention time of standard etheno derivatives.

2.7.5 Chromatographic conditions

Separations occurred via reverse-phase liquid chromatography with a constant flow rate of 0.8ml/min and injection volume of 40μl. The eluting solvents were: A) 0.2M phosphate buffer and B) acetonitrile (HPLC gradient grade, Fisher, UK) mobile phase. The following gradient was used: 0-31min 99.5%-0.5% B, 31-36min 88%-12% B, 36-37min 80%-20% B, after 37min until 45min return to initial 99.5%-0.5% B. The system was controlled to change solvent used gradually as defined by a linear gradient. UV spectra were collected from 200-400nm, while the fluorescence detection excitation wavelength was set to 290nm, the emission was set to 415nm and gain of 1x.

2.8 CD73 enzyme-linked immunosorbent assay (ELISA)

For the quantitative determination of CD73 concentrations in cell cultures supernatants and plasma, a commercial CD73 ELISA assay (Cusabio, UK) was used. The assay was run according to manufacturer's instructions, with the following method: after preparation of standards and samples, 100μl of either sample or standard was added per well and incubated for 2 hours at 37°C. Following this, 100μl of biotinylated-anti CD73 antibody was added to each well and incubated at 37°C for another hour. The plate was then washed several times with the wash buffer provided and thoroughly blotted. Horseradish peroxidise (HRP)-avidin (100μl) was then added to the plate and after incubation for an hour at 37°C, washed and blotted again. The subsequent incubation was with 90μl of tetramethylbenzide (TMB)-substrate, for 30min at 37°C, after which 50μl stop solution was added prior to reading the plate.

The optical density in each sample was determined using a microplate reader (Thermo Multiscan-ex, MA, USA) reading at 450nm after gently shaking the plate. Duplicate reading values for each standard, control, and samples were averaged and sample background control density subtracted from each sample reading. A standard curve was generated using a four-parameter logistic curve fit. All supernatants and samples were tested in duplicate.

2.9 Alkaline phosphatase (AP) assay

For the measurement of the activity of the enzyme AP in plasma and synovial fluid, the alkaline phosphatase assay kit (colorimetric) (Abcam, UK), was used according to the manufacturer's instructions. The kit measures the amount of dephosphorylation of the p-nitrophenyl phosphate (pNPP) as a substrate and the method utilized is described here briefly. Samples were diluted 1:10 and 1:100 with assay buffer, to these 50µl of 5mM pNPP was added and incubated for 60min at 25°C, protected from light. A standard curve was prepared by a 1:2 serial dilution of the 5mM pNPP solution with the assay buffer, to which 10µl of AP enzyme solution was added and left to incubate for 60min at 25°C, protected from light. All reactions were stopped by addition of 20µl of stop solution to both standards and samples reactions. The optical density was determined using a microplate reader (Thermo Multiscan-ex, MA, USA) reading at 450nm after gently shaking the plate. The duplicate readings for each standard, control, and samples were averaged and sample background control density subtracted from each sample reading. A standard curve was generated using Prism, which allowed estimation of concentration of pNPP in the sample. Final AP activity was calculated with the following formula: AP activity (U/ml): A x V x T with A being amount of pNPP dephosphorylated (turns yellow), V volume of sample added and T reaction time.

2.10 Cell culture

Cells were counted and resuspended at 1-2 $x10^6$ cells per ml in culture medium supplemented with 10% FBS and placed in an incubator at 37°C in a humidified 5% CO_2 atmosphere (Galaxy S$^+$, Biotech) for the time indicated. Cells were harvested at day 1, 3, and 5 of culture and counted for cell viability (section 2.1.4) and stained with antibodies for flow cytometry analysis.

2.10.1 Preparation of plate for T cell stimulation-B cell stimulation protocol

96 well cell culture round bottom plates from Costar (Corning) were coated with anti-CD3 mAb, clone UCHT1 (stock 0.5mg/m, R&D, UK) at a final concentration of 1µg/ml and anti-CD28 mAb clone CD28.2 (stock 0.5mg/ml, BD pharmingen, UK) at a final concentration of 5µg/ml in sterile Dulbecco's Phosphate Buffered Saline, (DPBS, Life Technologies, UK). Each well contained 60µl of this solution. The coated plate was then placed in an incubator at

37°C for 3hrs, after which the solution was removed and wells washed with PBS, before the addition of cells.

For B cell stimulation, soluble type B oligonucleotide CpG 2006 (Invivogen, USA) was added to the culture medium with cells at a final concentration of 1μM. Another method of B cell stimulation tested was coculture of PBMC with Chinese Hamster Ovary (CHO) cells that had been transfected with CD40Ligand (CD40L) or with control un-transfected CHO cells (kindly provided by Dr K.Nistala, University College London). For coculture experiments, CHO cells were initially left to expand for 3-4 days in culture, until they reached confluency of around 90%. CHO cells were irradiated for 38.5min at 70Gγ in 25cm^2 cell culture flasks, after having been treated with 1.5ml of prewarmed trypsin/EDTA solution for 3min at 37°C to detach cells. CHO cells were cultured with human PBMC at a ratio of 1:10 for 72hr at 37°C, both at 1M/ml. Another B cell stimulant tested was the Affinipure F(ab')$_2$ fragment of antibody directed against IgG and IgM surface immunoglobulin on the B cell from Jackson immunoresearch (USA) (kind gift of Prof. C. Mauri, University College London).This was added to cell culture at a final concentration of 10μg/ml.

Cell culture supernatants were processed, stored at -20°C and analysed either by HPLC (section 2.7) or ELISA (section 2.8).

2.10.2 Addition of recombinant cytokines and human plasma for cell culture

For analysis of cytokine effect on ectoenzyme expression, PBMC were cultured in the presence or absence of cytokines in 96 well plates, which had been previously coated with antibodies or had been left untreated. Recombinant cytokines tested were: TNF-α (BD pharmingen, UK), IL-1 (R&D, UK), IL-6 (BD Pharmingen, UK), TGF-β (R&D, UK), IFN-γ (Peprotech, UK) and IL-2 (Roche, USA). Cytokines were added either alone or in combinations as detailed in results sections, at final concentrations of: 10ng/ml for TNF-α, IL-1, IL-6 and IFN-γ, 2ng/ml for TGF-β and 100U/ml for IL-2.

Addition of human healthy plasma (VBP) or synovial fluid (SF) was tested on cell culture. Aliquots used were made from a pool of 5 healthy individual's plasma or 10 synovial fluid samples as described in section 2.1.7. These were added to the wells with cells which were cultured at either 10% plasma/SF in culture medium with no additional FBS, or at 5% of plasma/SF in culture medium with 5% FBS.

MTX polyglutamates (MTXGlu$_{3,4,5}$) (Schirck, Switzerland) were added to cell culture medium at final concentrations of 50nM for MTXGlu$_3$, 20nM for MTXGlu$_4$ and 10nM for MTXGlu$_5$.

2.11 Statistical analysis

For statistical comparison of multiple groups one-way ANOVA or in the case of nonparametric data, Kruskall-Wallis test with Dunn's multiple comparison post-tests was used. To analyse paired patient samples, paired T test or Wilcoxon matched pairs test was used. All data are expressed as median with interquartile range with stated p value, unless otherwise indicated. Statistical analysis was performed using GraphPad Prism version 5.03 (GraphPad software).

Chapter 3 Expression of CD73 and other ectonucleotidases on leukocytes from the inflamed site of human inflammatory arthritis and peripheral blood cells

3.1 Introduction

The biosynthesis and catabolism of the anti-inflammatory nucleoside adenosine, responsible for dampening of many immune cell responses, is regulated by a series of ectonucleotidases. These include CD39, which dephosphorylates pro-inflammatory ATP to AMP and CD73, which generates adenosine from AMP. Additionally, ADA (adenosine deaminase) terminates the effects of adenosine by converting it to inosine (Junger 2011). Extracellular release of ATP occurs by cell necrosis in addition to non-lytic mechanisms such as egress through connexin-43 gap junctions (Eltzschig et al. 2006a) and pannexin-1 hemichannels (Schenk et al. 2008) in response to inflammation, tissue damage and cell activation.

ATP receptors were first described by Burnstock, who referred to them as P2 receptors (Burnstock et al. 1978). These were divided into P2X receptors (ligand-gated ion channels) and P2Y receptors (G-protein coupled receptors) based on their chemical properties and structure. ATP binding to its P2 class of metabotropic and ionotropic receptors orchestrates the triggering of a series of pro-inflammatory effects, such as T cell proliferation and caspase-dependent secretion of IL-1 (Mariathasan et al. 2006; Schenk et al. 2008).

The effects of ATP are terminated by its phosphohydrolysis to AMP, mediated by the action of the ectonucleoside triphosphate diphosphohydrolase CD39. This ectoenzyme is also a lymphoid activation marker (Maliszewski et al. 1994) and has been previously found to be elevated on synovial T lymphocytes from JIA patients resulting in increased ATPase activity of these cells (Moncrieffe et al. 2010b). Dephosphorylation of AMP to adenosine occurs mainly by the action of the ecto-5'nucleotidase CD73 (Thompson et al. 1989), considered to be a critical regulator of the availability of extracellular adenosine. The nucleoside adenosine exerts its immunosuppressive effects by binding to its P1 class of transmembrane G-protein coupled receptors widely expressed on both immune and non-immune cells. Of the adenosine receptors A_1, A_{2A}, A_{2B}, and A_3 (previously described in Chapter 1), A_{2A} is the one most associated with the immunosuppressive function of adenosine.

Synthesis of adenosine during inflammation helps to alleviate the detrimental effects that arise after ATP release and for this reason, the production of adenosine by murine Treg via the coordinated action of CD39 and CD73 is considered to be one of their mechanisms of immunoregulation (Kobie et al. 2006; Deaglio et al. 2007). Adenosine is inactivated by the enzyme adenosine deaminase (ADA), which deaminates it to inosine, a purine capable of

inhibiting inflammatory cytokine production (Hasko et al. 2000). ADA is a cytosolic protein, bound to the cell surface after interaction with anchoring proteins, of which the cell membrane protein CD26 (also known as Dipeptidyl-peptidase 4) was the first to be identified (Kameoka et al. 1993).

As discussed in Chapter 1 and depicted in Figure 1.2, CD39, CD73 and ADA/CD26 are the key enzymes involved in the balance of the extracellular levels of available pro-inflammatory ATP and immunoregulatory adenosine. In order to assess the roles of these enzymes in childhood inflammatory arthritis, their expression on cells from the JIA joint was first analysed.

The aims of this chapter were to:

1) Characterise the protein expression of ectoenzymes involved in the adenosinergic pathway on leukocytes in inflammatory and non-inflammatory conditions;

2) Investigate a possible relationship between frequency of synovial T and B cells expressing CD73 and clinical subtype of oligoarticular JIA;

3) Understand the relationship between CD73 expression and effector cell markers;

4) Analyse the potential of human regulatory T cells to produce adenosine.

3.2 Results

3.2.1 CD73 protein is expressed by lymphocytes, but not by monocytes or granulocytes

The expression of CD73 on different leukocyte populations within peripheral blood was investigated by multi-colour flow cytometry. PBMC from healthy adult donors were stained, gated, and analysed as described in methods section 2.2.4 and Figure 2.1. Figure 3.1A shows that CD73 is expressed at the cell surface of both T and B-lymphocytes from healthy peripheral blood. $CD8^+$ T cells and $CD19^+$ B cells were consistently the lymphocyte cell populations with the highest percentage of CD73 positive cells with median and interquartile range (IQR) of 51% (IQR 47-59%) and 68% (IQR 62-76%) respectively. The relative proportion of $CD4^+$ $CD73^+$ cells was much smaller than that of $CD8^+$ $CD73^+$ with a median and IQR of 13% (8-15%) of $CD4^+$ T cells expressing CD73 (Figure 3.1A and C).

In contrast to T and B cells, monocytes from peripheral blood, as defined by surface CD14 expression, were found to be negative for CD73 expression (Figure 3.1B and C). Summary data for CD73 positive cells within the CD3, CD4 and CD8 T cell and CD19 B cell populations of 39 healthy individuals and of 4 healthy individuals for the CD14 population, are shown in Figure 3.1C.
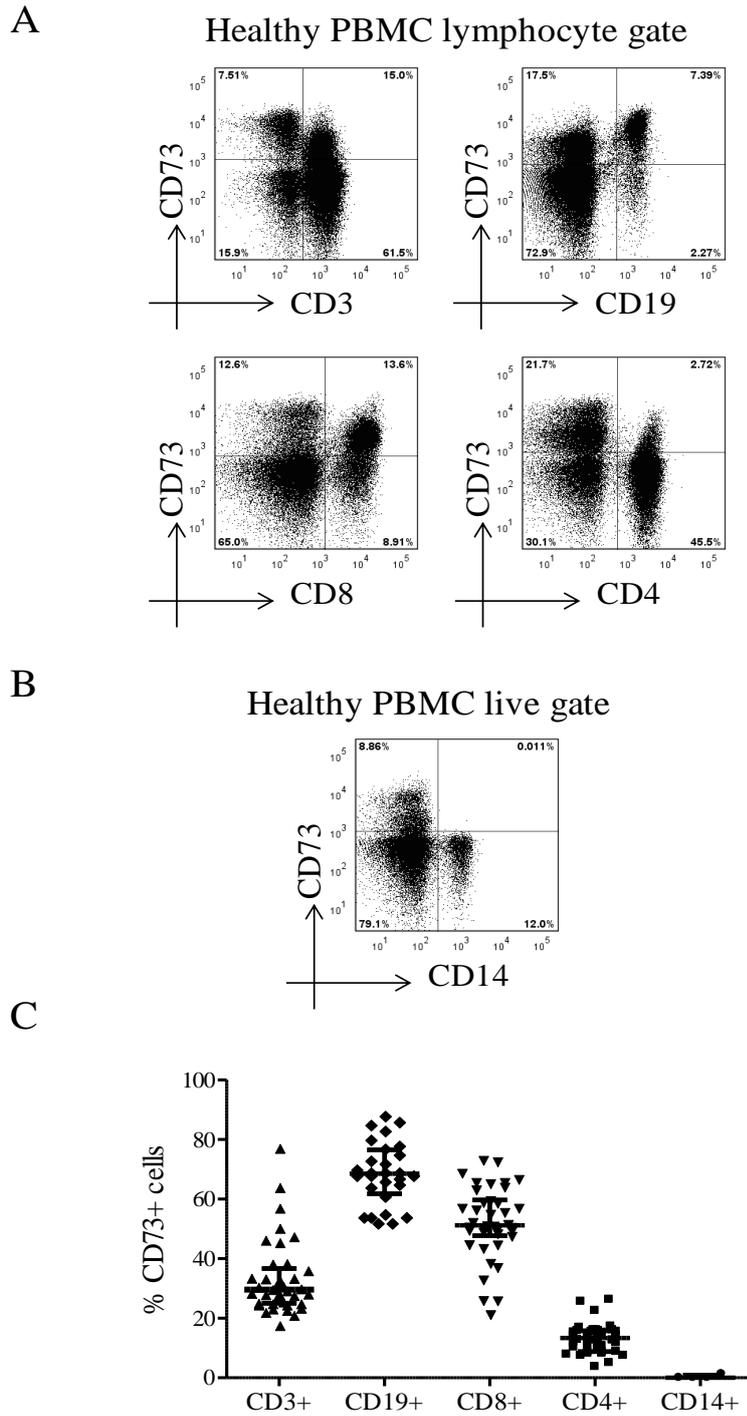
Figure 3-1 CD73$^+$ cells within populations of healthy PBMC

Flow cytometry dot plots of healthy PBMC gated on lymphocytes showing CD73 expression against CD3, CD19, CD8 and CD4 shown (A) and on live mononuclear cells showing lack of CD73 expression on CD14$^+$ monocytes (B). Summary data showing variability, between healthy individuals, in the percentage of CD3, CD4 and CD8 T cells and CD19 B cells (n=39) and monocytes (n=4) expressing CD73. Bars represent median with interquartile range.

In order to investigate CD73 expression on all immune cells and as granulocytes are lost during the preparation of mononuclear cells, CD73 expression was also tested in healthy adult whole blood. This was done after lysis of erythrocytes and data were compared to that obtained from freshly isolated JIA synovial fluid samples. Granulocytes, defined by the expression of CD66B, were analysed for CD73 expression on whole blood and whole synovial fluid cells. In parallel, to verify that lack of CD73 expression on monocytes within PBMC was not due to cell processing during the preparation of PBMC by density centrifugation, expression of CD73 was investigated on monocytes (CD14$^+$) within whole blood. As shown in Figure 3.2, granulocytes (CD66B$^+$) from both healthy blood and synovial fluid of patients with JIA lack CD73 expression. In addition the analysis of CD14$^+$ cells in whole blood and JIA synovial fluid, confirmed the lack of CD73$^+$ cells within the monocyte population (Figure 3.2, right hand panels). Interestingly, in Figure 3.2 a clear difference in the frequency of cells expressing CD73 was observed between healthy blood cells and synovial fluid cells in the CD14 and CD66B negative populations.
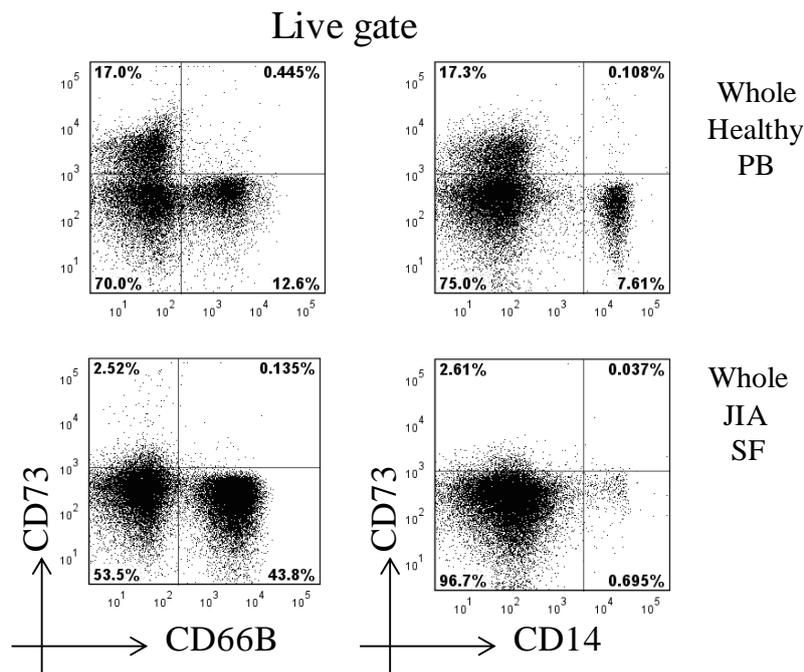
Figure 3-2 Lack of CD73 positive cells within the neutrophil and monocyte populations from whole healthy blood or synovial fluid of patients with JIA

Representative dot plots of flow cytometric analysis for CD73 expression on CD14± and CD66B± cells, gated on live cells, obtained from whole healthy peripheral blood [top] and whole JIA synovial fluid [bottom]. Proportions of cells are shown in quadrants.

3.2.2 Synovial T cells demonstrate a reduced frequency of CD73$^+$ cells and lower CD73 expression levels than T cells from peripheral blood

CD73 expression on T cells from peripheral blood of healthy adult and child controls was compared with expression on T cells from blood and synovial fluid of patients with JIA (Figure 3.3A-C). As shown in representative flow cytometry dot plots in Figure 3.3A, healthy child control CD3$^+$ T cells have a distinct population of CD73 positive cells, as is the case in JIA patient peripheral blood. However, the proportion of CD73 positive T cells was found to be much lower in JIA synovial fluid.

By analysing the CD4$^+$ and CD8$^+$ populations of T cells, it was clear that few CD4 T cells express CD73 in either healthy child blood or JIA patient blood (as also demonstrated in histograms, Figure 3.3B), consistent with the findings in adult healthy blood (Figure 3.1 and summary data in Figure 3.3C). This is also the case for synovial fluid (SF) CD4 T cells. In contrast, the majority of CD8 T cells in both healthy child and JIA patient blood are CD73 positive, whereas most CD8 T cells in synovial fluid are CD73 negative (Figure 3.3A).

Figure 3.3C shows summary data from patients and controls, consistent with the representative plots in Figure 3.3A and B. While there was no difference in CD73 expression on CD8$^+$ T cells from blood of healthy children, adults or patients with JIA [medians and IQR of 56% (34-69%), 51% (47-59%), 54% (49-59%) respectively] expression of CD73 on synovial CD8$^+$ T cells [median and IQR of 13% (8-19%)] was significantly reduced compared to both patient and control blood (Figure 3.3C). This decrease in CD73 expression in synovial fluid T cells was evident in both the proportion of CD8$^+$ T cells that were positive for CD73 and by protein expression on a cell basis as assessed by MFI (median fluorescent intensity) of CD73$^+$ cells (Figure 3.3C-D).

When paired samples of JIA PBMC-SFMC were analysed, the reduction of CD73 expression on synovial fluid CD8$^+$ T cells was highly significant ($p < 0.0001$) (Figure 3.3E). In contrast, when CD4$^+$ T cells of paired JIA patient blood and synovial fluid were compared for percentages of CD73 positive cells, there was only a slight decrease ($p=0.093$) for synovial CD4$^+$ T cells expressing CD73 compared to their blood counterparts (Figure 3.3F).
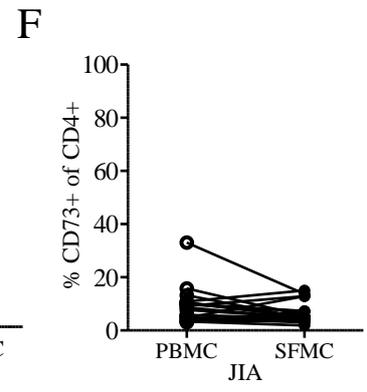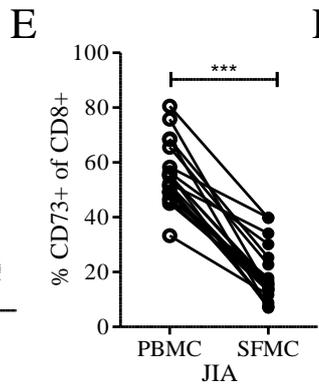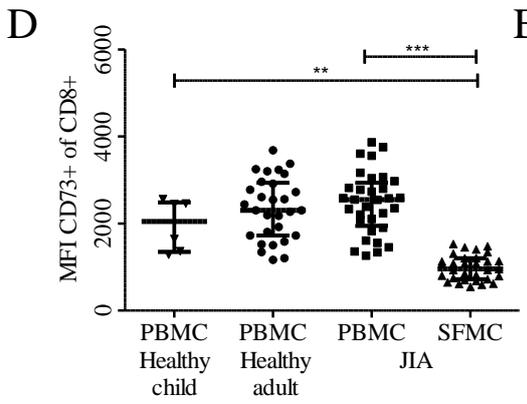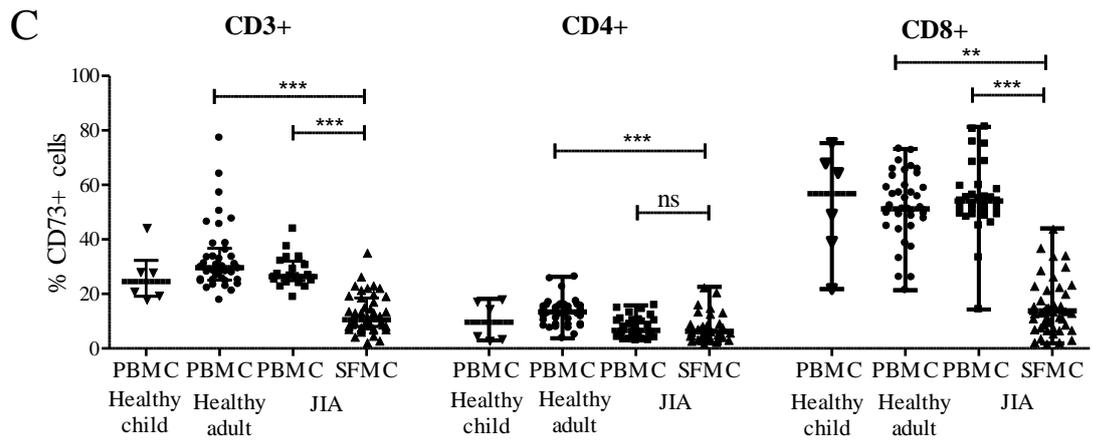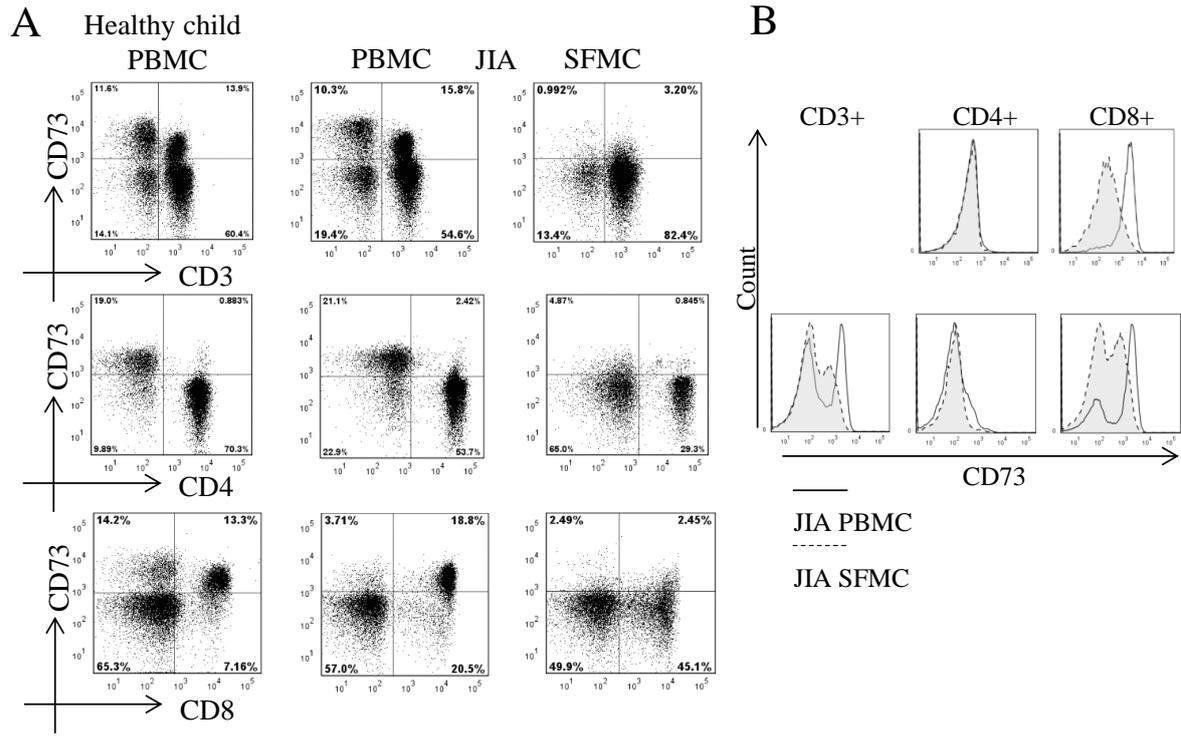
Figure 3-3 Decreased CD73 frequency and level on synovial T cells

A) Representative flow cytometry plots, set on lymphocyte gate, showing CD73 expression on $CD3^+$, $CD4^+$ and $CD8^+$ T cells from healthy child PBMC and paired JIA PBMC-SFMC samples. B) Histograms of CD73 expression on $CD3^+$, $CD4^+$ and $CD8^+$ T cells [left to right] of paired PBMC [open histogram] and SFMC samples from two JIA patients [dashed, shaded histogram] gated respectively on $CD3^+$, $CD4^+$ and $CD8^+$ T cells. C) Scatter plots illustrate percentage of CD73 positive cells within the $CD3^+$, $CD4^+$ and $CD8^+$ subsets in samples from healthy children (n=6), healthy adults (n=39) and PBMC (n=31) and SFMC (n=48) from children with JIA. D) MFI of CD73 expression of $CD8^+$ $CD73^+$ T lymphocytes from blood of healthy children (n=6), healthy adults (n=29) and PBMC (n=31) and SFMC (n=40) from children with JIA. E) Percentage of $CD73^+CD8^+$ cells of paired PBMC and SFMC samples (n=20) from children with JIA. F) Percentage of $CD73^+CD4^+$ cells of paired PBMC and SFMC samples (n=19) from children with JIA. Lines represent median values with interquartile range. Samples are from a combination of patients with extended and persistent oligoarthritis.

3.2.3 Reduced CD73 expression on synovial T cells is not due to intracellular retention of protein

To determine whether CD73 protein retention inside cells could explain the reduced CD73 expression observed on JIA SF T cells, CD73 protein expression at the cell surface and total CD73 protein levels were compared. In order to achieve this, samples were stained for CD73 before and after being fixed and permeabilized as described in methods section 2.2.5. When the CD73 antibody was added before the fixation/permeabilization step, protein expression was considered limited to the cell surface, whereas antibody added after fixation/permeabilization could enter the cell and bind simultaneously to surface and intracellular protein with expression identified as "total".

No significant difference between CD73 surface and total (surface plus intracellular) protein expression was observed on synovial fluid CD8$^+$ T cells, suggesting that protein retention inside the cell does not explain the reduced CD73 expression found on JIA SF T cells (Figure 3.4A-B). One representative example is shown in Figure 3.4A and summary data for n=11 samples are shown in Figure 3.4B.

To confirm the findings represented in Figure 3.4A-B, Western blots were performed to measure CD73 protein content in CD8$^+$ T cells. In contrast to healthy control or patient CD8$^+$ cells sorted from PBMC, CD8$^+$ bead-sorted SFMC had no detectable band at 70kDa, corresponding to the size of the CD73 protein monomer as shown in the representative example in Figure 3.4C. Therefore, it would appear that the Western blot assay is not able to detect low levels of CD73 protein within the conditions used.

A

JIA
SFMC

Surface

Total

CD73

CD8

B

% CD73+ of CD8+

ns

surface   total

total = surface + intracellular

C

Healthy
adult   JIA

kDa   PBMC   PBMC   SFMC

70

β-actin
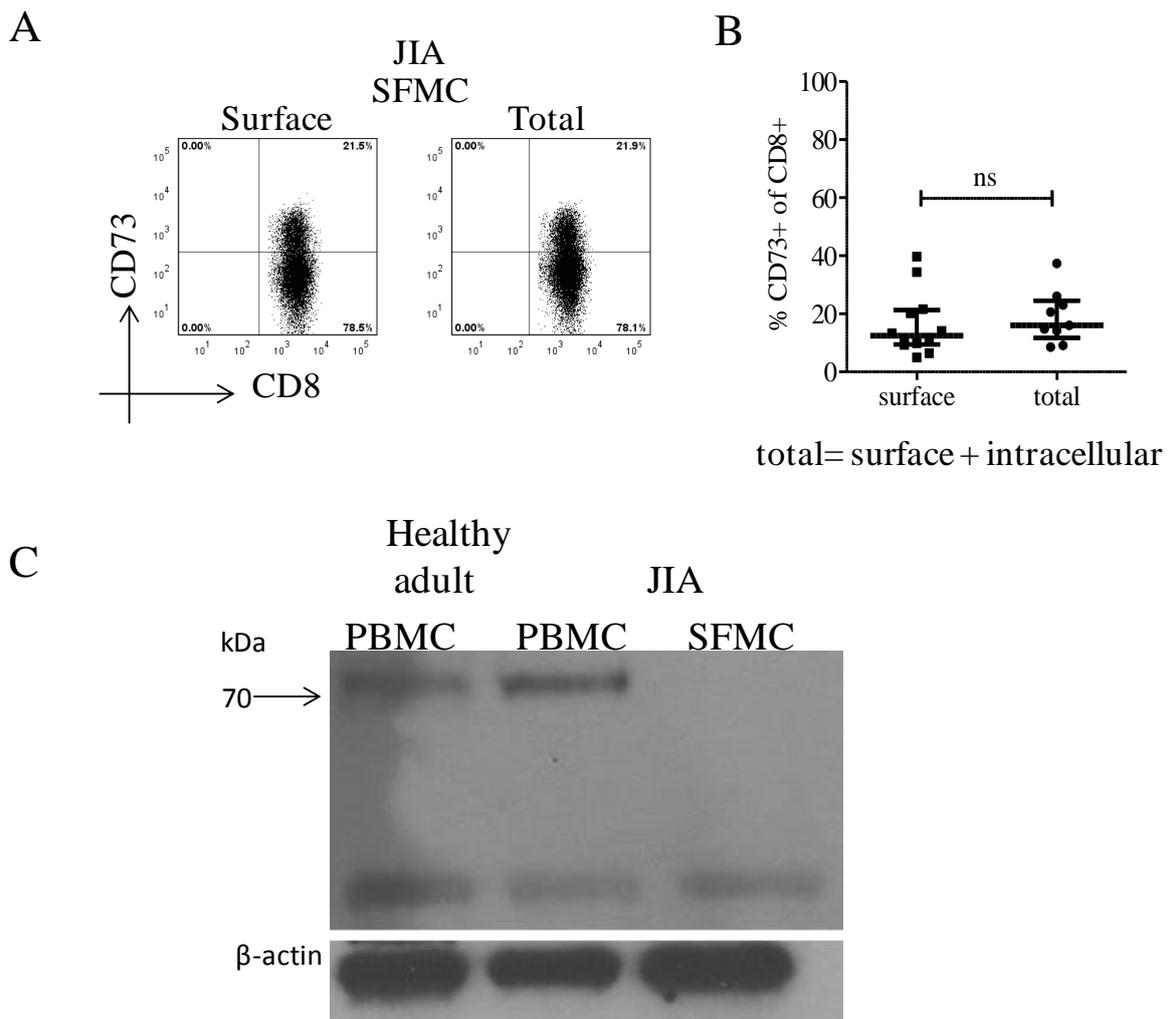
Figure 3-4 Comparison of levels of CD73 protein in CD8^{+} T cells from blood and synovial fluid by flow cytometry and Western blot analysis

Example of flow cytometry plots from one JIA patient (A) and summary plot for n=11 samples (B) of JIA SFMC gated on CD8^{+} T cells stained for CD73 either prior to, or after, fixation/permeabilization resulting in surface and total expression respectively. C) Western blot assay of CD73 protein in 1-2 x 10^6 CD8^{+} bead-sorted healthy adult PBMC and JIA patient PBMC and SFMC. β-actin was used as an internal control for protein loading.

3.2.4 Reduction in proportion and median fluorescence intensity of CD73^{+} synovial B lymphocytes

As previously shown in Figure 3.1C, the lymphocyte population with the highest proportion of CD73 positive cells was that of B cells. CD73 expression on B cells from synovial fluid of JIA patients was therefore analysed to determine whether there was any difference in CD73 expression on B cells from blood and synovial fluid similar to that shown for CD8^{+} T cells. These data are shown in Figure 3.5.

The percentage of CD73^{+} B cells and the MFI of CD73 protein on CD73^{+} B cells found in the synovial fluid was decreased compared to blood of JIA patients or healthy controls (Figure 3.5A-C). Thus, the median (IQR) proportion of CD73^{+} B cells within the B cell population was 60% (51-70%) for healthy child blood, 68% (62-76%) for healthy adult blood, 77% (72-83%) for JIA blood, and 49% (28-61%) for JIA synovial B cells respectively. The reduced percentage of synovial B lymphocytes expressing CD73 was particularly evident for CD19^{+} B cells from paired samples of patient blood and synovial fluid (p< 0.0001) (Figure 3.5D).

Of note, very low percentages of CD19^{+} B cells were found in the synovial fluid of JIA patients compared to peripheral blood of healthy controls and JIA patients (Figure 3.5E). This suggests that even if synovial B cells had expressed CD73 protein within the normal range, synovial B-lymphocytes may not have contributed significantly to adenosine production from AMP.
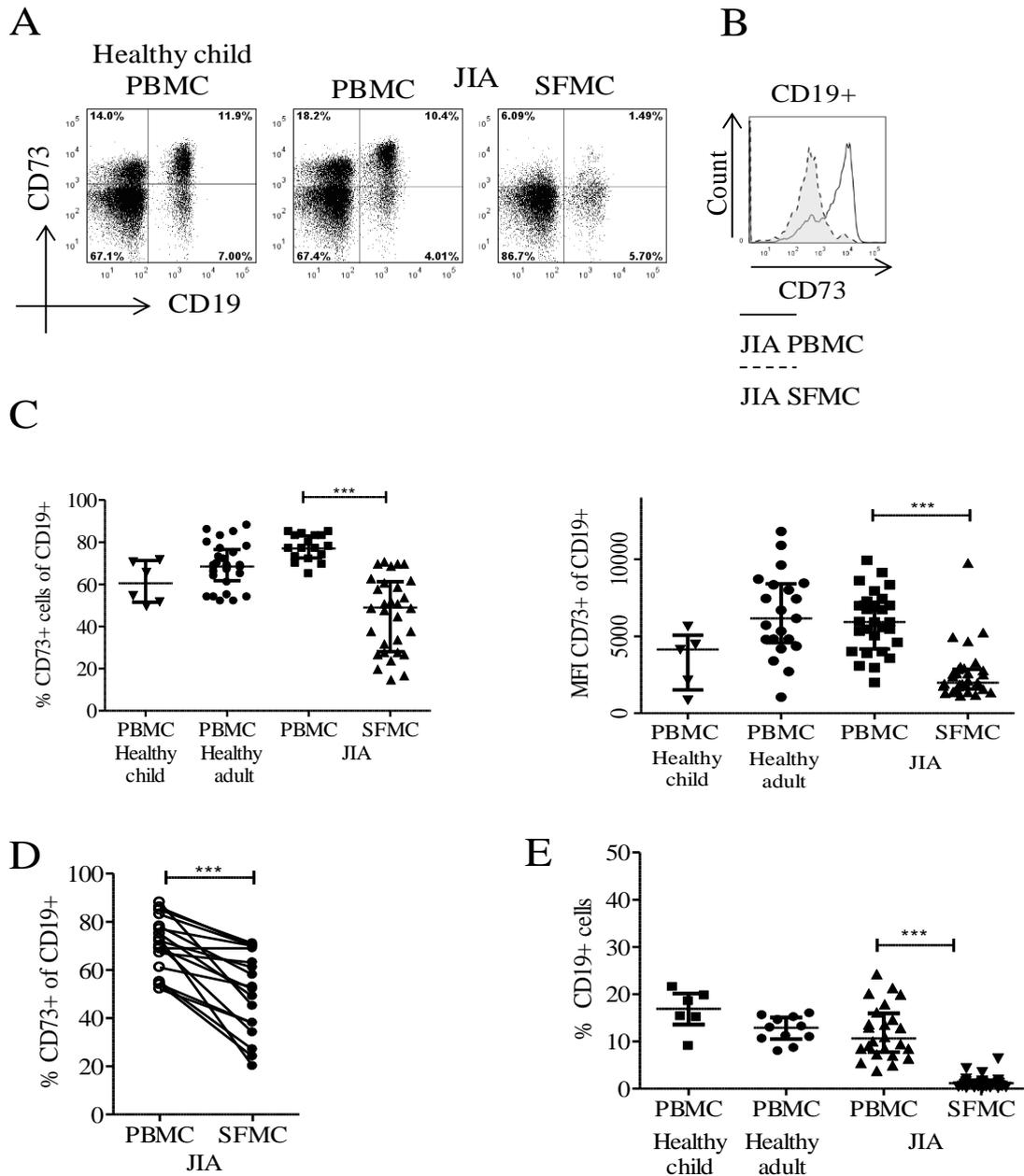
Figure 3-5 Reduced CD73 frequency and MFI of synovial B cells

A) Representative examples of CD73 expression on CD19^{+} B cells gated on lymphocytes from healthy child PBMC and paired PBMC-SFMC JIA samples. B) Histogram of CD73 expression on CD19^{+} B cells from paired JIA PBMC and SFMC samples gated on B cells. C) Scatter plot of percentage of CD73^{+} cells [left] and MFI [right] of PBMC CD19^{+} B cells from healthy children (n=6) and adults (n=17) and JIA PBMC (n=28) and SFMC (n=30). D) Percentage of CD73^{+}CD19^{+} cells of paired PBMC and SFMC samples (n=18). E) Frequency of CD19^{+} B cells in PBMC from healthy children (n=6), adults (n=11) and in JIA PBMC (n=24) and SFMC (n=30). Bars represent medians with IQR.

3.2.5 Analysis of CD73 expression on synovial CD8 T cells and B cells from patients with different severity of Oligoarticular JIA

In this study, a clear decrease has been demonstrated in the percentage of both T and B cells positive for CD73 in the joints of patients with oligoarticular JIA. In the analysis presented so far, samples were obtained from patients with persistent oligoarticular JIA (less than 5 joints affected) and the more severe extended oligoarticular JIA (5 or more joints affected). To determine whether the decrease in CD73 expression on synovial lymphocytes was related to disease severity, CD73 expression on $CD8^+$ T cells and $CD19^+$ B cells was compared between these two clinically distinct categories. A representative example of two of these samples, where CD73 expression has been analysed on CD8 cells, is shown in Figure 3.6A.

Although both clinical categories of patients had reduced CD73 expression on $CD8^+$ T cells in SFMC compared to blood, there was also a difference between the two clinical types at the inflamed site, with the more severe type showing a greater reduction in CD73 expression. Thus, a significantly higher proportion (p=0.0006) of $CD8^+$ T cells expressing CD73 was found in the inflamed joint of oligoarticular JIA patients whose disease had remained mild [median (IQR) 27% (14-37%)] compared to those whose disease had become more severe [median (IQR) 10% (2-14%)] (Figure 3.6B). A similar result was found for synovial B-lymphocytes (p= 0.0495), as shown in the scatter plot in Figure 3.6C. These results suggest that the decrease in CD73 expression is correlated with disease severity.
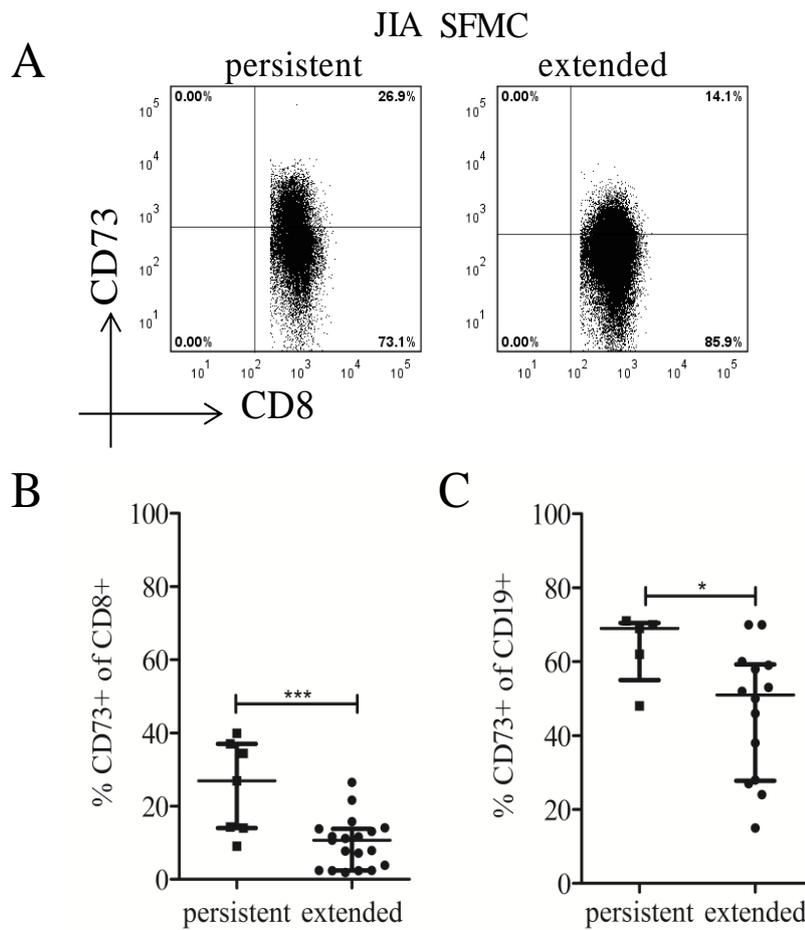
Figure 3-6 Lower CD73 expression on lymphocytes from extended oligoarticular JIA

A) Representative flow cytometry plots of CD73 expression on synovial CD8$^+$ lymphocytes from patients with persistent and extended oligoarticular JIA. B) Percentage of CD8$^+$ SFMC expressing CD73 from patients with the less severe persistent oligoarthritis (n=7) and from patients with extended oligoarthritis (n=19). C) Percentage of CD73$^+$CD19$^+$ SFMC from patients with the less severe persistent disease (n=5) compared to those with extended oligoarthritis (n=14). Bars represent medians with IQR.

3.2.6 Relationship between expression of CD73 on IFN-γ and perforin producing effector CD8 T cells

Upon antigen recognition, CD8$^+$ T lymphocytes can acquire effector functions mediated by release of cytokines (including IFN-γ) and perforin-dependent pathways. Since CD8$^+$ T lymphocytes have been found to express CD73 protein, which allows for breakdown of AMP into the immunosuppressive nucleoside adenosine, it was relevant to determine whether CD73 is expressed by activated CD8$^+$ T cells. To understand the relationship between the concurrent pro- and anti-inflammatory functions of CD8$^+$ lymphocytes, which may be important to disease pathogenesis in JIA, expression of IFN-γ, perforin and CD73 was analysed by flow cytometry (Figure 3.7).

IFN-γ production by blood CD8$^+$ T cells from both healthy controls and JIA patients, after stimulation with PMA and Ionomycin, was found predominantly within the CD73$^-$ population. Although IFN-γ was mostly synthesized by the CD8$^+$CD73$^-$ population in JIA synovial fluid, some SF CD8$^+$ T cells producing IFN-γ were present among both the CD73$^+$ and CD73$^-$ populations, suggesting a degree of dysregulation in the effector population in the joint (Figure 3.7A). This was a consistent pattern in a set of samples (summary data in right hand part of Figure 3.7A).

In both healthy and JIA blood, there was very low coexpression of CD73 with the cytolytic protein perforin (Figure 3.7B). Consistent with previous findings in rheumatoid arthritis patients (Cho et al. 2012), production of perforin was reduced for SFMC compared to PBMC, with neither the CD73 positive nor the CD73 negative SFMC populations producing perforin (Figure 3.7B). These results raise interesting questions about the suppressive and effector function of CD8 lymphocytes.
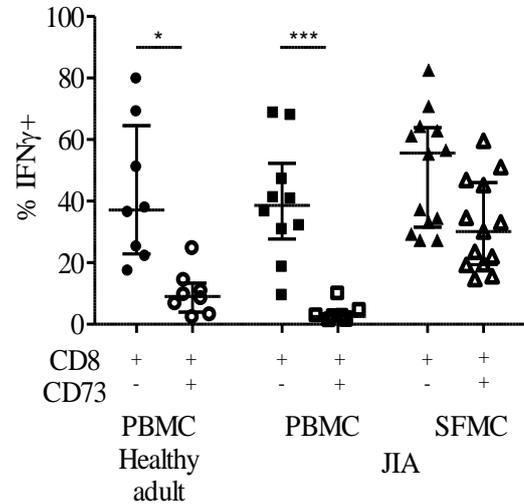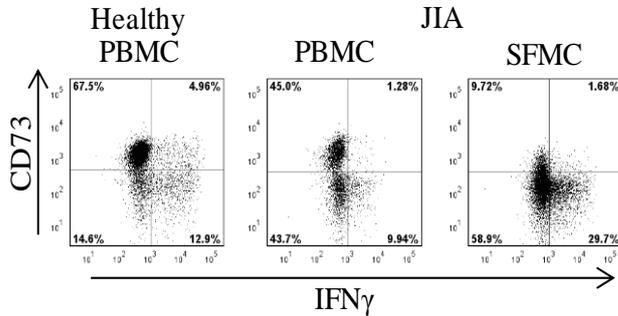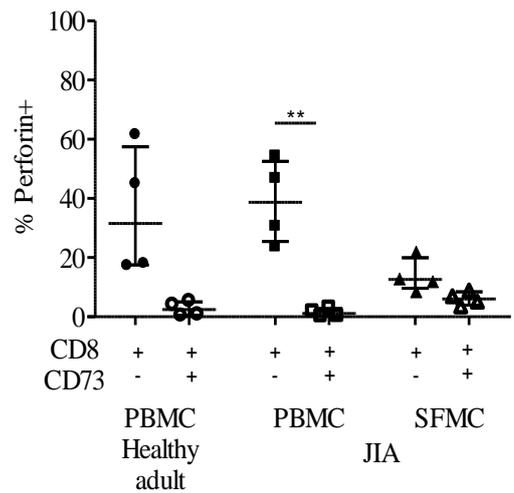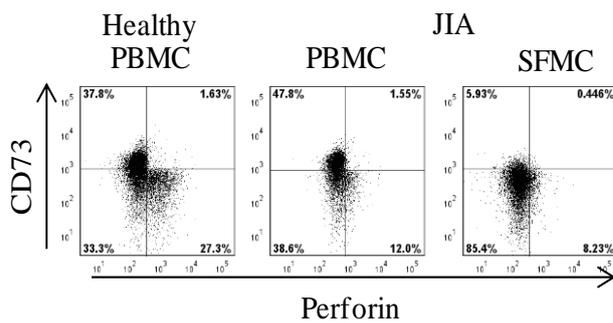
Figure 3-7 Lack of coordination between CD73 expression and IFN-γ and perforin release by CD8+ T lymphocytes

A) Representative flow cytometry plots [left] gated on CD3+CD8+ cells in healthy PBMC or paired JIA PBMC and SFMC samples stimulated with PMA/Ionomycin showing expression of CD73 and IFN-γ. The scatter plot [right] shows summary data for percentage of IFN-γ+ cells within the CD8 CD73 negative and CD73 positive populations [healthy PBMC, JIA PBMC and SFMC, n=8, 9 and 12 respectively]. B) Representative flow cytometry plots of CD73 and perforin expression from healthy PBMC and paired PBMC- SFMC gated on CD3+CD8+ cells [left]. Scatter plot (healthy PBMC, JIA PBMC and SFMC n= 4, 7, and 6 respectively) of proportions of CD8+CD73- and CD8+CD73+ cells positive for perforin [right]. Bars represent medians with IQR.

3.2.7 Expression of CD45RO and CD45RA on CD8$^+$ CD73$^+$ T cells

The leukocyte antigen CD45 is a membrane phosphatase, which can be found in different isoforms as the result of alternative splicing of the CD45 encoding mRNA. Peripheral blood T cells can be broadly distinguished into two populations according to their CD45 expression: a CD45RA$^+$RO$^-$ population known as naïve cells and a CD45RA$^-$RO$^+$ population identified as memory cells. It must be kept in mind however, that these two CD45 isoforms can also be partially coexpressed. T cells in the JIA joint show a higher proportion of CD45RO$^+$ cells compared to those from blood from patients (Silverman et al. 1993; Wedderburn et al. 2000), indicating an accumulation of memory cells in the synovium.

To understand whether CD73 expression is associated with the naive or memory phenotype of CD8$^+$ T cells, co-expression of CD73, CD45RA, and CD45RO on PBMC and SFMC was analysed by flow cytometry. As expected, a decreased proportion of CD45RA$^+$ naïve cells and an increased proportion of CD45RO$^+$ memory cells CD8$^+$ T cells were seen in JIA synovial fluid compared to blood (representative flow cytometry plots and summary data in Figure 3.8A and B). One outlier SFMC sample (seen in the summary data in Figure 3.8A and B) had 74.6% of its CD8$^+$ T cells expressing CD45RA, while 20.8% were CD45RO positive. However, these two isoforms were not highly coexpressed in this sample, as below 3% of its CD8$^+$ T cells were CD45RA$^+$CD45RO$^+$ (data not shown).

Regarding the relationship between CD73 and CD45, most of the CD8$^+$ CD73$^+$ T cells in healthy adult and JIA patient blood expressed the CD45RA isoform (Figure 3.8C). The median percentage of CD8$^+$ CD73$^+$ T cells from peripheral blood of JIA patients and healthy adult controls expressing CD45RA was 96.9% and 91.4% respectively. While only 3% and 5.9% of CD8$^+$ CD73$^+$ positive cells were CD45RO$^+$ in JIA and healthy blood, respectively (Figure 3.8D), suggesting that in blood the expression of CD73 is downregulated on memory CD8$^+$ T cells. This was not the case in the inflamed joint of patients with JIA, however, where this clear distinction between CD73 and CD45 expression was not present. In SF T cells, a median value of 36% of CD8$^+$ CD73$^+$ T cells were CD45RO$^+$, while the rest were CD45RA$^+$ (Figure 3.8C-D). These data suggest that in the joint, CD73 expression is reduced even in naive CD8$^+$ CD45RA$^+$ T cells and that the demonstration of reduced CD73 expression in SF T cells is not simply due to the enrichment of memory cells in the joint.

A

B

C

D
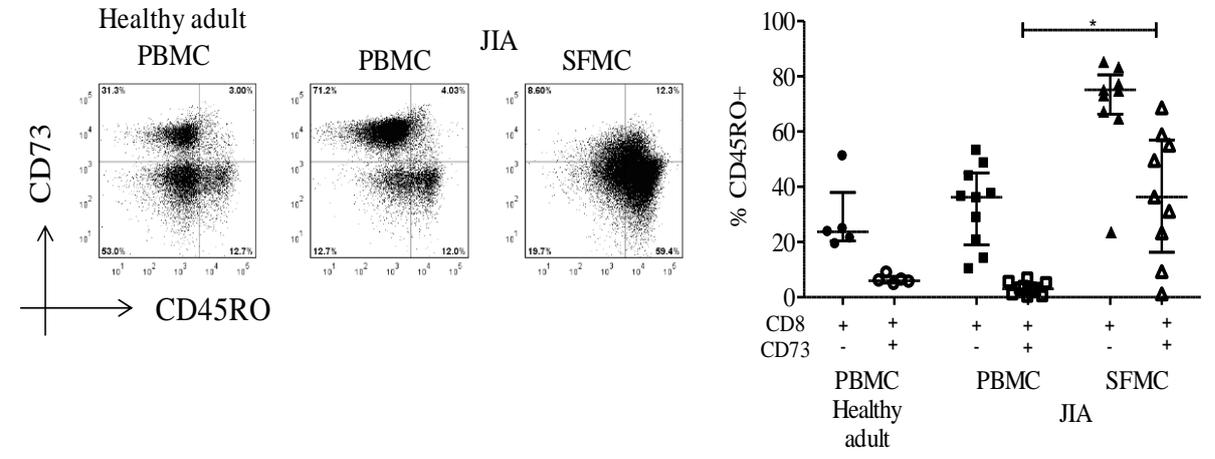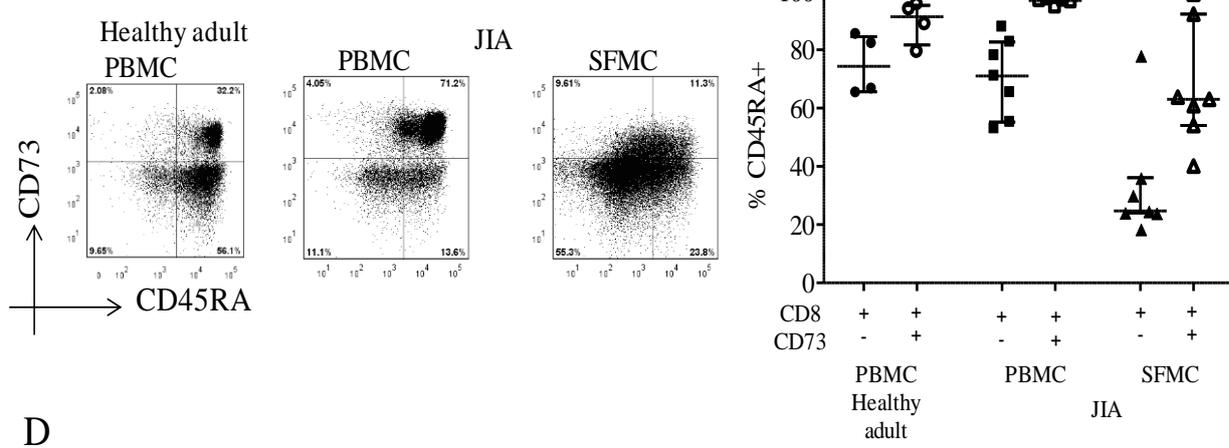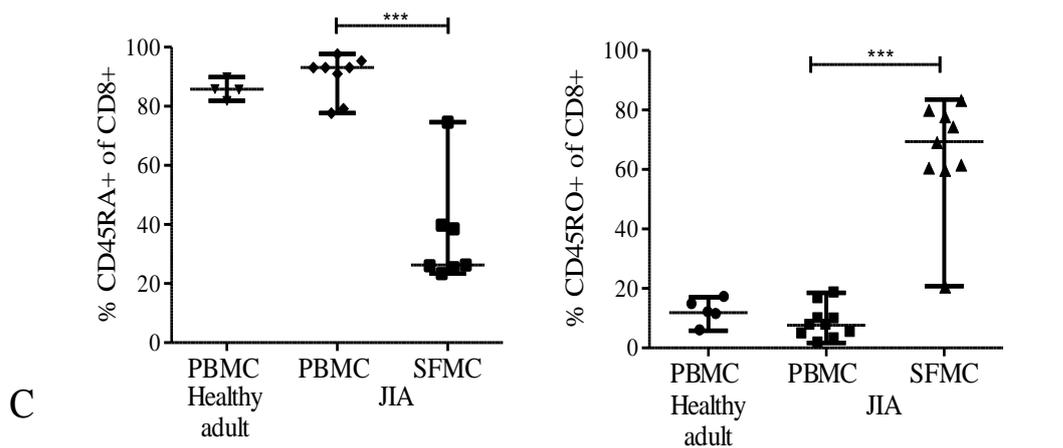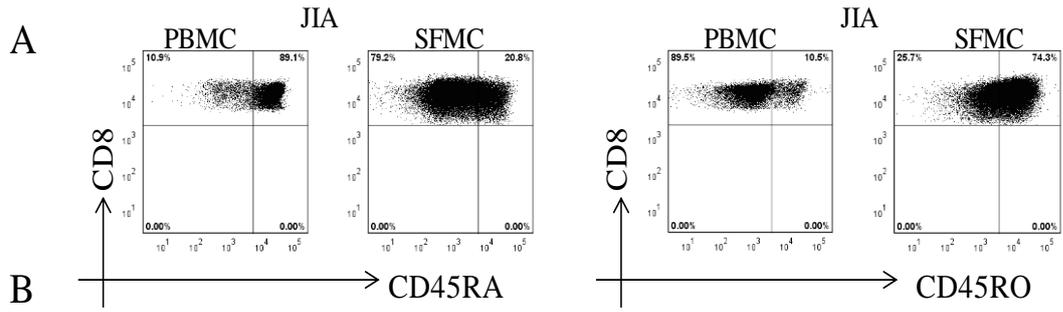
Figure 3-8 Expression of naive (CD45RA) and memory (CD45RO) markers on CD8$^+$CD73$^+$ T cells in the JIA joint

All data shown in this Figure is from the CD8 T cell gate. A) Representative flow cytometry plots showing expression of CD45RA and CD45RO on CD8$^+$ T lymphocytes from JIA PBMC or SFMC. B) Summary data of CD8$^+$ T lymphocytes positive for CD45RA [left] and CD45RO [right] expression from healthy PBMC, JIA PBMC and SFMC n=4,7,7 [left] and n=5,10,9 [right] respectively. C) Representative flow cytometry plots of relationship between CD73 and CD45RA expression in healthy PBMC and JIA PBMC and SFMC; scatter plot shows summary data for percentage of CD45RA$^+$ cells within the CD8 CD73 negative or CD73 positive population (healthy PBMC, JIA PBMC and SFMC n=4,7,7 respectively). D) Representative flow cytometry plots of CD73 and CD45RO expression by healthy PBMC and JIA PBMC and SFMC; scatter plot shows summary data for percentage of CD45RO$^+$ cells within the CD8 CD73 negative or CD73 positive populations (healthy PBMC, JIA PBMC and SFMC, n=5,10,9 respectively). Bars represent medians with IQR.

3.2.8 Increase in lymphocytes positive for CD39 in the inflamed joint and association with genetic variation in CD39 gene

Given the close relationship in function between CD73 and the ectonucleotidase CD39, the expression of CD39 was investigated in healthy control and patient samples in parallel to CD73. High frequencies of T cells positive for CD39 have been previously demonstrated for cells derived from the inflamed JIA joint (Moncrieffe et al. 2010b) as compared to those found in the peripheral blood.

Data shown in Figure 3.9A and summary data in Figure 3.9B reconfirm these findings demonstrating an increased percentage of JIA synovial T cells expressing CD39. Among T cells, CD8$^+$T cells were the population with the lowest percentage of CD39 positive cells (data not shown). The majority of healthy blood B cells express CD39 protein, but in this study, a new finding was a slight increase in the percentage of synovial fluid CD19$^+$ cells that were positive for CD39 expression. This percentage was significantly higher than that of B cells positive for CD39 in both healthy adult and child control PBMC. However, the percentage of B cells positive for CD39 expression in JIA SF showed only a trend to being higher than in the matched JIA PBMC (Figure 3.9B right). In addition to lymphocytes, CD39 is also highly expressed by monocytes (data not shown).

The data above showed that within the JIA inflamed joint, there is an increased percentage of T and B-lymphocytes expressing CD39. Since both CD39 and CD73 ectonucleotidases are required for hydrolysis of ATP to adenosine, their coexpression was next investigated by flow cytometry on T and B-lymphocytes. As Figure 3.9C-D shows, CD39, and CD73 ectonucleotidases were coexpressed by B cells from healthy adult blood, with a median percentage value of CD39$^+$CD73$^+$ B cells of 74.6%, whereas there was no co-expression on T cells. The percentage of synovial CD19$^+$ cells coexpressing these two ectonucleotidases (median 42.3%) was significantly decreased compared to both patients CD19$^+$ PBMC (median 64.6%) and healthy control CD19$^+$ PBMC (median 74.6%) (Figure 3.9D).
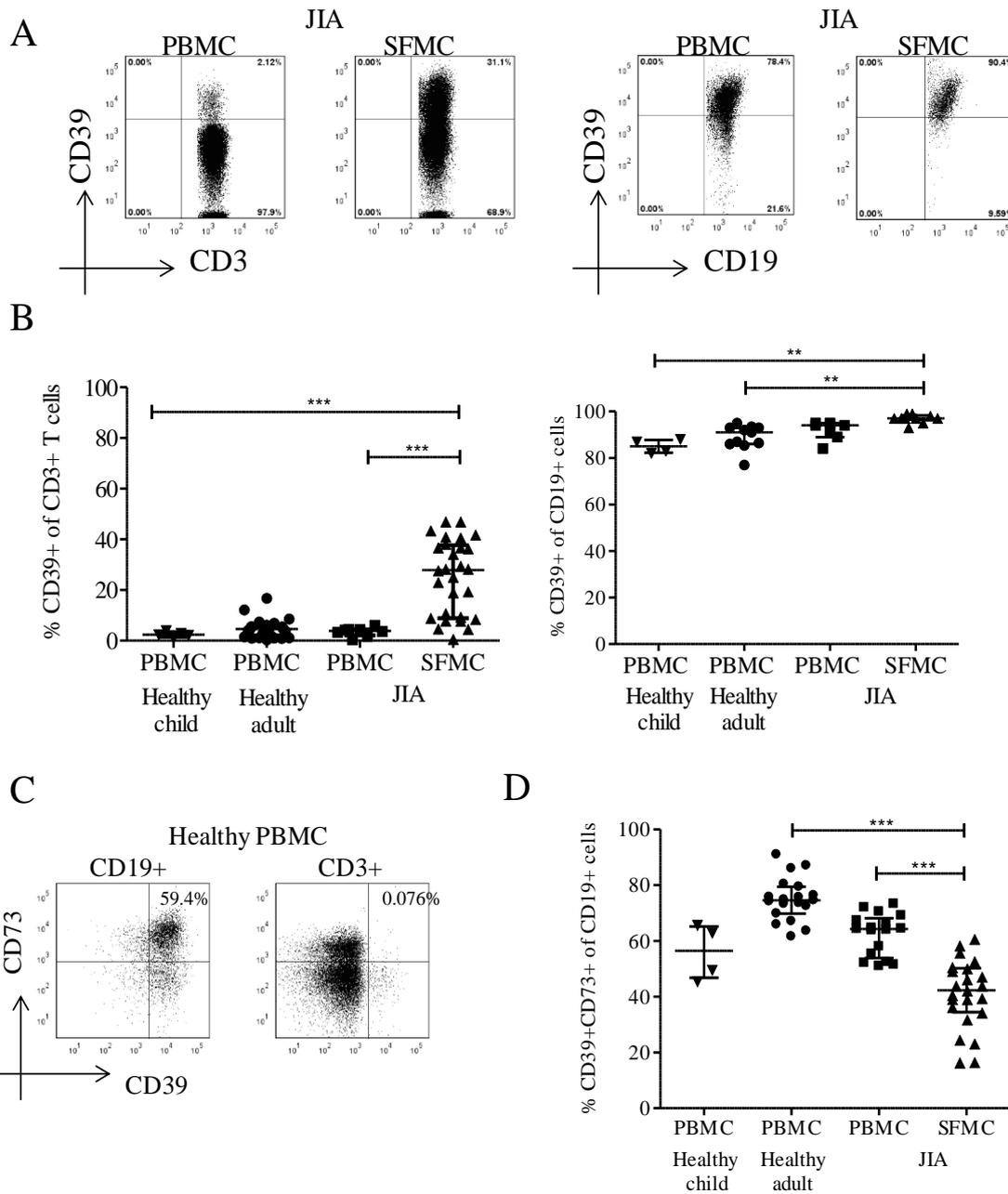
Figure 3-9 Enrichment of CD39⁺ T and B-lymphocytes in the inflamed joint.

A) Representative examples of CD39 expression on CD3⁺ T [left] and CD19⁺ B cells [right] from JIA blood (PBMC) or joint (SFMC). B) Scatter plots showing the percentages of CD3⁺ T cells [left] and CD19⁺ B cells [right] positive for CD39 in healthy child PBMC, healthy adult PBMC and JIA PBMC and SFMC n=5,30,8 and 28 [left] and n=4,11,7,10 [right]. C-D) Representative dot plots of healthy PBMC gated on B cells (left) and T cells (right), showing expression of CD73 against CD39 and summary plot for B cells in healthy child PBMC, healthy adult PBMC and JIA PBMC and SFMC (n=4,19,17,23) coexpressing CD73 and CD39. Data are represented as medians with IQR.

95

The CD39 gene has previously been shown to contain a single nucleotide polymorphism (SNP) rs10748643 close to the CD39 promoter, which results in a substitution of a G base with an A. This variation is associated with low CD39 expression and susceptibility for autoimmune Crohn's disease (Friedman et al. 2009). CD39 protein expression on patient synovial T cells was therefore assessed according to patient genotype (Figure 3.10).

Clear differences in the frequency of $CD3^+$ $CD39^+$ cells within SFMC were demonstrated in association with the SNP rs10748643. Thus, those individuals who were AA homozygotes had a significantly lower percentage of T cells positive for CD39 with a median of 8.5% compared to the 31% and 33.9% in patients with AG and GG genotype respectively (Figure 3.10). These data suggest that variation in the CD39 gene modulates expression of this protein on synovial T cells of JIA patients. The analysis of potential SNPs in the CD73 gene is discussed later in Chapter 4.
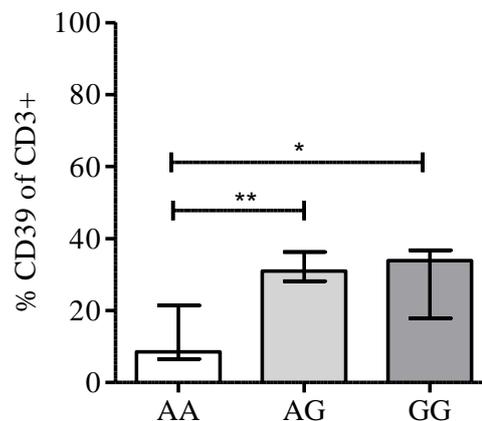
Figure 3-10 Autoimmune susceptibility SNP alters CD39 T cell protein expression

Bar graph of percentages of $CD3^+$ $CD39^+$ SFMC from patients with JIA with CD39 SNP rs10748643 AA (n=6), AG (n=7) and GG (n=5) alleles. Genomic DNA genotyped for CD39 SNP rs10748643 by Dr Simona Ursu. Data are represented as medians with IQR.

3.2.9 Reduced percentage of CD26$^+$ T cells, with elevated expression levels in JIA joint

Adenosine deaminase (ADA), the enzyme involved with adenosine breakdown to inosine is bound to the outside of the cell membrane by a variety of anchoring proteins, with CD26 being the most used as a surrogate marker for ADA expression (Kameoka et al. 1993). CD26 expression was analysed on T cells from blood and the JIA joint by flow cytometry. Results in Figure 3.11 A-B show that the percentage of synovial CD4$^+$ and CD8$^+$ T cells positive for CD26 expression was reduced compared to that of T cells from both healthy control and patient blood.

There was a significant difference ($p < 0.001$) when 7 pairs of JIA patient blood and synovial cells were compared for their percentages of CD4$^+$ and CD8$^+$ T cells positive for CD26 expression; with the median of CD4$^+$ CD26$^+$ cells in blood and synovial fluid of 85% and 47% respectively. Whilst that of CD8$^+$ T cells expressing CD26 among the CD8 T cell population in patient blood and synovial fluid was of 63% and 25% (Figure 3.11C). CD26 was not expressed by resting B cells from both blood and synovial fluid (data not shown). Interestingly, while there was a decrease in the proportion of synovial fluid T cells positive for CD26, the level of CD26 expression on CD26$^+$ cells, as expressed by MFI was significantly elevated as compared to blood of patients with JIA (Figure 3.11D).

97

A

Healthy adult
PBMC

JIA
PBMC   SFMC

B

CD3$^+$ CD4$^+$ CD8$^+$
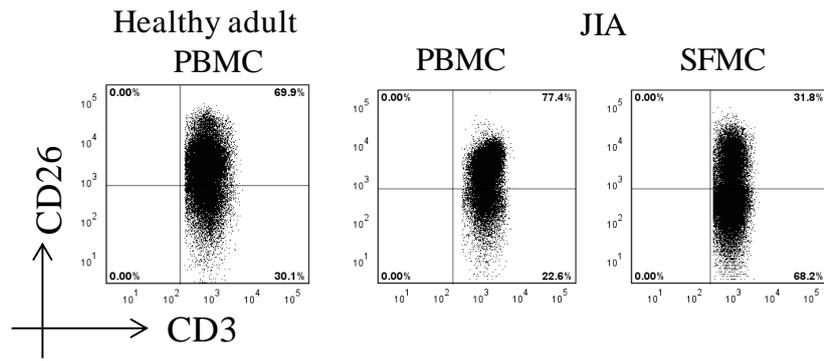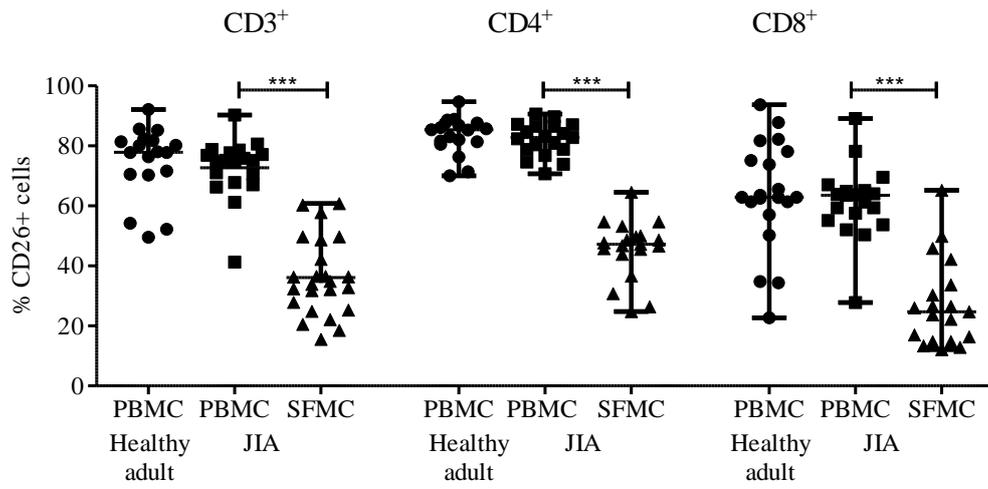
C
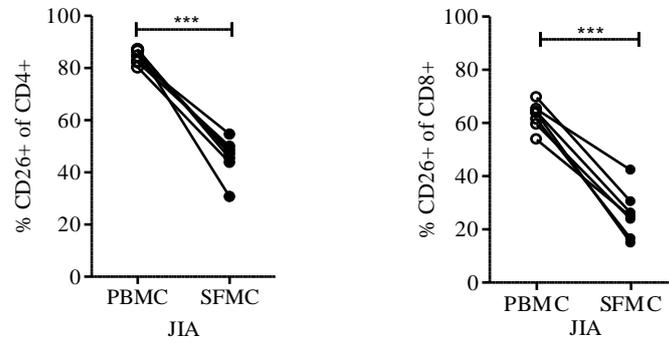
D
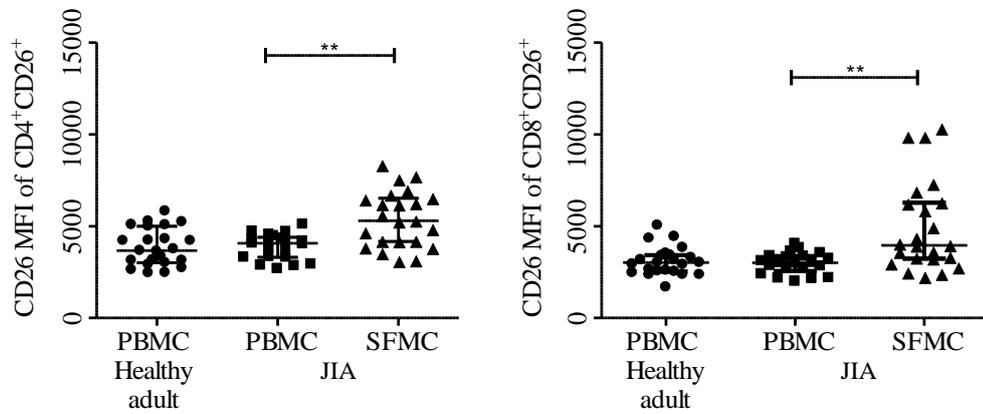
Figure 3-11 Reduced percentage of CD26$^+$ cells and elevated CD26$^+$MFI in the inflamed joint

A) Representative flow cytometry plots of CD26 expression of CD3$^+$ T cells from healthy adult control blood (PBMC) and paired blood (PBMC) and synovial fluid (SFMC) from a JIA patient. B) Scatter plots summarising percentages of CD3, CD4 and CD8 T cells positive for CD26 from healthy blood (n=18), patient blood (n=19) and synovial fluid (n=24). C) Comparison of percentage of CD4 and CD8 T cells positive for CD26 expression from paired samples of JIA patient blood and synovial fluid (n=7). D) MFI of CD26 expression on CD26$^+$ CD4$^+$ and CD26$^+$ CD8$^+$ T cells from healthy control (n=23), JIA patient blood (n=21) and synovial fluid (n=24). Bars represent medians with IQR.

3.2.10 Expression of ectonucleotidases on regulatory T cells (Treg)

Treg are involved in establishing and maintaining peripheral tolerance. Mechanisms used for this function include the release of suppressive factors such as IL-10 (Ring et al. 2011), TGF-β (Tran et al. 2007), generation of adenosine by the action of CD39 and CD73 (Deaglio et al. 2007), contact-dependent mechanisms which involve transfer of cAMP to responder cells (Bopp et al. 2007) and cytolysis of antigen-presenting cells (Grossman et al. 2004).

A variety of markers has been used to identify Treg, including the high expression of CD25, low levels of CD127 and the transcription factor Foxp3. To explore CD73 expression on Treg, different strategies were considered to identify Treg in blood and synovial fluid (Figure 3.12). The percentage of Treg among healthy blood and JIA blood and synovial fluid CD4$^+$ T cells was initially defined in four different ways: CD3$^+$CD4$^+$Foxp3$^+$, CD3$^+$CD4$^+$CD25^{hi}, CD3$^+$CD4$^+$Foxp3$^+$CD127^{lo} and CD3$^+$CD4$^+$CD25^{hi}CD127^{lo} cells. Comparing these strategies, the proportion of Treg among the healthy adult blood CD4 T cell population was very similar (range 6.3-6.9%), whichever detection strategy was used (Figure 3.12A), as was also found for JIA patient blood Treg (data not shown). In contrast, within JIA synovial CD4$^+$ T cells 10.6%-15.5% of CD4$^+$ T cells were demonstrated to express Treg markers (Figure 3.12B). These detection strategies have been employed in previous studies and may be detecting distinct or partially overlapping populations, particularly in the joint where it is known that the expression of CD25 and Foxp3 may be dissociated (Bending et al. 2014).

Using these different strategies, the percentage of healthy PBMC Treg that were positive for CD73 ranged between 9-11%, whilst that of JIA synovial CD73$^+$ Treg was between 2-3%, with the highest values of CD73 expression by Treg in the CD4$^+$CD25^{hi} gate (Figure 3.12C and D). No significant difference in the proportion of Treg expressing CD73 was observed using the different Treg markers. Accordingly, from here on Treg were defined as CD3$^+$CD4$^+$Foxp3$^+$ cells.

A Healthy adult PBMC

B JIA SFMC

C Healthy adult PBMC

D JIA SFMC

CD4⁺Foxp3⁺   CD4⁺CD25ʰⁱ

Foxp3⁺CD127ˡᵒ   CD25ʰⁱCD127ˡᵒ
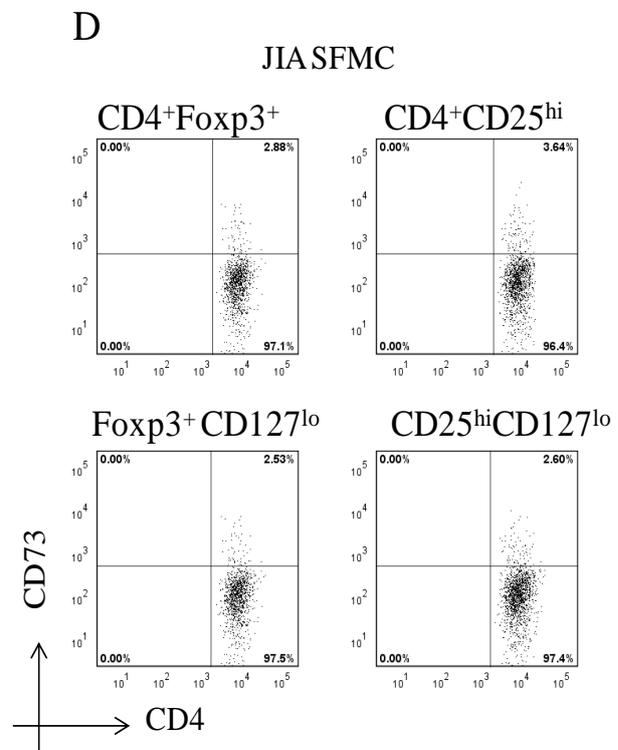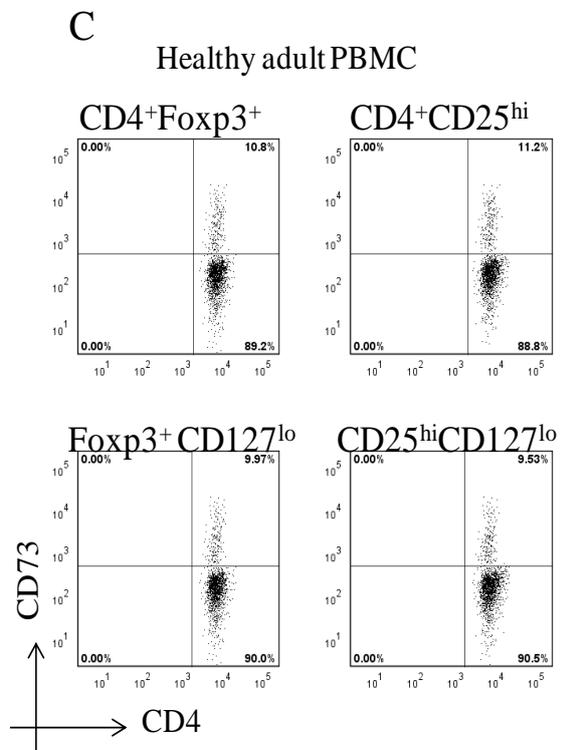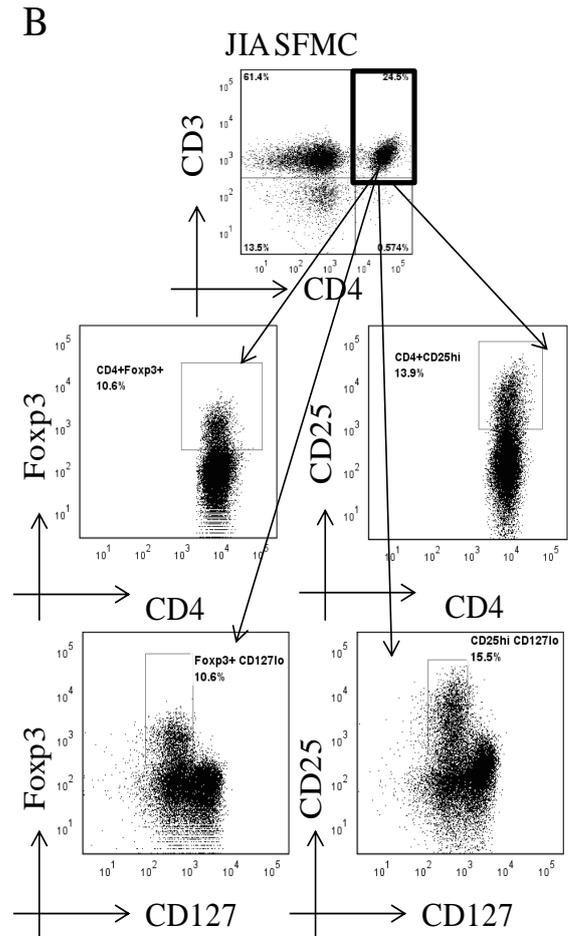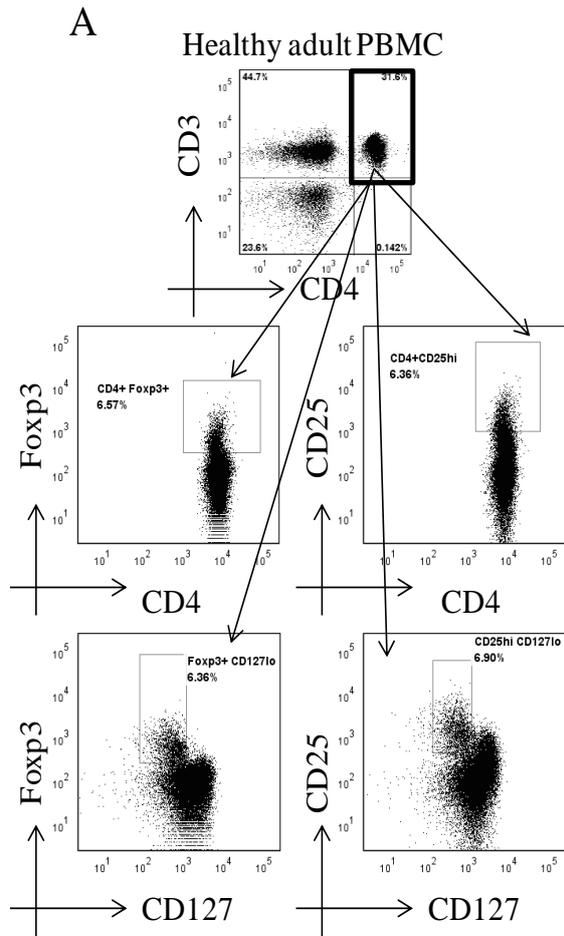
Figure 3-12 Strategies used to identify human regulatory T cells and their CD73 expression

Identification of Treg from one healthy individual blood (A) and one patient synovial fluid (B) by use of markers CD25, CD127 and Foxp3 measured by flow cytometry on CD3^{+}CD4^{+} T cell gate. The gates identifying Treg in (A) and (B), as indicated, were used in (C) and (D) to define CD73 expression on Treg from healthy control blood (C) and JIA synovial fluid (D). Plots shown are representative of three experiments, all of which gave similar results.

In agreement with previous findings of (de Kleer et al. 2004; Nistala et al. 2008; Ruprecht et al. 2005), in the JIA joint there was an elevated proportion of CD4$^+$ T cells expressing Foxp3 since a median of 8.7% of CD4$^+$ PBMC from JIA patients expressed Foxp3 compared to the median value of 23.6% of CD4$^+$ T cells from their inflamed joint (Figure 3.13A).

CD73 expression on CD4$^+$Foxp3$^+$ Treg from paired samples of blood and synovial fluid from one JIA patient is shown in flow cytometry plots in Figure 3.13B (left). In this patient, the median percentage of CD73 positive cells within the PBMC Treg (8.35%) was higher than within the synovial Treg of the same patient (0.515%).

There was a significant difference in the proportion of Treg expressing CD73 between healthy adult PBMC (n=27) and JIA SFMC (n=12), but no difference between healthy adult, child (n=5) and JIA PBMC (n=9) (Figure 3.13B, right). Although a reduction of SFMC Treg expressing CD73 was observed compared to patient Treg PBMC as represented in summary data in Figure 3.13A (right). This difference did not reach significance, possibly due to the small number of samples, particularly of paired samples, analysed.

In addition, as previously observed in the T cell population as a whole, there was a reduced percentage of a CD26$^+$ cells in the JIA patient synovial Treg population as compared to their percentage of blood Treg expressing CD26. Interestingly, again there were a few CD26$^+$ synovial fluid cells with a very high MFI for CD26 (Figure 3.13C flow cytometry plots and summary data).
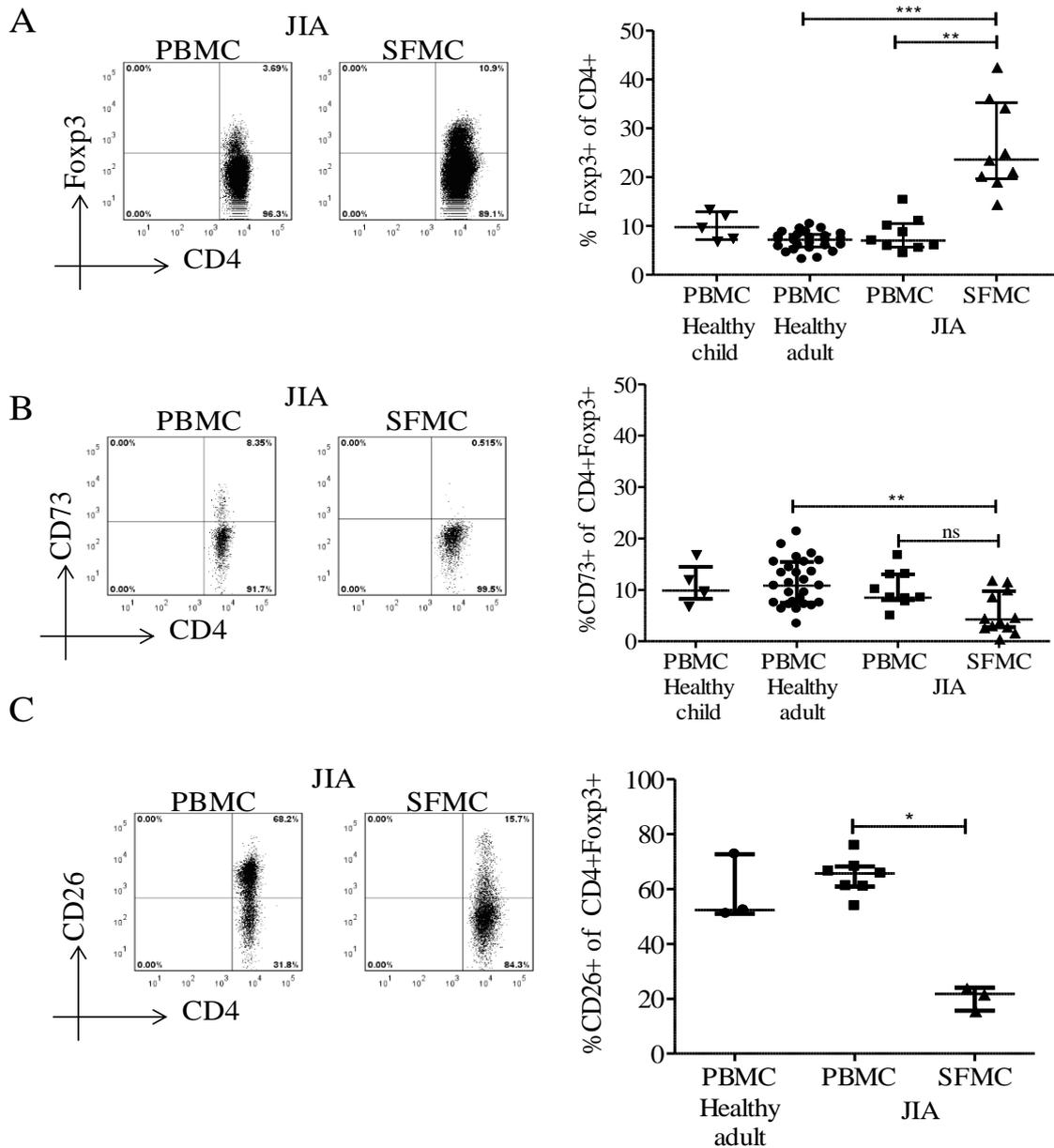
Figure 3-13 Expression of CD73 and CD26 Treg from blood and JIA SFMC.

A) Flow cytometry plots gated on CD4$^+$ T cells and summary data displaying the proportion of Foxp3$^+$ cells within the CD4$^+$ T population from healthy child and adult PBMC, patient PBMC and SFMC (n=5,27,9,11 respectively). B) Representative flow cytometry plots, gated on CD4$^+$Foxp3$^+$, of CD73 expression on CD4$^+$ T cells from same patient's blood and synovial fluid [left] and summary data of frequencies of CD73$^+$ cells within Treg from healthy PBMC (child n=5, adult n=27) and JIA patients PBMC (n=9) and SFMC (n=12) expressing CD73. C) Flow cytometry plots gated on CD4$^+$Foxp3$^+$ and summary data showing percentage of CD4$^+$Foxp3$^+$ Treg positive for CD26 from healthy adult blood (n=3), patient blood (n=7) and synovial fluid (n=3). Bars represent medians with IQR.

A suggested mechanism of action of murine Treg is adenosine production by hydrolysis of ATP via the coordinated action of ectonucleotidases CD39 and CD73, coexpressed on these cells (Kobie et al. 2006; Deaglio et al. 2007). Expression of both these ectonucleotidases was therefore measured by flow cytometry on Treg from patient's blood and JIA synovial fluid and compared to that of healthy controls (Figure 3.14).

A low percentage of both healthy blood and JIA synovial fluid Treg positive for CD73 expression was confirmed (Figure 3.14A), while high levels of CD39 expression was seen on Treg from the JIA joint compared to those from both healthy and patient blood as previously shown by (Moncrieffe et al. 2010b). No co-expression of CD39 and CD73 was seen on human Treg from the JIA inflamed joint, either patient or healthy blood (Figure 3.14A and B). Since few CD4$^+$ T cells express CD73 (Figure 3.1), this was expected. These results would suggest that human Treg are incapable of producing extracellular adenosine by breakdown of ATP via this hydrolytic pathway in isolation, unless CD73 expression on other cells could compensate for the lack on Treg.
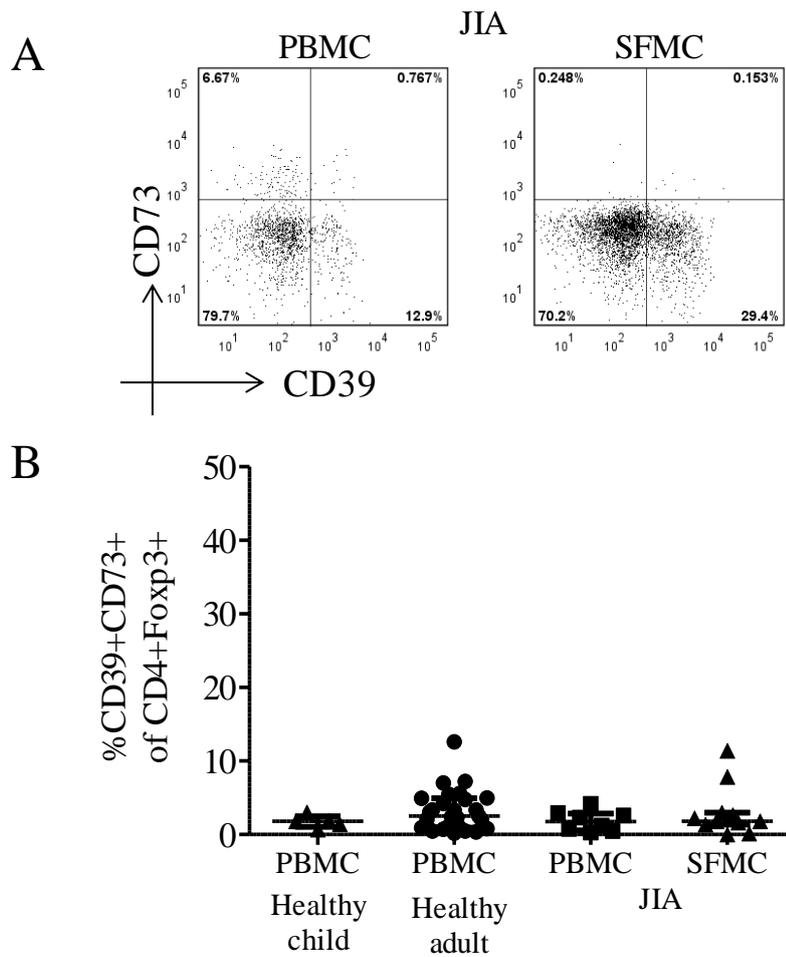
Figure 3-14 No co-expression of CD73 and CD39 on human Treg.

A) Representative flow cytometry plots gated on CD4$^+$Foxp3$^+$ T cells of CD73 and CD39 expression on Treg from blood (left) and synovial fluid (right) of the same JIA patient B) Proportion of CD39/CD73 coexpression on CD4$^+$ Foxp3$^+$ PBMC from healthy children (n=5), adults (n=27) and JIA patient PBMC (n=8) and SFMC (n=11). Data are expressed as medians with IQR.

3.3 Discussion

3.3.1 Reduced CD73 expression on lymphocytes from JIA joint

The pathophysiology of juvenile idiopathic arthritis involves swelling and destruction of the joints, particularly of the knee, characterised by infiltrating activated immune cells, including autoreactive T cells (Prakken et al. 2009) which release cytokines and chemokines to attract further cells to the site of inflammation (Pharoah et al. 2006). Potential mechanisms for suppressing inflammatory reactions include metabolic breakdown of the inflammasome-activator ATP by the enzyme CD39 and synthesis of the broad immunosuppressive regulator adenosine by the action of CD73.

Adenosine is known to inhibit the activation, proliferation and cytotoxic activity of T cells (Huang et al. 1997; Sitkovsky et al. 2004) and prevent generation and release of pro-inflammatory cytokines such as TNF-α (Sajjadi et al. 1996) to regulate inflammation. To dampen down the inflammatory response, adenosine can also increase the release of regulatory IL-10 (Haskó et al. 1996; Linnemann et al. 2009). By dampening activation-induced cell death of $CD4^+$ T cells (Himer et al. 2010) and preventing neutrophil-mediated injury to endothelial cells by suppression of their superoxide anion release (Cronstein et al. 1986), adenosine can regulate immune homeostasis (Cekic et al. 2013) and attenuate tissue damage, mostly by activating the A_{2A} receptor (Ohta et al. 2001).

Since CD73 is the main ectoenzyme responsible for extracellular generation of adenosine by dephosphorylation of AMP, investigation of its expression is important to understand the inflammatory process in the context of autoimmune diseases, such as JIA. The expression of CD73 is of particularly interest to investigate in JIA, since CD39 or ENTDP1, which generates AMP from ATP has previously been determined to be highly expressed by JIA synovial T lymphocytes compared to their blood T cells (Moncrieffe et al. 2010b), therefore resulting in high levels of AMP produced in the JIA joint. AMP can be considered to have net pro-inflammatory effects, as it is an agonist of the A_1R, (Rittiner et al. 2012) a purine receptor coupled to the G_i protein resulting in depletion of anti-inflammatory cAMP by inhibition of adenylate cyclase (Eltzschig et al. 2012). The action of CD73 is therefore important for both decreasing pro-inflammatory AMP and elevating extracellular regulatory adenosine levels.

Consistent with previous reports (Resta et al. 1997; Eltzschig et al. 2006a), with the latter study also revealing the absence of AMPase activity of healthy blood neutrophils, a lack of CD73 expression on both monocytes and neutrophils was observed in this study (Figure 3.2). These findings are in contrast to those from a mouse model of collagen induced arthritis (CIA) (Flögel et al. 2012), where not only did the peripheral blood neutrophils and monocytes express CD73, but a profound upregulation of CD73 and $A_{2A}R$ positive cells among neutrophils and monocytes derived from the mouse inflamed knee was observed. This observation, together with the finding in the synovium of RA patients of A_{2A} being the least expressed receptor (Stamp et al. 2012), suggests that CIA is not a good model for all aspects of human inflammatory arthritis.

As shown in Figure 3.1, $CD19^+$ B cells and $CD8^+$ T cells were the populations with the highest proportions of CD73 positive cells, in healthy adult peripheral blood, with values in accordance with those previously published by (Thompson et al. 1990). In contrast to the findings in blood, a clear downregulation of CD73 was observed on both JIA T and B synovial lymphocytes: both in their percentages and in protein expression level per cell for CD73 positive cells, expressed as median fluorescence intensity (MFI). A particularly significant difference was noted when comparing paired samples of PBMC and SFMC. The consequences of this downregulation and the potential reasons behind this will be investigated in the following chapters.

CD73 has previously been found to be abnormally low or absent on lymphocytes from patients with the immunodeficiency diseases: SCID, Wiskott-Aldrich syndrome, common variable immunodeficiency, selective IgA deficiency and congenital X-linked agammaglobulinemia (Thompson et al. 1984; Thompson et al. 1987). Even CD8 peripheral blood lymphocytes from patients with the acquired immunodeficiency AIDS, had both reduced nucleotidase activity (Salazar-Gonzalez et al. 1985) and reduced frequency of CD73 positive cells (Tóth et al. 2013), independent of disease status. The proportion of $CD8^+CD73^+$ cells in AIDS patients was observed to be negatively correlated with HLA-DR and PD-1 expression (Tóth et al. 2013), suggesting that CD73 downregulation on these cells was correlated with cell activation and exhaustion. This raises an interesting point of whether the observed reduction of CD73 expression on synovial lymphocytes is connected to cell activation and replication. This hypothesis is further investigated in this thesis (as shown in Chapter 6), the concept of cell exhaustion during JIA would be consistent with the reported

increase in telomeric erosion and age-inappropriate T cell senescence reported in JIA patients (Prelog et al. 2009).

It was possible that the reduced CD73 expression on synovial lymphocytes observed in this study was due to protein retention inside the cell. Extensive recycling of CD73 from the cytosol on to the cell surface of rat cells has been previously observed (Stanley et al. 1980). Therefore expression of surface and total CD73 of synovial CD8$^+$ T lymphocytes was compared by flow cytometry, where however no difference was found between the two (Figure 3.4). When CD73 protein was assessed by Western blot, using cell lysates of CD8$^+$ bead-sorted healthy PBMC, patient PBMC and SFMC, no band at the CD73 position was found for SFMC, in contrast to PBMC, indicating that these cells had no detectable CD73 protein at the limit of detection for the Western blot. These results suggest that the low proportion of CD73 positive synovial cells is not due to the retention of the protein inside the cell.

In addition to low CD73 expression on synovial B cells, a much smaller proportion of B cells among lymphocytes were found in the JIA inflamed joint compared to that found in both healthy and patient blood (Figure 3.5E). Together these results suggest that B cells have only a minor role in the production of adenosine from AMP within the inflamed JIA joint.

JIA synovial B cells do however have a different phenotype from that of peripheral blood B cells with high levels of expression of co-stimulatory markers CD80, CD86 and the activation marker CD69 (Corcione et al. 2009; Morbach et al. 2011), which could contribute to the JIA pathogenesis via antigen-presenting activity to T cells. It is interesting that CD73 expression was reduced on B cells from the JIA inflamed joint, despite previous findings that JIA synovial B cells are mostly CD27$^+$ IgA$^+$ IgG$^+$ class-switched memory cells (Corcione et al. 2009) and that this cell subset typically expressed the highest levels of CD73 in peripheral healthy blood (Schena et al. 2013).

A question arises of whether transitional B cells have a role in JIA, in the context of CD73 expression. These cells have migrated from the bone marrow to the periphery, but have not yet completed maturity, as they can still undergo negative selection and have a short half-life (Tussiwand et al. 2009). This subset of B cells defined by the phenotype CD19$^+$CD24^{hi}CD38^{hi}, have regulatory capacities by production of the anti-inflammatory cytokine IL-10 and inhibition of Th-1 differentiation *in vitro*, but are functionally impaired in

patients with systemic lupus erythematosus (SLE) (Blair et al. 2010). Transitional B cells are almost entirely absent among JIA synovial B lymphocytes, while at the same time being elevated in JIA patient blood compared to healthy controls (Corcione et al. 2009). Because of their low proportion in the JIA synovium, transitional B cells were not explored further in this study.

3.3.2 CD73 expression correlates with JIA clinical phenotype

JIA is a heterogeneous disease with many clinical subtypes (Petty et al. 2004) and the oligoarticular subtype is further divided in persistent and extended oligoarthritis, depending on the number of joints affected from 6 months after diagnosis. One of the aims of this study was therefore to define whether CD73 expression by lymphocytes from the JIA joint was correlated with clinical phenotype.

The reduction of CD73 protein expression on both CD8$^+$ T cells and CD19$^+$ B cells was indeed found to be greater in patients with the more severe extended oligoarticular subtype compared to those with persistent oligoarthritis (Figure 3.6). This would suggest that the potential to produce adenosine, which inhibits proinflammatory mechanisms by repressing T cell functions, could contribute to preventing the disease turning to severe and destructive in milder cases of JIA. These data are also supported by a previous study from our group of gene expression in so called "extended-to-be" patients, in which transcription of CD73 (NT5E) was lower in those cases who went on to the more severe disease (Hunter et al. 2010).

Persistent and extended oligoarthritis have quite distinctive disease courses. The first group of patients, persistent oligoarthritis have disease that is milder, frequently self-remitting, and requiring less treatment, whereas the second is more aggressive, less likely to go into remission and necessitating further treatment. The relationship between CD73 expression by synovial lymphocytes and more detailed clinical characteristics of patients such as disease duration and number of joints affected are therefore of importance and are investigated in the following chapter.

3.3.3 Dissociation between cytotoxic/effector and memory marker expression and CD73 expression by synovial CD8$^+$ T lymphocytes

A low CD4:CD8 ratio has been found previously within the synovial compartment due to a predominance of CD8$^+$ T cells in the inflamed joint (Hunter et al. 2010), suggesting that CD8

cells may have a pathogenic role in the disease. Since synovial CD8$^+$ T lymphocytes present an activated phenotype, (Black et al. 2002) they can be presumed to have encountered and recognised antigen at some stage and therefore to have converted to an effector phenotype characterised by release of IFN-γ and specific cytolysis by perforin-dependent pathways (Pittet et al. 2001).

When analysing the relationship between expression of regulatory protein CD73 and of effector molecules IFN-γ and perforin on CD8$^+$ T lymphocytes, differences were found between CD8$^+$ T cells in healthy blood and JIA synovial fluid. In healthy conditions, the expression of both IFN-γ and perforin was mostly restricted to CD8$^+$ CD73$^-$ cells, with almost no CD73 positive cells expressing these effector markers. While for lymphocytes derived from the inflamed synovium there was less distinction between cells positive for CD73 and IFN-γ/ perforin. This was more of the case for IFN-γ than perforin, as perforin was found to be produced at low levels by JIA SFMC, as previously observed for RA SFMC (Cho et al. 2012). The reduction of perforin expression by JIA SFMC would suggest that they are less cytotoxic. It is not known whether the lack of CD73 on IFN-γ producing cells is due to downregulation of CD73 on effector cells after stimulation or whether they present this phenotype prior to activation.

CD45RA is a high molecular weight isoform found on naïve leukocytes that then transform to the low molecular weight CD45RO marker once development into a mature memory T cell is complete. In-between this transformation, there is a transitional stage of dual CD45RA/RO coexpression. The expression of these two isoforms is broadly used to define naive and memory cells which have different requirements for activation and differentiation (de Jong et al. 1991). In healthy blood, CD73 expression has previously been found mostly limited to naive T cells, with over 75% of CD73 positive cells being naive CD45RA$^+$ T cells (Dianzani et al. 1993). This study therefore investigated whether this was also the case for synovial T cells, studied in conjunction with healthy and patient blood T cells.

The results observed here for both healthy and JIA patient CD8$^+$PBMC were in line with those previously published. This was not the case however for SFMC, where both CD8$^+$CD45RO$^+$ and CD8$^+$CD45RA$^+$ cells expressed CD73. The reason why CD73 was not limited to naive cells in the joint is unknown, but as CD45RO$^+$ T cells are accumulated in the inflamed joint (Wedderburn et al. 2000), these results show that the decrease in CD73

positive cells in the joint, observed in this study, was not simply due to the increase of memory cells.

It must be kept in mind however, that some CD45RA$^+$ cells are actually memory cells, which have re-expressed CD45RA, after TCR-induced activation and have downregulated their CD28 expression (Tomiyama et al. 2002). This pattern is most commonly observed on CD8$^+$ T cells. The expression of CD27 is up-regulated upon TCR stimulation, but is then lost on chronically stimulated T cells which have been subject to persistent antigen stimulation. Even when CD27 is down-regulated, the expression of CD45RA is unaltered (Hamann et al. 1999). Another protein which would have been worth analysing together with CD73 expression is CD57 protein, another marker of chronic immune activation, expressed by T cells which have become proliferation incompetent, after many cell divisions (Palmer et al. 2005). These so-called "revertant" cells are CD28$^-$CD27$^-$ T cells and are a caveat, as they make it more difficult to define a cell clearly as being either naive or memory.

The results shown here about the relationship between the expression of CD73 and CD45RA/RO in the joint showed that CD73 is present on both naive and memory synovial CD8$^+$ T cells. Bearing in mind the caveat that CD45RA and CD45RO are not definitive markers for these two populations, the distinction could have been made more ultimate with the use of antibodies against CD27, CD28, and CD57 proteins.

3.3.4 Opposite effects of joint environment on CD39 and CD26 expression

In this study, the finding of (Moncrieffe et al. 2010b) of an increase of synovial T cells positive for CD39 expression as compared to blood, was confirmed. Additionally, a slight upregulation of synovial B cells expressing CD39 was also established compared to healthy control blood (Figure 3.9B). In contrast, no difference was found in the proportion of B cells expressing CD39 between JIA patient blood and synovial fluid, potentially because already more than 90% of peripheral blood B cells expressed CD39.

A decrease in the proportion of B cells coexpressing CD39 and CD73 was observed in both blood of healthy and JIA children, as well as cells derived from the synovial fluid compared to adult blood cells (Figure 3.9D). The reason for this decrease could be because in the blood of children there is a smaller proportion of class-switched memory B cells compared to that of adults (Kruetzmann et al. 2003), and because this subset of blood B cells has previously been observed to have the highest levels of CD39 and CD73 expression (Schena et al. 2013).

Because of these results, fewer B cells in children would be expected to co-express CD39 and CD73.

Downstream of the enzymatic activity of CD73 is the enzyme adenosine deaminase (ADA) which metabolizes adenosine to inosine. CD26, the first ADA binding protein to be discovered (Kameoka et al. 1993), is used as a surrogate marker of ADA expression, as this protein is generally cytosolic and is able to bind to the cell membrane only by interacting with membrane proteins. Interestingly expression of CD26 the "anchor" protein for ADA was reduced on synovial T lymphocytes compared to their blood counterparts in this study. This is surprising as CD26 has suggested to be an activation marker (Dong et al. 1996) and synovial T cells have an activated phenotype (Black et al. 2002). The finding is in contrast with previous findings in RA, of increased percentages of $CD3^+$ $CD26^+$ cells in SF compared to blood T cells (Mizokami et al. 1996).

However, for those synovial T cells positive for CD26 protein, higher expression levels per cell was observed measured as MFI which is in agreement with the increased CD26 density of synovial $CD4^+$ lymphocytes from patients with RA (Ellingsen et al. 2007). The reason for the discrepancy between these two autoimmune diseases is unknown, but could be associated with the exhaustion of T cells in RA, characterized by loss of CD28 and gain of CD57 expression (Schmidt et al. 1996; Wang et al. 1997).

3.3.5 Reduced potential for immunosuppression for JIA synovial Treg

Treg are the main cells involved with immune regulation and suppression, which unpredictably, have been found at a higher frequency in the JIA inflamed synovia compared to blood of either patients or healthy controls (de Kleer et al. 2004; Ruprecht et al. 2005). These synovial Treg were revealed to have suppressive ability, preventing effector cell proliferation and release of cytokines (Vercoulen et al. 2009) and to be elevated in the joints of patients with the less severe persistent oligoarthritis (de Kleer et al. 2004), suggesting they are related to clinical severity.

In this study, a variety of markers were initially investigated to define Treg such as Foxp3, $CD25^{high}$ and $CD127^{low}$ expression, as none of them are entirely specific for this population. Treg have been found to express low levels of the IL-7Rα chain (CD127) (Liu et al. 2006) and high levels of the IL-2 receptor CD25 (Baecher-Allan et al. 2001). Use of these markers does have caveats, as it is difficult to separate high and low expression and the expression of

both Foxp3 (Wang et al. 2007) and CD25 (Ruprecht et al. 2005) can be increased on non-regulatory CD4$^+$ T cells upon activation. Expression of Treg in the inflamed joint has additional complications, as a population of Foxp3$^+$CD25$^-$ CD4 T cells has previously been reported (Nistala et al. 2008), together with the finding of Foxp3$^-$CD127$^-$ CD4 T cells in RA patients (Aerts et al. 2008). This suggests that CD25 and CD127 may not be ideal markers for Treg in the joint. For this reason, CD4$^+$Foxp3$^+$ were defined as Treg for the rest of the analysis of CD39 and CD73 expression.

ATP hydrolysis to the immunosuppressive nucleoside adenosine by the action of CD39 and CD73 has been suggested to be an important mechanism of murine Treg suppression (Kobie et al. 2006; Dwyer et al. 2007). In contrast, human CD4$^+$FOXP3$^+$ cells expressed only low levels of CD73 and no coordinated expression with CD39 was present on either cells from blood or the synovium, suggesting that human Treg are unable to synthesize adenosine from ATP through these ectoenzymes. The reduction of JIA synovial Treg expressing CD73 found in this study has recently also been found in the joint of patients with RA (Herrath et al. 2014), suggesting that it is a feature of autoimmune diseases.

Previous reports investigating the immunosuppressive ability of synovial Treg towards effector cells, observed an impairment of the response to effector cells to suppressive effects of Treg. This result correlated inversely with the expression of CD69 and HLA-DR by effector cells, which only partially responded to cytokine secretion by SF Treg, (Haufe et al. 2011) and appeared to be mostly associated with hyperactivation of effector cells (Wehrens et al. 2011) and not a defect in SF Treg, which are fully functional.

3.3.6 Summary and Conclusion

Taken together, the data presented in this chapter show that the ectoenzymes that form part of the adenosinergic pathway behave differently in inflammatory conditions, with the proportion of cells positive for CD39 elevated, while that of CD73 and CD26 cells decreased in the inflamed joint.

The upregulated CD39 expression and subsequent increased ATPase activity (Moncrieffe et al. 2010b), would suggest that synovial lymphocytes have a high capacity for metabolizing the elevated levels of pro-inflammatory ATP found in tissues after inflammatory stimuli (Eltzschig et al. 2006a; Schenk et al. 2008). The supposed elevated ATP would therefore be metabolized to AMP by CD39 and if the low levels of CD73 observed, correspond to

decreased AMPase activity, there would be a build-up of AMP, with no adenosine synthesized. Because of the essential role of adenosine in regulating tissue damage, by circumventing cytokine and chemokine production by many different immune cells, its absence in the JIA joint, would result in the blockade of tissue homeostasis.

The reduced proportion of CD26 positive cells should suggest a reduction of ADA expression and potentially ADA activity. Lower ADA activity could be a consequence of regulation, to permit the extracellular adenosine, synthesized by CD73, to have a longer half-life to increase its likelihood of binding to adenosine receptors. Activation of these receptors, particularly of A_{2A} receptor, which mediates most of the cytoprotective effects of adenosine, would prevent the T cell receptor-triggered effector functions of T cells (Huang et al. 1997). This together with the effect of expanding Treg with increased suppressive function (Ohta et al. 2012) would overall limit the collateral damage of cell activation known to be associated with inflammation in JIA joint. The possible outcomes of the changes in ectonucleotidase expression in the synovium will be examined in the following chapters, by measuring their enzymatic activity.
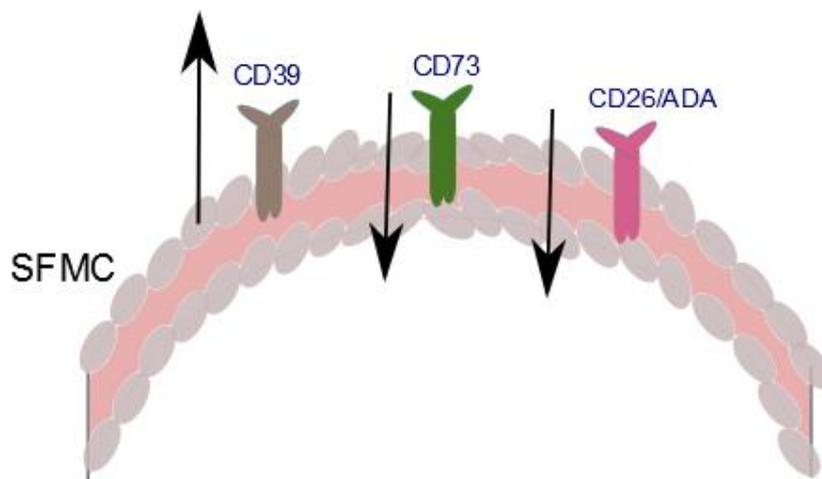
Figure 3-15 Summary of the alterations of ectonucleotidase expression on SFMC

Depiction of how the expression of the main ectoenzymes altering the extracellular levels of adenosine is altered on synovial lymphocytes of JIA patients compared to peripheral blood lymphocytes. There is an upregulation of CD39^{+} synovial lymphocytes, and a downregulation of both CD73^{+} and CD26^{+} synovial lymphocytes.

Chapter 4 Association of CD73 with clinical characteristics of JIA patients and treatment with Methotrexate

4.1 Introduction

Results described in the previous chapter showed that JIA synovial T and B lymphocytes are characterised by a decreased proportion of CD73 positive cells compared to both healthy control and patient peripheral blood lymphocytes. The observed reduction was less significant among synovial exudates of patients with the more moderate and treatable form of JIA, persistent oligoarthritis, in contrast to those with the more severe type of disease, extended oligoarthritis. The expression of CD73 by synovial lymphocytes from patients with polyarticular JIA, a form of arthritis where children have 5 or more joints involved in the first 6 months of disease (Petty et al. 2004), will also be explored in this chapter.

Analysis of CD73 expression is interesting, because it is the main enzyme responsible for generation of adenosine, a cytoprotective nucleoside able to inhibit and counteract the damaging effects of chronic inflammation that characterises the JIA joint. The observed reduction of CD73 in the joint could also have implications for treating JIA. This is because methotrexate (MTX), the first and major course of treatment for JIA (after simple intra articular joint injections), has been reported to convert AMP to adenosine via the action of CD73. This theory was tested in a study of CD73-deficient mice (Montesinos et al. 2007), where injection of MTX was not able to counteract the elevation in leukocyte count and TNF-α release in the air pouch model of inflammation (carrageenan-induced) or increase adenosine levels as effectively as wild-type mice in the same model. If CD73 is required for this anti-inflammatory action of MTX, it is possible that the loss of CD73 expression at the site of inflammation in JIA could lead to the failure of arthritis to respond well to MTX treatment.

The aims of the experiments described in this chapter were to:

1) Determine whether treatment with methotrexate is a potential confounder in the analysis of the association between CD73 expression and clinical phenotype of oligoarthritis patients;

2) Investigate whether treatment with methotrexate itself influences the expression of CD73 and other ectonucleotidases by lymphocytes from peripheral blood of JIA patients;

3) Determine any associations of genes involved with the adenosinergic pathway with JIA disease pathogenesis and response to methotrexate.

4.2. Results

4.2.1 Association of CD73 expression with clinical subtype

In Chapter 3, the decrease in CD73 expression on synovial lymphocytes of JIA patients with persistent oligoarthritis was found to be less than that observed in patients with the more severe extended oligoarthritis. This result suggests that the expression of CD73 may be correlated with the clinical phenotype of oligoarthritis. As persistent oligoarthritis is frequently self-remitting (Prakken et al. 2011), disease duration (from the time of disease presentation to the time of synovial fluid withdrawal) is generally shorter compared to the extended form of the disease. This was also the case for JIA patients analysed in this thesis, with persistent oligoarthritis patients having an average disease duration of 2.5 years compared to 6.3 years for extended oligoarthritis patients (p<0.001) (Table 2.1, Material and Methods, Chapter 2).

In order to test whether the reduced CD73 expression on synovial lymphocytes of patients with the more severe extended oligoarthritis is a function of longer disease duration, CD73 expression by synovial CD8$^+$ T cells and CD19$^+$ B cells from both subtypes of oligoarthritis patients was evaluated against disease duration at time of sample. As shown in Figure 4.1A, the frequency of CD8$^+$ T cells and CD19$^+$ B cells expressing CD73 protein was not correlated with length of disease. This was also the case when disease duration was tested as a whole with both the persistent and extended JIA oligoarticular subtypes together (for CD8, r= -0.22, p=0.15 and for CD19 r=-0.09, p=0.61, data not shown).

In addition to comparing CD73 expression by synovial lymphocytes from patients with persistent and extended oligoarticular JIA, CD73 expression on synovial lymphocytes from patients with polyarticular JIA (>5 joints during the first 6 months of disease) was also analysed. Despite this group of patients having very similar disease duration to that of patients with persistent oligoarticular arthritis (Table 2.1), there was still a lower percentage of synovial CD8$^+$ T cells and CD19$^+$ B cells expressing CD73 from the joints of polyarticular arthritis patients compared to those in patients with persistent oligoarthritis (Figure 4.1B). This suggests that the observed differences in CD73 expression were related to the different types of JIA, and not disease duration.

118

Furthermore, the number of joints affected, (employed as a surrogate of severity) up to the date when the synovial fluid sample was taken, was tested against CD73 expression by JIA synovial CD8 T cells or B cells. As shown in Figure 4.1C, a trend towards an inverse relationship between CD73 expression and cumulative joint count was observed. This result was not unexpected, since the separation of oligoarthritis into its two subtypes (persistent and extended) depends on the number of joints affected up to the time of analysis.

4.2.2 Analysis of CD73 expression in treatment naive patients

Treatment with the DMARD methotrexate (MTX) could be a potential confounder for analysis of CD73 expression, since more patients with extended oligoarthritis were on this drug compared to those with persistent oligoarthritis (Table 2.1). Expression of CD73 by synovial T and B-lymphocytes in patients who were naive to treatment with both steroid and MTX was therefore analysed, and this was compared in the two clinical subtypes of oligoarthritis.

In this analysis of patients who had never received medication, a clear difference was seen with a greater decrease in CD73 expression on synovial lymphocytes from patients with extended oligoarticular arthritis being observed (Figure 4.2), similar to that seen for the group as a whole, which included patients on treatment. The percentage of synovial $CD8^+$ $CD73^+$ T cells in the extended oligoarthritis group was significantly lower than that from persistent oligoarthritis patients. A decrease was also seen for $CD19^+CD73^+$ B cells, but this did not reach statistical significance, possibly due to the small number of patients in the B cell analysis. These results suggest that the observed reduction in CD73 levels on synovial T and B cells was not due to effects of the drugs.

A

Persistent:
r -0.25
P value 0.34

Extended:
r -0.06
P value 0.73

□ Persistent
▲ Extended

Persistent:
r -0.17
P value 0.54

Extended:
r 0.24
P value 0.33

□ Persistent
▲ Extended

B

C

| Spearman r | -0.3613 |
| P value (two-tailed) | 0.0160 |

| Spearman r | -0.4266 |
| P value (two-tailed) | 0.0119 |
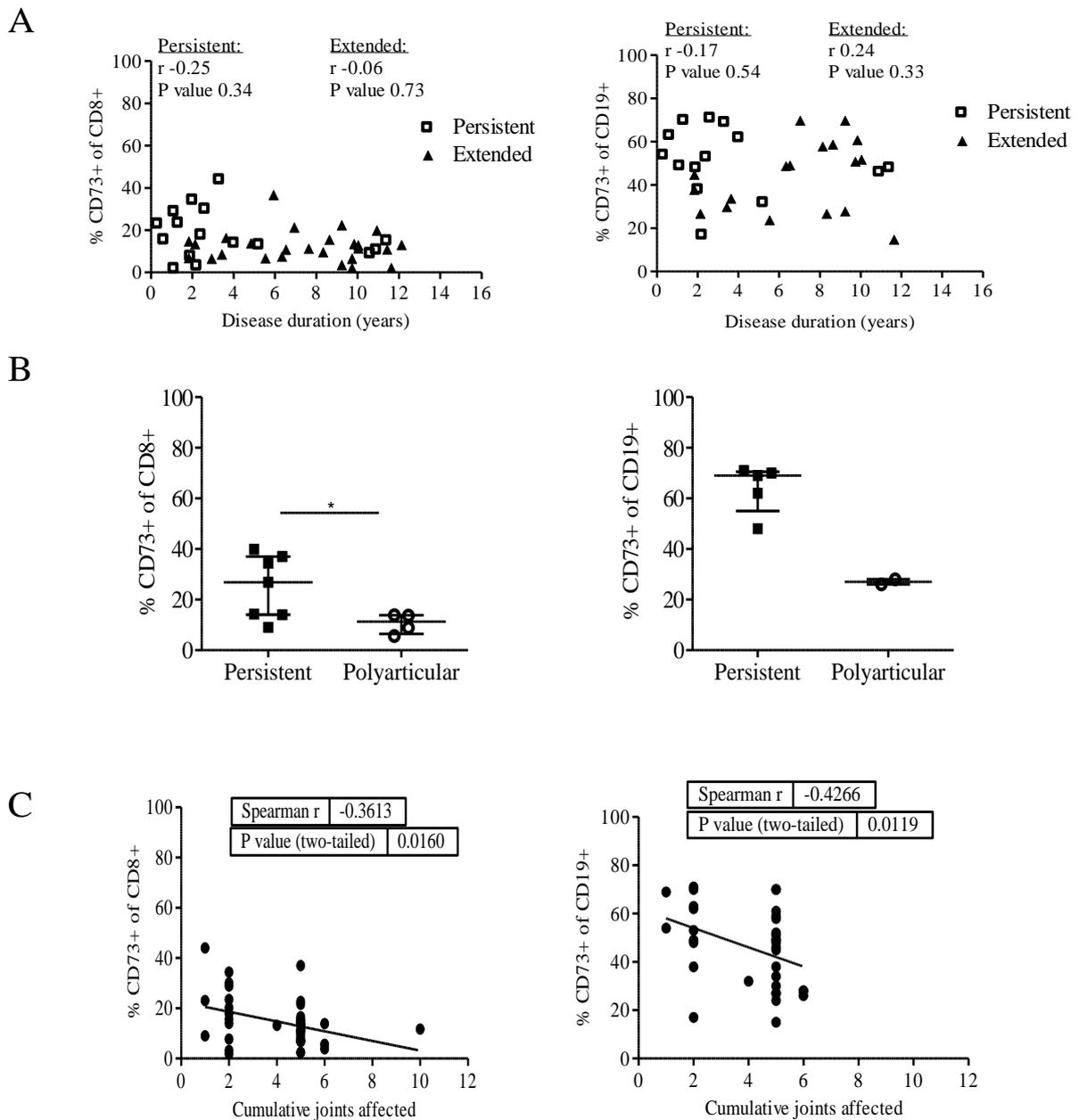
Figure 4-1 CD73 expression on JIA synovial lymphocytes is associated with joint count, but not disease duration

A) Lack of correlation between percent of CD8^{+} SFMC expressing CD73 (n=42 samples) and of CD73^{+}CD19^{+} SFMC (n=32 samples) with JIA disease duration. B) Comparison of the proportion of synovial fluid CD8 T cells [left] expressing CD73 from persistent oligoarticular and polyarticular arthritis patients (n=7,4 respectively) and of CD19 B cells [right] expressing CD73 from the two subtypes (n=5,2 respectively). C) Correlation of CD73 expression by JIA CD8 [left, n=44 samples] and CD19 [right, n=34 samples] synovial lymphocytes with cumulative joint count of patients with oligoarthritis. Data are represented as medians with IQR.
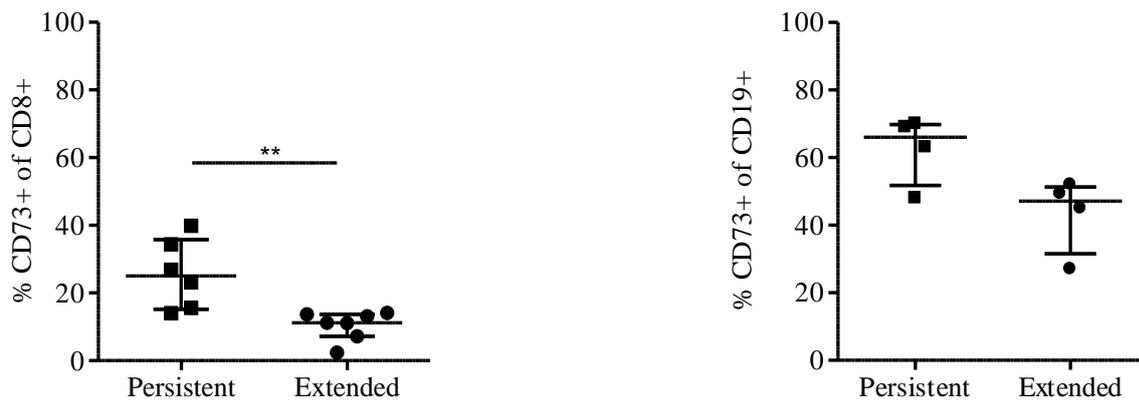
Figure 4-2 CD73 expression by synovial lymphocytes from untreated JIA oligoarthritis

Comparison of the proportion of synovial fluid CD8 T cells (left) and CD19 B cells (right) expressing CD73 from persistent (n=6,4 for CD8,CD19 respectively) and extended (n=7,4 for CD8, CD19 respectively) oligoarthritis patients, who were naïve to methotrexate and steroid treatment up until the time of sample. Data are represented as medians with IQR.

4.2.3 Effect of methotrexate treatment on patient leukocyte ectonucleotidase expression

Low-dose methotrexate (MTX) is considered the mainstay treatment for juvenile idiopathic arthritis (Ravelli et al. 2007) and has been observed to exert anti-inflammatory effects by increasing release of adenosine through the action of CD73 (Montesinos et al. 2007). For this reason, the expression of CD73 together with that of CD39 and CD26 was evaluated on blood lymphocytes of JIA patients prior to, and after 6 months of treatment with MTX (Figure 4.3). This analysis was to determine whether treatment with MTX affects ectonucleotidase expression of PBMC. Since it takes an average of at least 8-12 weeks of continuous MTX therapy to observe its clinical effects in patients (Ravelli et al. 2000), a uniform time point of 6 months was chosen after the start of MTX to analyse the effects of the drug on ectonucleotidase expression of PBMC.

As shown in Figure 4.3A, the proportion of JIA peripheral blood $CD3^+$ T cells, $CD8^+$ T cells and $CD19^+$ B cells expressing CD73 was unaffected by treatment with MTX. In contrast, the proportion of $CD3^+$ T cells expressing the CD39 ectonucleotidase was slightly decreased after treatment, while that of B cells was slightly increased (Figure 4.3B). As described in Chapter 3, resting B cells do not express CD26 and no difference was observed in the proportion of $CD3^+$ and $CD8^+$ T cells expressing CD26 before and after treatment with MTX (p=0.681,p=0.769, for CD3 and CD8 respectively, data not shown). The proportion of Treg expressing CD39, CD73, and CD26 was unaltered by treatment with MTX (Figure 4.3C).

Not all JIA patients treated with MTX respond well to the drug, as many develop adverse effects to the drug (Schmeling et al. 2014) and about 30% of cases do not respond at all to treatment (Becker et al. 2011), as determined using the JIA definition of improvement (Giannini et al. 1997). This definition relies on the use of six core set variables: the ESR (erythrocyte sedimentation rate), the physician's global assessment, the parent global assessment, the childhood health assessment questionnaire (CHAQ), the number of restricted joints, and the number of joints with active arthritis. Together these variables are used to calculate response using the ACR (American College of Rheumatology) criteria. A patient is said to have reached a 30% improvement from baseline or ACR30, when there is at least 30% improvement in minimum 3 core set variables, with no more than one variable getting 30% or more worse. Similarly a patient is said to have reached a 70% improvement from baseline or

ACR70, when there is at least 70% improvement in minimum 3 core set variables with no more than one variable getting 30% or more worse.

In order to test for any potential association between expression of ectonucleotidases and responder/non-responder status of a patient to MTX, 10 pairs (5 pairs each) of non-responders (NR, defined as not having reached 30% improvement) and responders (R, those that have reached ACR70 or above of response) were analysed pre- and post-treatment with MTX (Figure 4.3D). No significant difference was found in the proportion of $CD8^+$ T cells expressing CD73 (Figure 4.3D) or CD39 (data not shown) between responders and non-responders, both pre- and post-treatment.
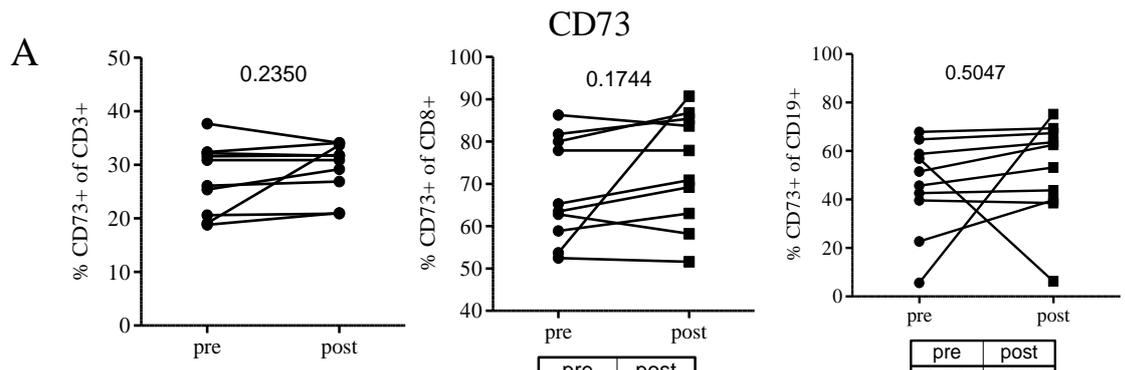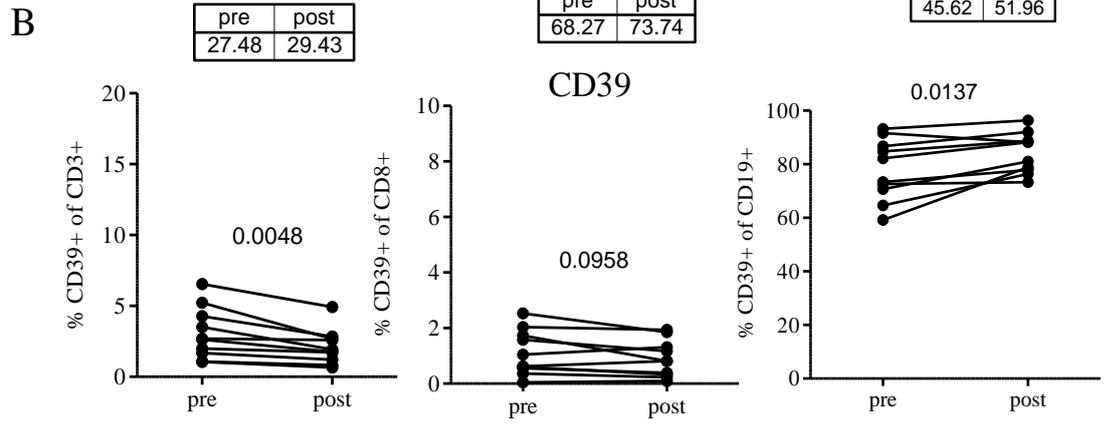
A

CD73

% CD73+ of CD3+ — 0.2350

pre	post
27.48	29.43

% CD73+ of CD8+ — 0.1744

pre	post
68.27	73.74

% CD73+ of CD19+ — 0.5047

pre	post
45.62	51.96

B

CD39

% CD39+ of CD3+ — 0.0048

pre	post
3.064	2.115

% CD39+ of CD8+ — 0.0958

pre	post
1.112	0.8939

% CD39+ of CD19+ — 0.0137

pre	post
77.90	84.10

C

Treg

% CD39+ of CD4+ Foxp3+ — 0.7932

pre	post
26.01	25.09

% CD73+ of CD4+ Foxp3+ — 0.8029

pre	post
11.26	11.93

% CD26+ of CD4+ Foxp3+ — 0.2990

pre	post
59.73	64.73

D

MTX response status

Pre treament — 0.3095 — % CD73+ of CD8+ — NR, R

Post treament — 1.0000 — % CD73+ of CD8+ — NR, R
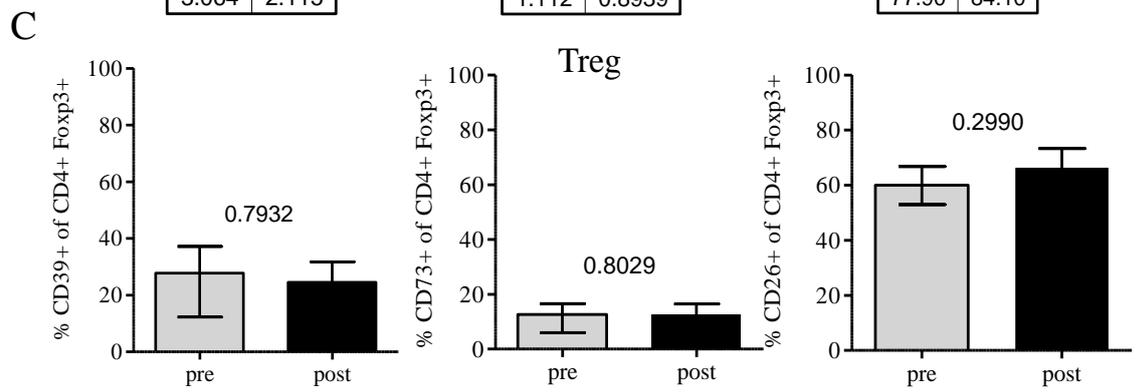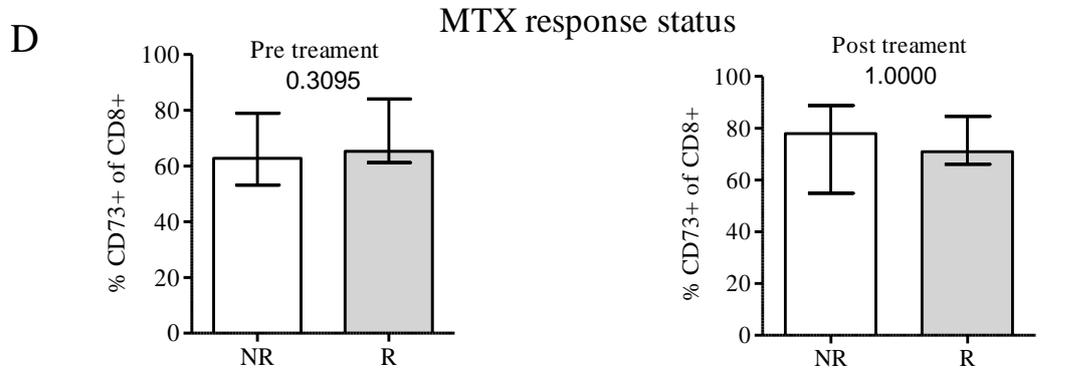
Figure 4-3 Expression of ectonucleotidases on JIA patient blood lymphocytes prior to and after 6 months of MTX treatment

A) Summary data showing percentage of CD73 positive CD3 and CD8 T cells and CD19 B cells from peripheral blood of JIA patients prior to the start and after 6 months of treatment with MTX (n=10). Numbers under plots represent mean of each group. B) Summary data showing percentage of CD39 expression by CD3, CD8 and CD19 cells from peripheral blood of JIA patients prior to the start and after 6 months of treatment with MTX (n=10). C) Summary data of the proportion of $CD4^+Foxp3^+$ Treg expressing CD39, CD73 and CD26 from peripheral blood of JIA patients prior to the start and after 6 months of treatment with MTX (n=10). D) Proportion of CD8 CD73 positive cells from blood of 5 non-responders (NR) and 5 responders (R) prior to the start (left) and after 6 months of (right) treatment with MTX. Bar graphs represent data as medians with IQR and values on top of each graph represent p values.

4.2.4 Genetic association study of enzymes involved in the adenosinergic pathway

JIA is a complex disease, as both genetic and environmental components contribute to JIA susceptibility (Ravelli et al. 2007). Previous case control studies, in which JIA patients samples were collected together with a series of control individual samples and tested for genetic variations that may confer risk of developing the disease, found some very interesting results. These include susceptibility loci in the PTPN22, STAT4, IL-2RA and the IL-2RB gene regions (Hinks et al. 2009; Hinks et al. 2013). Many of these risk loci for JIA are involved in pathways of T cell activation, development, and differentiation. Additionally, many of these susceptibility loci were found to overlap highly with RA arthritis patients (Hinks et al. 2010; Hinks et al. 2012).

Genetic polymorphism variations have been investigated in JIA patients looking for differences between responders and non-responders to methotrexate, to identify genetic associations with response to the drug. Significant differences have been found within the ITPA (inosine triphosphate pyrophosphatase) gene, the ATIC gene (as described in the introduction, leads to AICAR accumulation and subsequent higher extracellular adenosine levels), the solute carrier gene SLC16A7 and the GGH (glutamyl hydrolase) gene (Moncrieffe et al. 2010a; Hinks et al. 2011; Yanagimachi et al. 2011). All of these are part of the MTX metabolic pathway. More recently a genome wide association study of response to MTX in JIA has identified 31 novel genetic loci which are different between responders and non-responders (Cobb et al. 2014). These associations require further investigation, as they have not yet been validated.

It has been described that a single nucleotide polymorphism (SNP) rs10748643 that lies in close proximity to the CD39 gene promoter and results in the substitution of a G base with an A, is associated with decreased CD39 expression and increased susceptibility to Crohn's disease (Friedman et al. 2009). As described in Chapter 3, this SNP also affected CD39 expression by JIA synovial T cells. For this reason, eight genes (including the ENTPD1 gene encoding for CD39), encoding proteins forming part of the adenosinergic pathway, (Figure 1.3) were selected for SNP genotyping, initially in patients from all JIA disease subtypes to identify other potential SNPs that could contribute to JIA pathogenesis and MTX response. The 8 genes selected for this analysis are shown in Table 4.1.

Enzyme commission number	Protein	Gene name	Location	Accession number
3.1.3.5	CD73	NT5E	6q14-q21	NM002526.3
3.6.1.5	CD39	ENTPD1	10q24	NM001098175.1
3.4.14.5	CD26	DPP4	2q24.3	NM001935.3
3.5.4.4	ADA adenosine deaminase	ADA	20q 13.12	NM000022.2
3.1.3.1	AP alkaline phosphatase	ALPL	1p36.12	NM000478.4
2.4.2.1	PNP purine nucleoside phosphorylase	PNP	14q13.1	NM00270.3
2.7.4.3	AK1 adenylase kinase	AK1	9q34.1	NM000476.2
2.7.1.74	DCK deoxycytidine kinase	DCK	4q13.3-q21.1	NM000788.2

Table 4.1 Candidate genes for genetic association analysis

Genotyping and data analysis was carried out by Dr J Cobb and Prof W Thomson (University of Manchester), as part of a collaborative study with S Botta Gordon-Smith and L Wedderburn. This analysis was designed to determine whether variants in these genes were associated with JIA disease pathogenesis (susceptibility analysis) or response to MTX (MTX response analysis) by first comparing all JIA samples with healthy controls (WTCCC2: Wellcome trust case control consortium 2) and then, within JIA cases for whom response status to MTX was known, by comparing ACR70 responders with non-responders to MTX treatment. The JIA cases available for this analysis were detailed in Chapter 2, section 2.6.

The initial genotype analysis was carried out by analysing a large region selected around each candidate gene, based on the Illumina gene annotation file. A SNP was analysed if the annotation file found the SNP to be located within the gene of interest or intergenic to it. This resulted in different sizes of regions around genes and after quality control, the identification of 249 SNPs. Data from this analysis are shown in Table 4.2A. Only modest p values (range 0.05-0.004) for association between these SNPs and JIA as a whole or response to MTX treatment, were found in both the susceptibly analysis between JIA patients and healthy controls, and in the MTX response analysis between JIA responders and non-responders to MTX. None of these p values reached genome-wide significance, currently estimated at $p<5\times10^{-8}$ (Int. HapMap Consortium, 2005). However, since this was a candidate gene study, far fewer SNPs were investigated and this significance threshold might not be strictly applicable. Performing multiple tests at once by interrogating multiple SNPs and/or candidate genes can result in an increased false positive rate. To avoid this problem, the Bonferroni correction can be applied where the exact number of tests is adjusted by dividing the significance level by the number of polymorphisms tested. In this study, this would result in

0.05/249=0.0002, therefore p<0.0002 could be considered the significance threshold in this case. Table 4.2A is the summary of those SNPs that reached association with a significance of p<0.05 in the susceptibility to JIA analysis, suggestive of a difference between JIA patients and healthy controls. When comparing responders to non-responders in the MTX response analysis, a few SNPs were found within the ALPL (AP) gene, but associations were weak with p values of around 0.04. No SNPs were found to be significantly different within the NT5E gene (CD73) between all JIA samples and controls, or between JIA non-responders and responders to MTX (data not shown). However, several SNP frequency differences that were both intergenic and in an intron of NT5E were found between healthy controls and CHARMS samples (Table 4.2A).

For a more systematic investigation, the analysis was next repeated by mapping just a 5kb region upstream and downstream of each gene. This was achieved by determining gene coordinates using the publically available UCSC Genome Browser database and subtracting 5kb from start and adding it to end coordinates to determine region size, including any SNPs genotyped 5kb downstream and 5 kb upstream of the gene transcript. Since the genes of interest varied in length, the regions varied in size from ~19kb-175kb. The results from this second analysis are shown in summary data in Table 4.2B-C.

Again, in the MTX response analysis, no differences were found in the NT5E (CD73) gene SNPs between MTX responders and non-responders. While, some differences in the ALPL (AP) and DPP4 gene regions (CD26) were observed between MTX responders and non-responders (Table 4.2B). The same SNP variations found when comparing JIA samples (limited to CHARMS samples) to healthy controls in the NT5E (CD73) and DPP4 (CD26) in the first analysis (Table 4.2A), were found again in the analysis restricted to the ± 5kb region (data not shown).

Since much of the work in this thesis has focused on samples from patients with oligoarticular arthritis, a further genetic association analysis focused on this JIA subtype, in particular to analyse potential differences between patients with persistent and extended oligoarthritis (Table 4.2C).

A

	CHR	BP	SNP	Gene region	Protein encoded	SNP position	Minor allele	NMISS	Odds ratio	P value	Analysis
Susceptibility analyses	1	21826566	rs6667242	ALPL	AP	intergenic	G	6661	0.85	0.021	all JIA samples vs WTCCC2
	1	21837755	rs16825455	ALPL	AP	intron	G	6660	0.83	0.010	all JIA samples vs WTCCC2
	2	162867791	rs2909450	DPP4	CD26	intron	A	5877	0.85	0.036	CHARMS vs WTCCC2
	2	162872492	rs2268890	DPP4	CD26	intron	A	5890	1.13	0.047	CHARMS vs WTCCC2
	4	71937445	rs11941079	DCK	DCK	intergenic	G	5890	0.79	0.040	CHARMS vs WTCCC2
	6	86032269	rs313178	NT5E	CD73	intergenic	G	5891	1.19	0.041	CHARMS vs WTCCC2
	6	86042869	rs9444325	NT5E	CD73	intergenic	G	5878	1.14	0.028	CHARMS vs WTCCC2
	6	86060171	rs9450248	NT5E	CD73	intergenic	A	5894	1.15	0.025	CHARMS vs WTCCC2
	6	86067547	rs505923	NT5E	CD73	intergenic	G	5889	0.86	0.015	CHARMS vs WTCCC2
	6	86168265	rs6942065	NT5E	CD73	intron	A	5893	0.77	0.005	CHARMS vs WTCCC2
	20	43312397	rs912914	ADA	ADA	intergenic	A	5893	1.19	0.032	CHARMS vs WTCCC2

B

	CHR	BP	SNP	Gene region	Protein encoded	SNP position	Minor allele	NMISS	Odds ratio	P value	Analysis
MTX response analyses	1	21839690	rs975000	ALPL	AP	intron	G	284	1.44	0.04581	non-responders vs ACR70
	1	21840129	rs869179	ALPL	AP	intron	A	284	1.51	0.02549	non-responders vs ACR70
	1	21840498	rs1472563	ALPL	AP	intron	A	283	0.70	0.04669	non-responders vs ACR70
	1	21877074	rs885813	ALPL	AP	intron	A	283	0.66	0.02893	non-responders vs ACR70
	2	162929300	rs1861975	DPP4	CD26	intron	C	284	1.64	0.006774	non-responders vs ACR70

C

	CHR	BP	SNP	Gene region	Protein encoded	SNP position	Minor allele	NMISS	Odds ratio	P value	Analysis
Susceptibility analyses	1	21837755	rs16825455	ALPL	AP	intron	G	5421	0.70	0.04799	ExtOligo vs. WTCCC2
	2	162921135	rs3788976	DPP4	CD26	intron	A	616	1.36	0.01808	ExtOligo vs. PersOligo
	2	162921135	rs3788976	DPP4	CD26	intron	A	5423	1.32	0.01167	ExtOligo vs. WTCCC2
	6	86161269	rs3734442	NT5E	CD73	intron	G	5579	1.61	0.01945	PersOligo vs. WTCCC2
	6	86168265	rs6942065	NT5E	CD73	intron	A	5423	0.70	0.02696	ExtOligo vs. WTCCC2
	10	97618580	rs3181121	ENTPD1	CD39	intron	G	617	0.58	0.0427	ExtOligo vs. PersOligo

Table 4.2 Genes and associated SNPs investigated within the adenosinergic pathway

CHR, chromosome; SNP single nucleotide polymorphism, NMISS number of samples analysed, BP base pairs, CHARMS: Childhood arthritis response to medication study (which recruits children who are about to start treatment with MTX); WTCCC2, Wellcome trust case control consortium 2. ALPL: tissue alkaline phosphatase; ADA adenosine deaminase; DCK Deoxycytidine kinase. Coordinates relate to NCBI37 assembly genome build.

Summary table of SNP analysis between groups with p value differences <0.05 A) Susceptibility analysis from large region around gene. B) MTX response analysis from selected region of 5kb around each candidate gene. C) Susceptibility analysis limited to oligoarthritis patients.

In contrast to the difference found in CD73 protein expression by flow cytometry between synovial samples of persistent and extended patients, no significant variations were found around the NT5E (CD73) gene between the two subtypes of oligoarthritis in the susceptibility analysis (Table 4.2 C).

When each oligoarthritis subgroup was compared against healthy controls (WTCCC2), the persistent oligoarthritis samples were found to have a difference in SNP frequency of rs3734442, which lies in an intron within the NT5E (CD73) gene region, with an odds ratio of 1.61. This result suggests that carriage of the rs3734442 SNP increases the odds of being a persistent oligoarthritis JIA patient compared to healthy controls. In contrast, in extended oligoarticular JIA patients, the SNP rs6942065 located in an intron within NT5E (CD73), had a protective effect, compared to controls, with an odds ratio of 0.70 (Table 4.2 C). A variant is considered protective when it reduces the probability of people developing a disease, in this case extended oligoarticular JIA.

In the comparison between extended and persistent oligoarthritis patients, a SNP in DPP4 (CD26) was more common in extended oligoarthritis patients (p=0.01), whilst that in the ENTPD1 (CD39) gene region was more frequent among persistent oligoarthritis patients (p=0.04) (Table 4.2 C). This result was also unexpected, as no difference in expression of these two proteins had previously been found by flow cytometry between these two patient groups.

4.3 Discussion

In the analysis of CD73 expression by JIA synovial lymphocytes presented in the previous chapter, the reduction in CD73 positive cells appeared to be associated with clinical subtype of oligoarthritis. This is because less reduction in CD73 positive synovial lymphocytes was observed for samples derived from the joints of patients with moderate disease (persistent oligoarthritis) compared to those with the more severe extended oligoarthritis.

As cell activation was found to result in down regulation of CD73 expression (which will be presented in Chapter 6 of this thesis) and the JIA joint is known to present activated leukocytes (Black et al. 2002), it was hypothesized that the greater reduction in CD73 positive synovial lymphocytes observed for extended oligoarthritis compared to those with milder disease could be related to longer-lasting cell stimulation *in vivo.* For this reason, an association between CD73 expression by synovial CD8 and CD19 lymphocytes and disease duration was tested, but none was found (Figure 4.1A). In contrast, a significant difference was observed in the comparison between CD73 positive synovial lymphocytes from patients with persistent oligoarthritis and those with polyarticular JIA. This result is interesting as these two groups of patients have very similar (almost identical in this study) disease duration, but very different clinical outcomes as polyarthritis typically affects both large and small joints and can be very erosive and damaging, whilst persistent oligoarthritis is mostly self-remitting (Ravelli et al. 2007). The lack of association between CD73 expression and disease duration in a particular joint, would suggest that factors other than cell activation are influencing the proportion of CD73 positive synovial lymphocytes.

The significant association found between CD73 expression by synovial T and B lymphocytes and the total number of joints affected up till the time of sampling was not unexpected (Figure 4.1C), since the diagnosis of persistent and extended oligoarthritis is based upon the number of joints affected.

Since methotrexate, the main treatment for JIA, has been previously reported to increase adenosine levels in a mechanism dependent on CD73 activity (Montesinos et al. 2007), the relationship between treatment with this anti-inflammatory drug and CD73 expression was investigated. Firstly, the higher proportion of synovial T and B lymphocytes expressing CD73 from persistent oligoarticular patients compared to those with extended oligoarthritis was maintained in patients naïve to treatment with both MTX and steroids, suggesting that

medication is not a confounder in the analysis of the two groups. In addition, there was no difference in CD73 expression by T and B cells from blood of JIA patients before and after treatment with MTX (Figure 4.3). No difference was also found between CD73 expression by T and B peripheral blood lymphocytes from JIA patients of MTX responders (ACR70) and non-responders, either prior to or after-treatment, suggesting that the proportion of CD73 positive cells does not affect how patients respond to MTX. It must be kept in mind however, that the previously observed downregulation of CD73 positive cells from JIA patients was limited to synovial lymphocytes, which would have been the ideal cells to analyse in this study. Unfortunately, the cohort of samples measuring changes in protein levels before and after medication only includes blood and not synovial fluid samples. Synovial samples are rarely obtained after use of MTX in children who have responded well to MTX. Therefore, it was not possible to measure whether the proportion of JIA synovial fluid lymphocytes expressing CD73 was affected by MTX treatment.

Allelic variations of genes coding for enzymes involved in purine metabolism were then investigated, as they could affect the transcriptional control, protein expression and enzymatic activity of these ectonucleotidases. This could therefore potentially result in changes to the amount of available nucleotides and nucleosides. The genes analysed for SNPs were ENTD1, NT5E, DDP4, ADA, DCK, ALPL, PNP and AK1. In the genetic association study, few associations were found in both the susceptibility and MTX response analysis. Different SNP variations were found between WTCCC2 controls and CHARMS samples in the susceptibility analysis, which were instead not found between controls and all JIA samples (Table 4.2A). The explanation for this difference could be due to the different breakdown of the subtypes.

SNPs for ALPL (AP) were mostly overrepresented in healthy controls compared to JIA patients, which would fit well with the immunoregulatory function of the protein, involved with the dephosphorylation of nucleotides to nucleosides. Further investigation into the enzymatic activity of alkaline phosphatase is described in the next chapter. The only statistically significant differences in genotype frequencies found between persistent and extended oligoarthritis patients were in the DPP4 and ENTDP1 gene regions, encoding CD26 and CD39 respectively (Table 4.2C).

When potential SNPs were researched in the NT5E (CD73) gene region, no association was found between persistent and extended oligoarthritis patients. This could be the result of a lack of power due to small sample sizes. An association cannot be ruled out with the available numbers. However, the SNP rs3734442 was found to be more common in persistent oligoarthritis patients compared to healthy controls. In contrast, the SNP rs69420651 was found more commonly in healthy controls compared to extended oligoarthritis patients (Table 4.2C). It is not all clear at this stage, how these variations would affect expression of the CD73 protein, especially as both these SNPs are found within introns. Intronic SNPs have regulatory potential and can possibly alter transcript splicing. There is the possibility, therefore, that the first SNP could promote higher CD73 expression, whilst that the second could induce reduced CD73 expression.

The task of identifying "functional SNPs" that may be associated with disease phenotype is rendered particularly difficult by the fact that most SNPs are part of a larger region of linkage disequilibrium (LD). That is, the non-random association of two loci due to their being close to each other in the genome. When two SNPs have a high LD, they are more likely to be inherited together, which makes it more complex to define precisely which SNP has a biological effect (Bryzgalov et al. 2013). Additional challenges arise because a vast amount of SNPs, found both in this study and in GWAS studies in general, are in non-coding regions. Data obtained from the ENCODE (Encyclopedia of DNA elements) consortium suggests that because of their location, the underlying mechanism through which SNPs can have a phenotypic effect may be mostly regulatory (Schaub et al. 2012).

Previous genetic association studies investigating the effect of genotype on MTX response, found genetic differences between responders and non-responders to MTX, by focusing on genes that influence MTX transport and metabolism, some of which also affected adenosine levels. These included SNPs in the SLC16A7 gene (Moncrieffe et al. 2010a) a transporter for lactate and pyruvate, which can function as a minor scavenger of harmful hydrogen peroxide (Sitkovsky et al. 2005) and in the ITPA and ATIC gene (Hinks et al. 2011), both involved in the purine pathway. While SNPs in the purine and pyrimidine synthesis pathway including again ATIC, ADORA2 (which codes for the A_{2A} adenosine receptor) and TYMS (thymylidate synthase) were found to be associated with the three clusters of MTX polyglutamation, considered a useful predictor of drug response (Becker et al. 2011).

In contrast, in this study, the only SNP polymorphisms established between MTX responders and non-responders were within the ALPL (AP) and DPP4 (CD26) gene regions, with weak associations. Neither of the proteins encoded by these genes is considered however to have a particularly important role in the purine pathway. Dipeptidyl-peptidase 4 (also known as CD26), cleaves dipeptides off polypeptides and has no nucleotidase activity of its own, whilst it is an anchoring protein for ADA (adenosine deaminase) (Kameoka et al. 1993), which is responsible for adenosine deamination, but for which no SNPs were found in the MTX response analysis. ALPL (encodes AP) on the other hand, could influence the amount of available adenosine, at it can dephosphorylate tri-nucleotides to nucleosides. Because of its low affinity for AMP, however, and as alkaline phosphatase does not function optimally at neutral pH (Millán 2006), this enzyme is not considered to have a major role in purine metabolism.

It must be also kept in mind that a small p value seldom confirms an association, because of the very high likelihood of false positives; it merely represents a signal that must be validated in subsequent analyses, but at the same time that a non-significant result could also be due to a lack of power.

Further work would require validation on new cohorts, but also include experimental lab work to confirm the predicted effects of the SNPs on protein expression and function, together with gene expression and methylation analysis of synovial fluid cells and T cells particularly important in JIA, considering that gene expression/regulation is cell-type specific. The performance of additional genotyping to increase sample numbers would also increase the power to detect associations.

4.3.1 Summary and Conclusion

The main findings presented in this chapter are that:

- The reasons and mechanisms behind differences in CD73 expression by synovial lymphocytes observed between the two subtypes of oligoarthritis (persistent and extended) remain to be clarified, but are apparently not due to disease duration or medical treatment with MTX or steroids.

- Treatment with methotrexate for 6 months does not appear to alter ectonucleotidase expression by patient peripheral blood lymphocytes.

- Ectonucleotidase expression by patient PBMC does not seem to influence responder status to treatment with methotrexate.

- Only weak associations were found between genes that form part of the purine metabolic pathways and JIA susceptibility. The patterns observed were also not linked to those found in expression levels by flow cytometry.

Chapter 5 Purine nucleotide generation by ectonucleotidases expressed by leukocytes from the inflamed JIA joint and by peripheral blood cells

5.1 Introduction

ENTDP1 or CD39 is the ectonucleotidase functioning upstream of ecto-5'-nucleotidase, which hydrolyses ATP to ADP and then AMP. It is an integral membrane protein expressed by T and B lymphocytes, endothelial cells, monocytes-macrophages, neutrophils, subsets of activated NK cells (Enjyoji et al. 1999) and by human Treg (Borsellino et al. 2007). It is also recognised as a lymphoid cell activation marker (Maliszewski et al. 1994).

Ecto-5'-nucleotidase (CD73) is the key plasma membrane enzyme responsible for hydrolysing nucleotide monophosphates, by release of phosphate in the presence of water. The enzyme is expressed on most tissues in the body, but not on all cell types. As described in Chapter 3, CD73 expression is particularly high on $CD19^+$ B cells and $CD8^+$ T cells, whilst lacking on monocytes and neutrophils.

High CD39 expression on synovial T and B cells in the JIA joint has previously been described (Moncrieffe et al. 2010b) and was confirmed in Chapter 3. Elevated expression of this protein results in increased ATPase activity and therefore higher breakdown of ATP to AMP by synovial T cells compared to blood T cells (Moncrieffe et al. 2010b), ensuring fast removal of ATP, and potential accumulation of AMP or metabolites in the inflamed joint.

Alkaline phosphatase is another nucleotidase capable of hydrolysing nucleotides. Significantly, it is the only ectonucleotidase which can sequentially dephosphorylate nucleoside triphosphates to nucleosides, therefore synthesizing adenosine directly from ATP, but it has lower affinity for AMP compared to CD73 and does not function optimally at neutral pH, preferring an alkaline environment (Millán 2006).

Adenosine deaminase (ADA) deaminates adenosine to inosine in both the intracellular and extracellular environment despite being a cytosolic protein, as it can be anchored to the cell surface via a range of membrane proteins including CD26 (Kameoka et al. 1993), also known as dipeptidyl peptidase IV. ADA has low affinity for adenosine, with a Km ranging from 50-100μM (Fredholm et al. 2001).
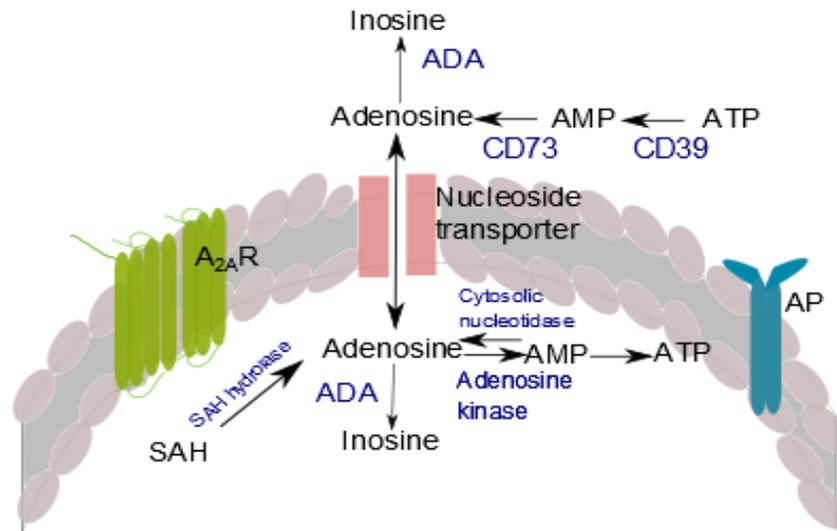
Figure 5-1 Depiction of main enzymes and proteins involved in the adenosinergic pathway

ADA adenosine deaminase, AP alkaline phosphatase, SAH *S*-adenosyl-homocysteine. Summary of the enzymes and proteins involved in altering the extracellular and intracellular levels of adenosine and its metabolites.

As Figure 5.1 shows, there are many enzymes involved in the adenosinergic pathway, regulating the intra- and extracellular levels of ATP and its derivatives. Among adenosine receptors, $A_{2A}R$ is the receptor subtype with the most immunosuppressive roles. Ligand binding to this receptor leads to intracellular accumulation of immunosuppressive cAMP metabolised from ATP via activation of the enzyme adenylate cyclase (Regateiro et al. 2013), functioning as a nonredundant regulator of activated T cells.

The aims of the experiments presented in this chapter were to:

1) Measure CD73 AMPase of synovial lymphocytes and synovial fluid, together with the activity of alkaline phosphatase in the same fluid and plasma;

2) Investigate the relationship between CD73 protein expression and adenosine-generating ability;

3) Test whether CD39-CD73 co-expression on the same cell is required for hydrolysis of ATP to adenosine;

4) Study the activity of adenosine-consuming enzyme adenosine deaminase;

5) Investigate $A_{2A}R$ expression in the joint.

5.2 Results

5.2.1 AMPase activity of PBMC is due to CD73

Reverse phase high-pressure liquid chromatography (HPLC) was used to study the enzymatic activity of CD73, measured by hydrolysis of E-AMP (Etheno-AMP) to E-adenosine. In this experimental system, E-AMP was broken down to E-adenosine by healthy PBMC (Figure 5.2A). E-adenosine is identified as a second peak on the chromatogram that runs at the retention time of 20min, while E-AMP appears at around 12min. To test whether this AMPase activity was due to CD73, PBMC were incubated with E-AMP in the presence of the CD73 specific inhibitor APCP. In this condition, there was negligible E-adenosine production, demonstrating that the observed breakdown was in fact due to CD73 activity (Figure 5.2B).

A
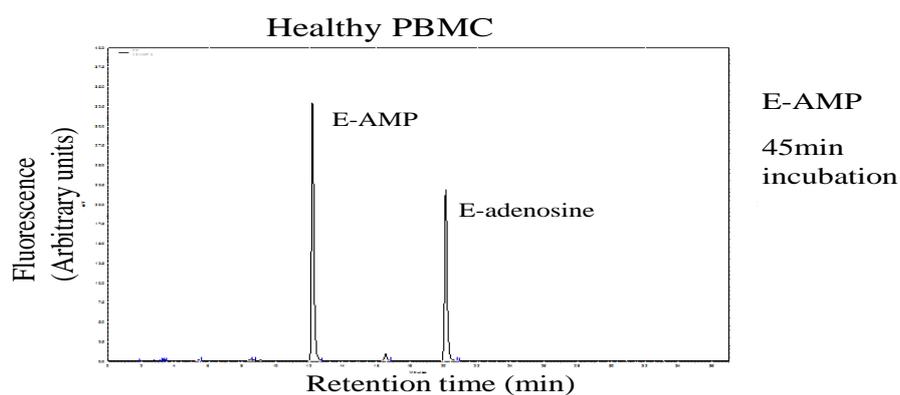
B
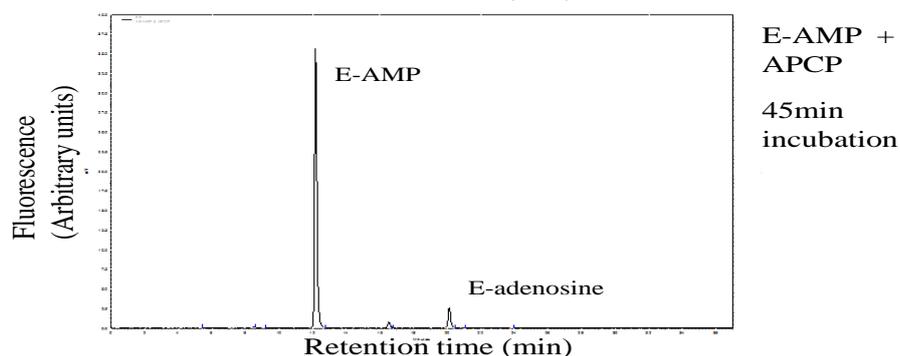
Figure 5-2 CD73 is responsible for the observed AMPase activity of PBMC

Representative HPLC chromatograms of the supernatants of healthy PBMC incubated with 25μM E-AMP for 45min at 37°C, in the absence (A) or presence (B) of 10μM APCP.

5.2.2 Reduced AMPase activity of SFMC

The HPLC assay was next used to determine whether the observed reduction in CD73 expression on synovial lymphocytes from the inflamed JIA joint (Chapter 3) resulted in reduced AMPase activity, and hence lower adenosine generation. Representative HPLC chromatograms of healthy PBMC, JIA patient PBMC and SFMC incubated with E-AMP show that synovial fluid mononuclear cells from the JIA joint had reduced AMPase activity as revealed by their decreased ability to produce E-adenosine (Figure 5.3A). This difference was observed in whole SFMC and purified synovial CD8^{+} cells, both of which generated lower levels of E-adenosine compared to whole PBMC and CD8^{+} sorted PBMC. In contrast, the AMPase activity of JIA PBMC did not differ from that of PBMC of healthy controls (Figure 5.3B). In order to study the kinetics of CD73 activity, both healthy PBMC and JIA SFMC were incubated with E-AMP for a range of periods of 30 to 60 minutes. As shown in Figure 5.3C, JIA SFMC metabolized E-AMP to E-adenosine at a slower rate compared to PBMC.

To test whether there was a direct correlation between the amount of CD73 positive cells and their CD73 enzymatic activity; the proportion and MFI of CD73^{+} cells was measured by flow cytometry in parallel with HPLC on the same samples and compared for CD8^{+} sorted samples (Figure 5.3 D). For healthy PBMC, no significant correlation (p=0.1071) was found between CD73 positive cells and their E-adenosine generation (Figure 5.3D, left), while there was a significant correlation between MFI of CD73 positive healthy CD8^{+} T cells and the amount of E-adenosine generated (p=0.0238) (Figure 5.3D, right). A lack of correlation between both the percentage of CD73 positive CD8^{+} T cells and the MFI for CD73 of CD8^{+}CD73^{+} cells and E-adenosine generated by JIA SFMC or JIA PBMC (Figure 5.3D) was observed.

A
Healthy PBMC | JIA PBMC | JIA SFMC

B

C

D
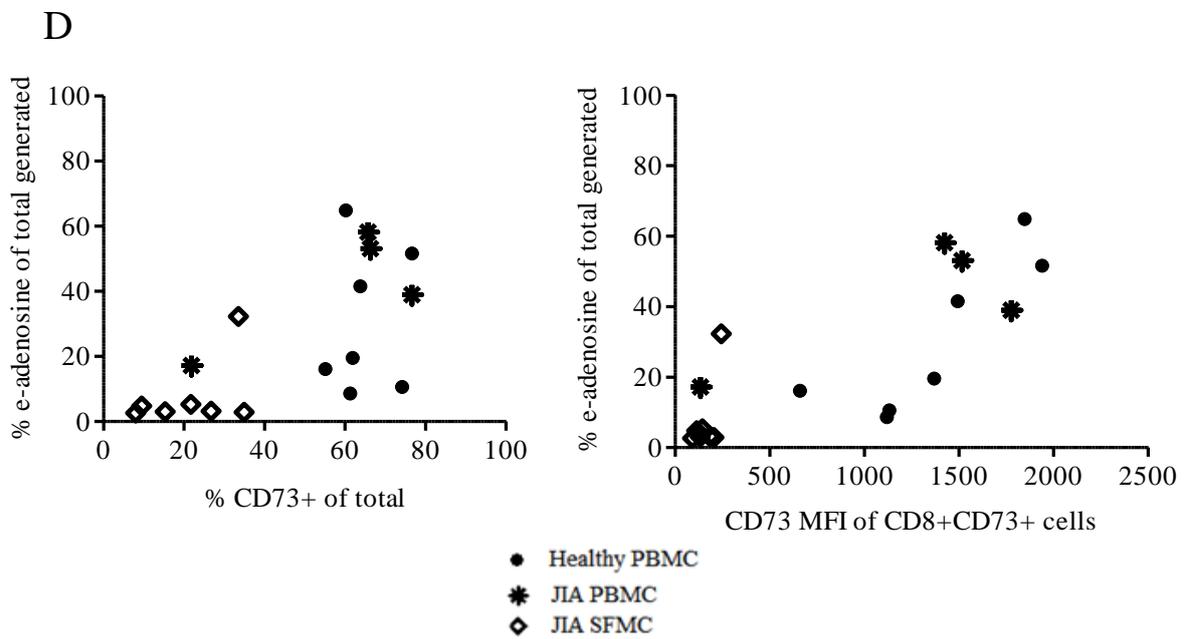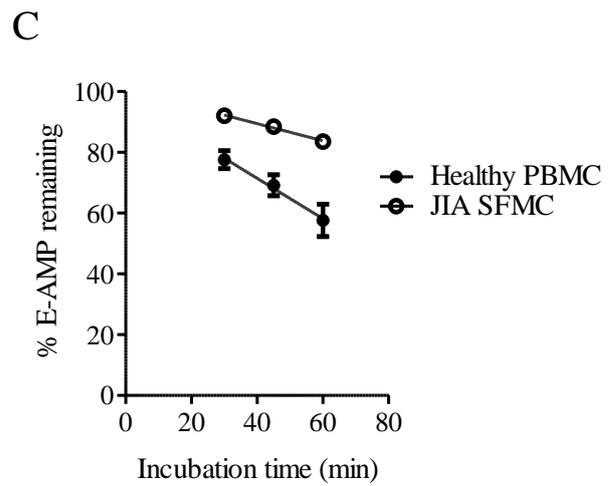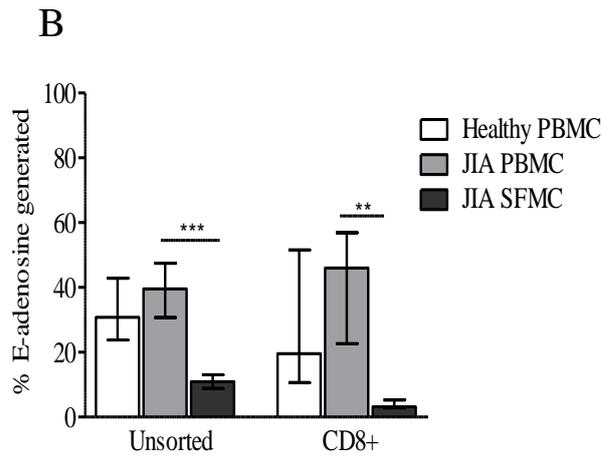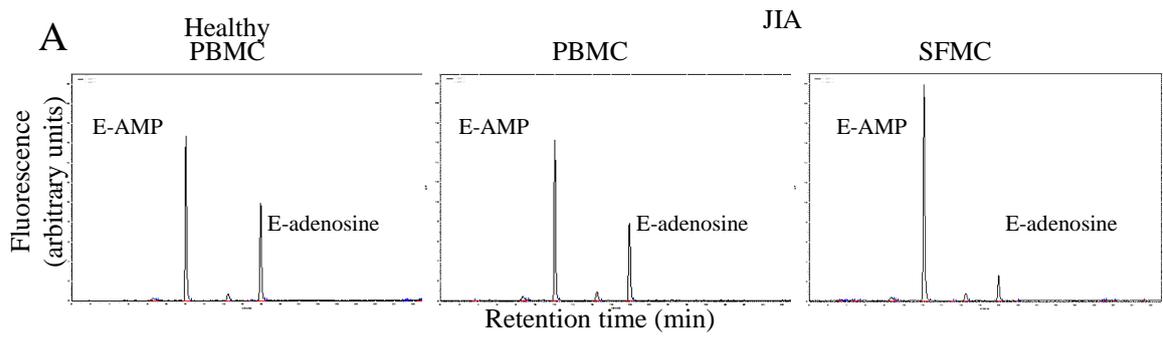
Figure 5-3 Lymphocytes from JIA joint are less able to hydrolyse E-AMP and generate adenosine

A) Representative HPLC chromatograms showing breakdown of E-AMP to E-adenosine by healthy control PBMC, JIA PBMC and SFMC incubated with 25μM E-AMP for 45min. B) Graph shows summary data for E-adenosine generated by healthy PBMC, JIA PBMC and JIA SFMC, (n=17, 5, 10 respectively) and CD8$^+$ bead-sorted healthy PBMC, JIA PBMC and JIA SFMC (n=7, 4, 7 respectively); statistical significance tested by ANOVA. Bars represent medians, error bars IQR. C) Comparison of the rate of breakdown of E-AMP by SFMC (n=5) and healthy PBMC (n=5) incubated for 30, 45 or 60 minutes. D) Correlation of the percentage of CD73 positive cells (left) and of CD73 MFI of CD8$^+$CD73$^+$ cells (right) with E-adenosine generation for healthy PBMC (n=7), JIA PBMC (n=4) and JIA SFMC (n=7).

5.2.3 No AMPase activity observed in synovial fluid or plasma

In order to test the hypothesis that the reduced CD73 expression observed on synovial lymphocytes may be due to shedding of CD73 protein from the cell surface, measurement of CD73 protein levels and activity was tested in synovial fluid (SF) itself (after removal of cells). A commercially available ELISA kit for CD73 was used to determine the amount of CD73 protein present in a pool of synovial fluid and compared to that found in a pool of healthy plasma. The preparation of these pools is described in Chapter 2, section 2.1.7. This showed that levels of CD73 protein in synovial fluid were higher than in plasma from healthy controls (Figure 5.4A). This result could suggest that indeed, some of the loss of CD73 protein on JIA synovial lymphocytes is due to shedding into the synovial fluid.

However, although soluble CD73 protein was detected in both plasma and synovial fluid, there was no formation of E-adenosine from E-AMP, after incubation with this substrate, indicating a lack of AMPase activity (Figure 5.4 B). This result suggests that either soluble CD73 (detectable by ELISA) is not able to hydrolyse E-AMP to E-adenosine, or that the levels of CD73 present in the fluids tested were too low to be enzymatically active.

A
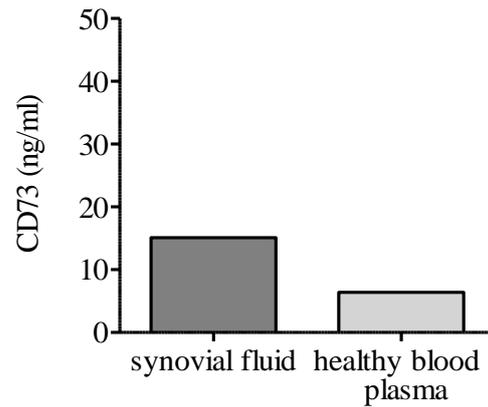
B
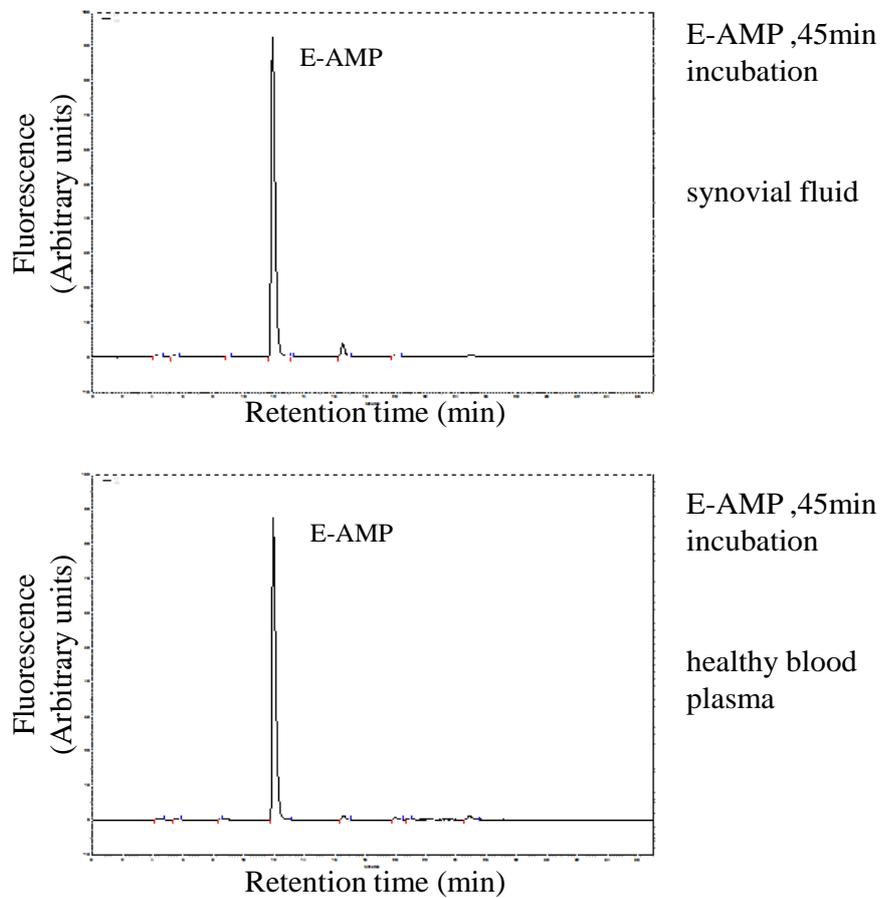
Figure 5-4 Measurement of CD73 protein content and activity in plasma and synovial fluid

A) CD73 concentrations were determined by ELISA in pools of plasma and SF, made from 5 healthy blood and 10 synovial fluid samples respectively. Each pooled sample was tested in duplicate. Bars represent the mean of duplicate measurement. B) Plasma and synovial fluid pools were incubated with 25μM E-AMP for 45min and supernatants assayed by HPLC.

5.2.4 Coordinated ATP and AMPase activity is specific to B cells and not to T cells

Inflammatory environments, such as the JIA joint, are associated with elevated extracellular release of nucleotides such as ATP from immune cells, in both a disordered fashion and by controlled molecular pathways (Idzko et al. 2014). Because both ectonucleotidases, CD39 and CD73, are required for hydrolysis of ATP to adenosine, their protein coexpression was investigated by flow cytometry (Chapter 3). This investigation found B cells to coexpress CD39 and CD73, while T cells did not.

To investigate the functional capacity of T and B-lymphocytes to breakdown ATP to adenosine, healthy PBMC were sorted by flow cytometry, as described in Methods section 2.3.2, into CD73 positive and negative CD19 and CD8 cell populations. The average purity for these sorted populations was >90%.

When sorted $CD19^+CD73^+$ cells from samples of healthy blood PBMC (n=4) were incubated with E-ATP they were found able to generate E-adenosine (Figure 5.5A), consistent with coexpression of CD39 and CD73. The levels of adenosine generation were relatively low after incubation with 25µM E-ATP for 45 min, but could have potentially been raised by increasing incubation periods. In contrast, healthy PBMC $CD8^+$ T cells (n=4) were not able to generate E-adenosine from E-ATP (either $CD73^+$ or $CD73^-$ $CD8^+$ cells), consistent with their lack of CD39 expression and therefore ATPase activity (Figure 5.5B).
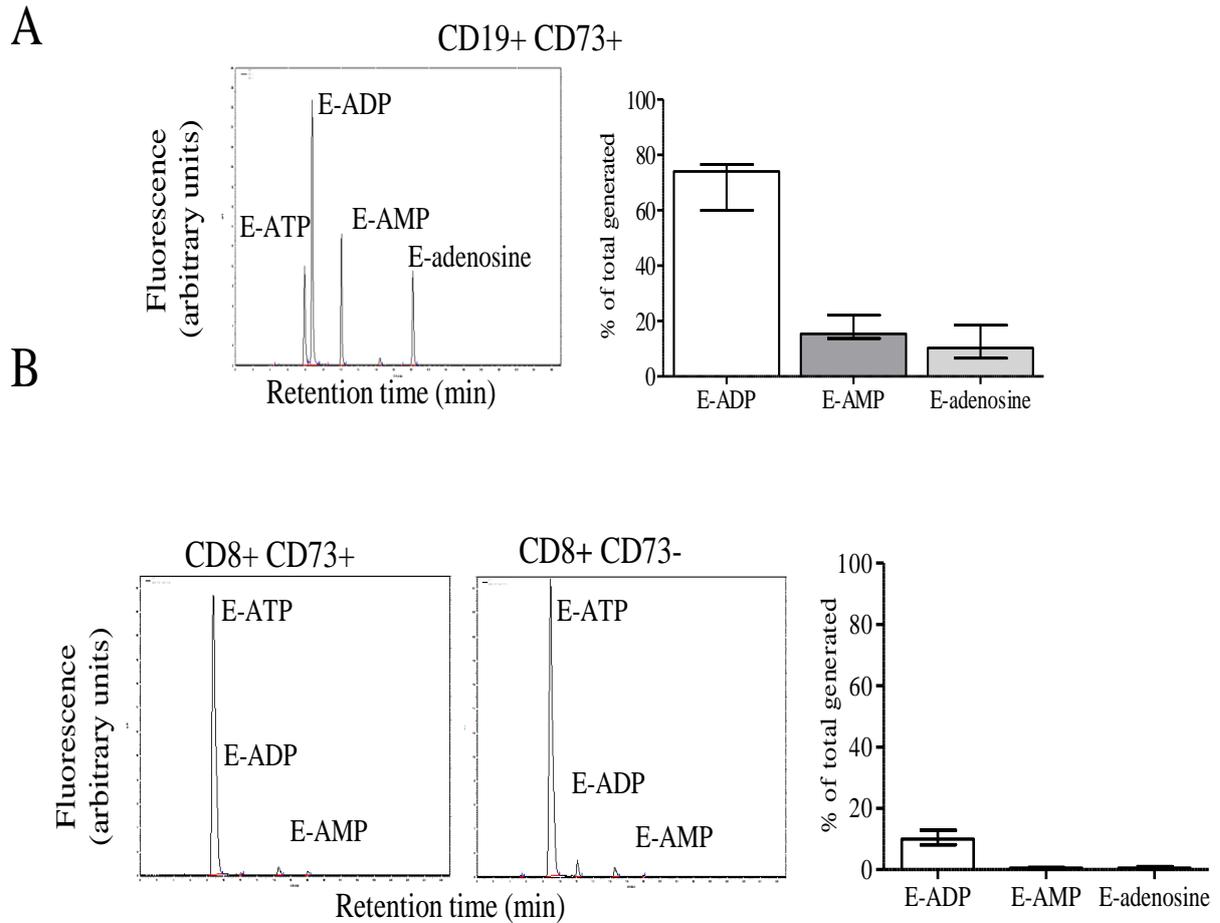
Figure 5-5 CD19$^+$ cells are able to generate adenosine from ATP.

A) Representative HPLC chromatogram of the supernatant of sorted healthy CD19$^+$CD73$^+$ cells incubated with 25μM E-ATP for 45min and summary data of nucleotide and nucleoside generation by these cells (n=4). B) Representative HPLC chromatograms of sorted healthy CD8$^+$CD73$^+$ and CD8$^+$CD73$^-$ cells incubated with 25μM E-ATP for 45min and summary data of generation of E-ATP metabolites by CD8$^+$CD73$^+$ cells (n=4). Bars represent medians, error bars IQR.

5.2.5 Expression of CD39 and CD73 on the same cell is required for adenosine generation

The data above suggested that B cells are the only lymphocyte subset that expresses both CD39 and CD73, and which are able to hydrolyse ATP directly to adenosine. However, B cell proportions are found in restricted proportions in the JIA joint (Chapter 3, section 3.2.4). It was therefore necessary to test whether coexpression of CD39 and CD73 proteins on the same cell is required for adenosine generation, or whether nucleotide dephosphorylation can occur with CD73 and CD39 on different cells acting cooperatively.

In order to test this, cell populations, which express each protein separately, were required. $CD8^+$ T cells express high levels of CD73 with E-AMPase activity, but do not have detectable ATPase activity (Figure 5.5B), because of their lack of CD39 protein (Chapter 3). In contrast, monocytes, which are CD73 negative (Chapter 3) and have no AMPase activity, express very high levels of CD39 and present high ATPase activity as measured by breakdown of E-ATP to E-AMP *in vitro* by HPLC (Figure 5.6). $CD8^+$ $CD73^+$ T cells and $CD14^+$ $CD39^+$ monocytes from PBMC were therefore sorted and co-cultured in the presence of E-ATP and the resulting culture supernatants analysed by HPLC. E-AMP was produced in the supernatants from these co-cultures (monocytes and $CD8^+CD73^+$ cells), showing that CD39 was able to mediate its effect, whereas no E-adenosine was generated by the combination of $CD39^+$ and $CD73^+$ cells (Figure 5.7A). In contrast, and as a positive control, unsorted PBMC from the same individual were able to generate E-adenosine (Figure 5.7B). This result demonstrates the importance of CD39 and CD73 coexpression on the same cell for generation of adenosine from ATP and a lack of complementation between $CD39^+$ and $CD73^+$ cells.

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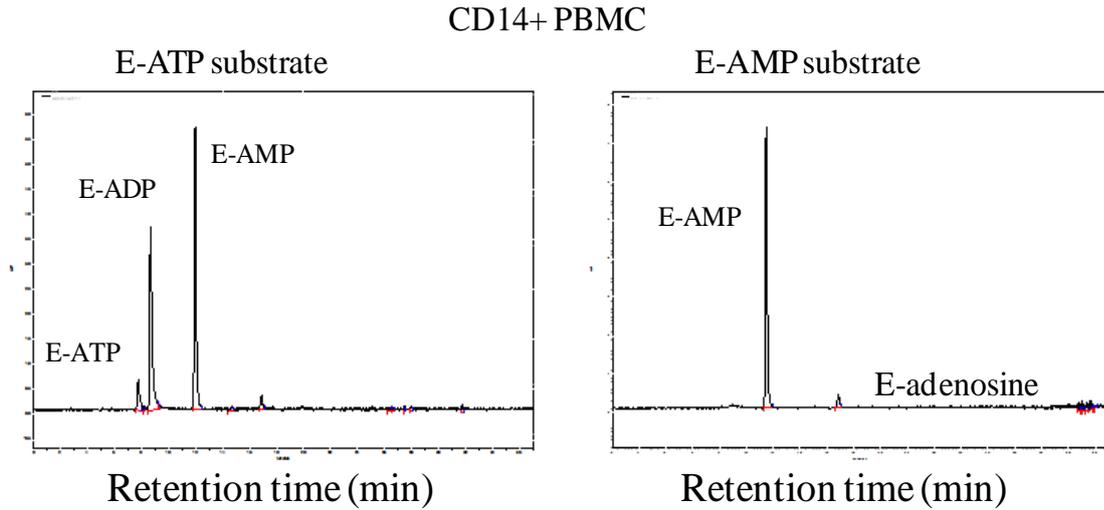
Figure 5-6 High ATPase and lack of AMPase activity of monocytes

Representative HPLC chromatograms of the supernatant derived from flow cytometry sorted CD14$^+$ monocytes incubated either in the presence of 25µM E-ATP (left) or 25µM E-AMP (right) for 45min, after which the reaction was stopped by the addition of 15mM HCl.

A

CD8+CD73+ co-culture with CD14+CD39+
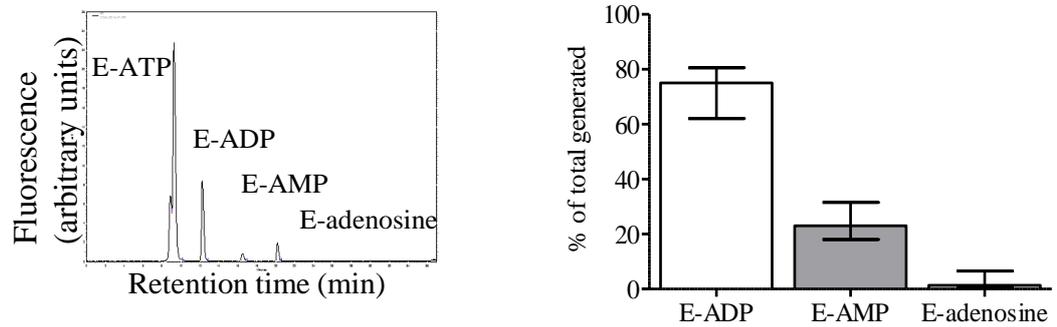
B

Total PBMC
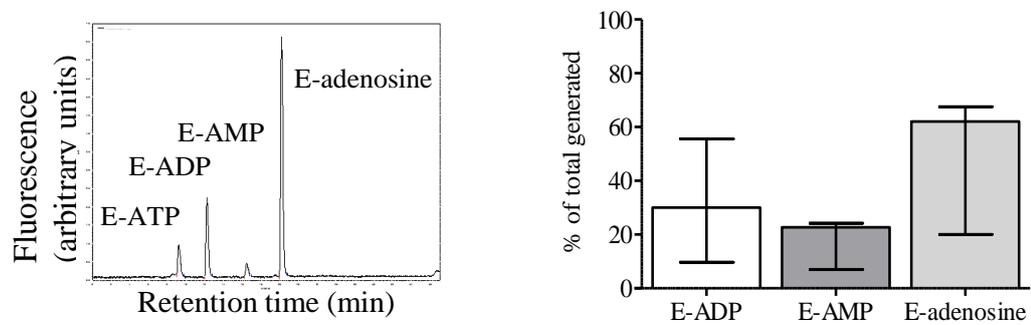
Figure 5-7 ATP hydrolysis to adenosine requires CD39-CD73 co-expression on same cell

A) Representative HPLC chromatograms and summary bar graphs of supernatant derived from CD8$^+$CD73$^+$ and CD14$^+$CD39$^+$ cells co-incubated with 25μM E-ATP for 45min (n=5). B) Representative HPLC chromatograms and summary bar graphs of unsorted PBMC (n=4) incubated with 25μM E-AMP for 45min. Bars represent medians, error bars IQR.

5.2.6 Alkaline phosphatase activity is not increased in synovial fluid

Alkaline phosphatase (AP) is another ectonucleotidase, which is capable of generating adenosine from adenosine monophosphate (AMP) (Millán 2006). Unlike CD73 however, AP can also metabolize 5'-tri and di- phosphates. This enzyme therefore has the combined action of CD39 and CD73, and can metabolize ATP and ADP directly to adenosine. The activity of AP was measured in synovial fluid to determine whether another enzyme was capable of compensating for the lack of CD73-generating adenosine on synovial lymphocytes.

P-nitrophenylphosphate (pNPP) is the most common substrate to measure the activity of AP. This phosphatase substrate generates the yellow coloured *p*-nitrophenol (*p*NP) after dephosphorylation by AP. The amount of *p*NP generated in this colorimetric assay was used to determine AP activity (see Chapter 2, section 2.9).

The activity of AP was measured in patient synovial fluid and compared to that in both JIA patient and healthy control blood plasma (Figure 5.8). This shows decreased AP activity in healthy adult plasma samples, with a median of 51 U/ml, compared to the 240U/ml and 252U/ml found in JIA patient plasma and healthy child plasma, respectively. Decreased AP activity was also found in JIA patient synovial fluid, compared to JIA plasma, with a median AP activity of 129 U/ml in SF samples, illustrating that there is no increase in AP action in the inflamed joint compared to blood (Figure 5.8).
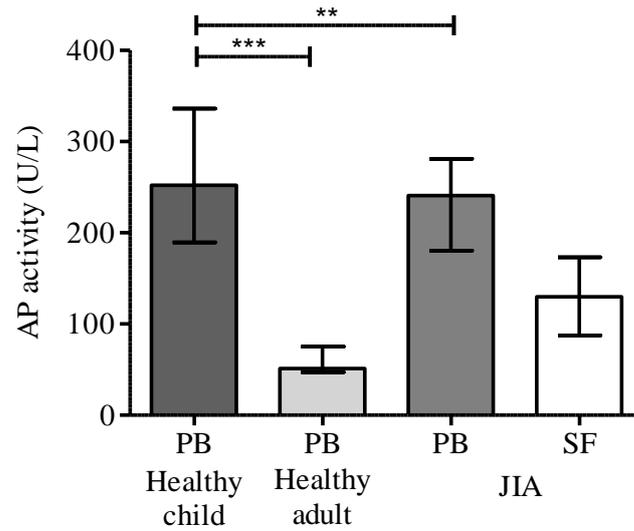
Figure 5-8 Alkaline phosphatase activity in plasma and synovial fluid

Generation of p-nitrophenol (pNP) by dephosphorylation of substrate p-nitrophenyl phosphate (pNPP), as an indicator of AP activity in blood plasma of healthy children (n=6), healthy adults (n=8), JIA patients (n=7) and patient synovial fluid (n=10). Data are medians with interquartile range, statistically analysed by Kruskal-Wallis test with Dunn's multiple comparison.

5.2.7 Adenosine deaminase activity

Next, an attempt was made to measure ADA (adenosine deaminase) activity in order to determine how much of the adenosine generated is metabolized to inosine. This enzyme deaminates adenosine to inosine both intracellularly and extracellularly and is anchored to the cell membrane by CD26 (Kameoka et al. 1993). The surface expression of CD26 can be used as a surrogate marker of ADA expression, the results of which obtained in this study for PBMC and SFMC were presented in Chapter 3, section 3.2.9.

After conversion to their etheno forms using the method previously described (Larson et al. 2001) (Figure 5.9) (see Chapter 2, section 2.7.3), ATP, ADP, AMP, adenosine and adenine can be very sensitively detected as their fluorescent etheno analogues
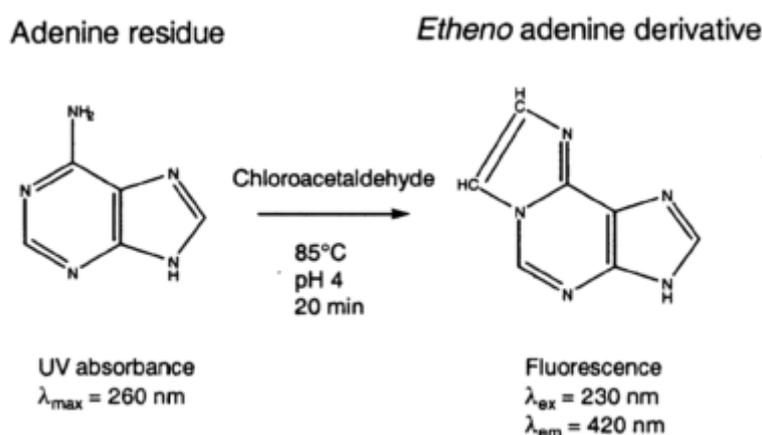
Figure 5-9 Formation of etheno adenine derivatives

Due to the lack of an adenine ring in the inosine structure however, inosine does not form etheno derivatives and must be detected together with the other nucleotides/nucleosides by UV absorbance, which does not allow for such specific and sensitive detection. Although an attempt to establish such a method was made, there were considerable problems with developing this method.

In reversed phase HPLC, more polar compounds (i.e. ATP) are eluted fast. In order to separate these compounds, it was necessary to use an ion-pairing reagent, TBAHS, which neutralises the charges on the nucleotides and therefore results in a reversed order of elution (i.e. ATP elutes last). The adenine compounds have an absorbance maximum at about 255nm, while inosine has an absorbance maximum at 248nm. Thus, it was not possible to distinguish which of the two nucleotides composed the fourth peak in the chromatogram in

Figure 5.10A. Further method development would be necessary in order to accurately measure inosine generation. An additional problem to this method would be that since the inosine is not etheno derivatized, it is not possible to be certain that it is originated from adenosine and not from other sources. The inosine could be due for example to inosine already present in the fluid and not a product of adenosine deaminase activity; proof of the lack of specificity of a non-etheno method.
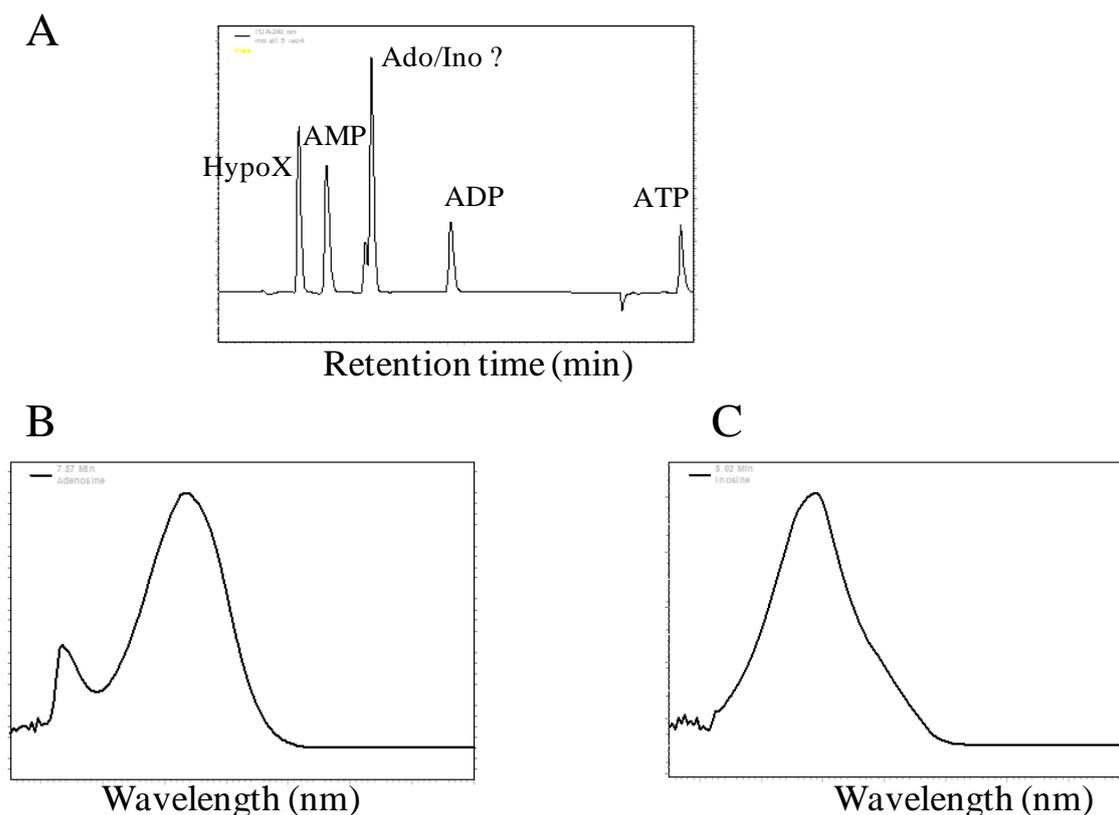
Figure 5-10 Attempts of running non-etheno standards

Representative HPLC chromatogram of non-etheno standard mix runs with eluents 0.2M KH$_2$PO$_4$, 5mM TBAB run with an acetonitrile gradient. Mix of non-etheno standards was composed of ATP, ADP, AMP, adenosine, inosine, and hypoxanthine. The quantities of purines were detected by absorption at 240nm. Ado=Adenosine, Ino=Inosine, HypoX= Hypoxanthine. Absorbance spectra peak of adenosine (G) at 257nm and inosine (H) at 248nm.

5.2.8 $A_{2A}R$ expression in the joint

Due to rapid fluctuations in local adenosine levels and limitations in the current analytic technologies, no definitive measurement of extracellular adenosine concentrations and its upstream nucleotides can be obtained currently. This is a major limitation in the field of purine research. As receptor availability has an important part in determining the potential effect of a ligand, expression of adenosine receptor on blood and synovial lymphocytes was investigated instead. A_{2A} receptor was chosen for this experiment, as it plays a key role in the inhibition of the inflammatory process.

Figure 5.11 shows that $CD8^+$ bead-sorted PBMC and SFMC had the same amount of $A_{2A}R$ protein (MW 45kDa), implying that in presence of the same amount of adenosine these two groups of cells should have the same ability to respond to adenosine.

While for unsorted cells, SFMC had more β-actin than PBMC, suggesting less overall $A_{2A}R$ in SFMC compared to patient and healthy control PBMC. The reason for the different number of bands between and unseparated and separated cells is unknown.
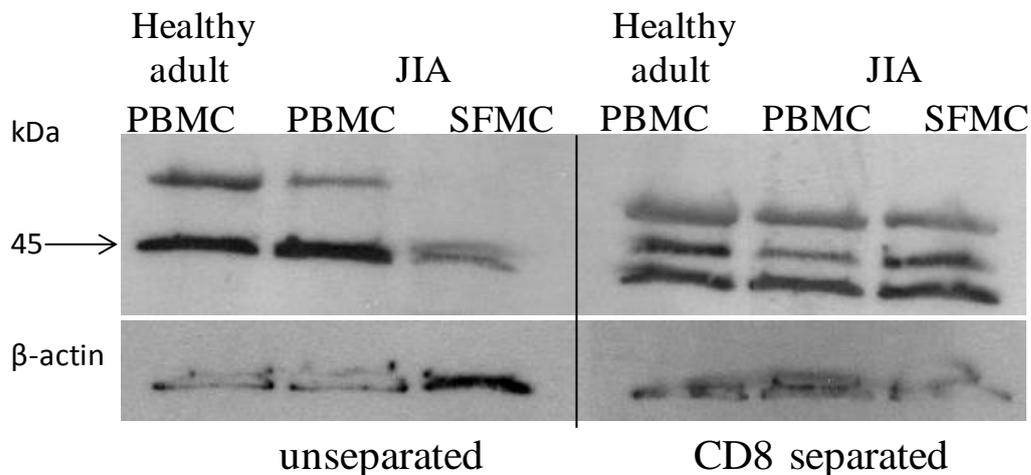
Figure 5-11 $A_{2A}R$ protein expression in PBMC and SFMC

Western blotting experiment showing $A_{2A}R$ expression, using an $A_{2A}R$ specific antibody, in total (left) and $CD8^+$ bead-sorted PBMC and SFMC (right) from healthy adult and JIA patient as shown. β-actin was determined in parallel and used as an internal standard.

5.3 Discussion

In Chapter 3, alterations in the expression of CD39, CD73 and CD26 (a marker for ADA) by JIA synovial lymphocytes compared to lymphocytes from both patient and healthy control blood were observed. The proportion of synovial lymphocytes expressing CD39 was found to be increased, whilst that of cells positive for CD73 and CD26 was decreased. The focus of the experiments discussed in this chapter was therefore to examine whether the observed changes in protein expression also affected the ability of the enzymes to metabolise nucleotides. As summarised in Figure 5.1, these enzymes, together with alkaline phosphatase, are the main extracellular regulators of the breakdown of ATP, and of the generation and metabolism of adenosine.

Analysis of the conversion of ATP to adenosine is of interest, particularly at the site of inflammation, because these two purines are generally considered to exert opposing biological effects in the context of immune homeostasis. ATP is regarded as being mostly pro-inflammatory, a danger signal released following tissue damage, with chemotactic effects on phagocytes (Elliott et al. 2009), able to trigger NF-κB activation (de Oliveira et al. 2014). Whilst adenosine is referred to as a safety signal that dampens and attenuates the damaging effects of cell activation by inhibiting TCR-triggered events, such as upregulation of CD25 expression (Huang et al. 1997) and differentiation of naive CD8^{+} T cells (Linnemann et al. 2009). Experimental studies in which this conversion was inhibited, by knocking out CD73 and impairing adenosine production, resulted in increased leukocyte traffic to the site of inflammation, exacerbation of tissue damage and impairment of feedback regulation (Eltzschig et al. 2004; Louis et al. 2008; Takedachi et al. 2008). These results provide evidence of the protective effects of adenosine signalling.

At sites of inflammation such the JIA joint, where the tissue is damaged and inflamed and the infiltrating leukocytes are activated (Black et al. 2002), it could be supposed that there is an elevation of release of ATP (Schenk et al. 2011). Due to this potential elevated ATP and the previously observed elevation in the proportion of CD39^{+} cells and ATPase activity of JIA synovial lymphocytes (Moncrieffe et al. 2010b), the levels of AMP in the JIA joint are presumed to also be elevated. Measurement of CD73 AMPase activity in the joint was therefore important to understand whether AMP would be converted to adenosine at the site of inflammation.

The experiment in which PBMC were incubated with E-AMP solution to test their AMPase activity, either with or without the CD73 competitive inhibitor APCP, which binds to the catalytic site of CD73 without being hydrolysed, confirmed the activity observed to be due to CD73. It was therefore investigated whether the reduced CD73 expression resulted in a corresponding decrease in enzyme activity. This was indeed the case, as both whole SFMC and CD8$^+$ bead-sorted SFMC show reduced E-adenosine generation from E-AMP. This reduced AMPase activity was also observed over several time points, indicating a slower rate of E-AMP breakdown for SFMC.

For healthy PBMC, a correlation was found between the amount of adenosine generated and CD73 median protein expression level per CD73$^+$ cell. As shown in Figure 5.3D, there was no correlation between CD73 protein and enzyme activity of JIA SFMC as shown by the fact that even though some CD73 expression was detectable, there was almost no generation of E-adenosine by these cells. This would suggest that in addition to the low proportion of CD73 positive SFMC, there was potentially a further dysfunction. It must also be considered that E-AMP is an analogue of the natural AMP and so it is assumed that CD73 is equally able to metabolize the etheno form. Interestingly, ethenoadenosine is not a substrate for adenosine deaminase (Jamal et al. 1988), suggesting that in this experiment it does not get further metabolised to inosine.

Despite having found a reduction of CD73 positive cells for both CD8$^+$ T cells and CD19$^+$ B cells (Chapter 3), because of the low proportion of B cells in the joint, with an average <1% of SFMC, it was not possible to sort SFMC for CD19$^+$ cells to test their AMPase activity.

Results shown in Figure 5.4 would appear to suggest that cellular CD73 has a nonredundant role, as no AMP breakdown was observed in either healthy control plasma or JIA synovial fluid, despite having detected the presence of soluble CD73 protein in both fluids. Previous studies in RA patients have observed increased 5'-nucleotidase activity in synovial fluid compared to both synovial fluid of other arthritis patients such as osteoarthritis and the serum from RA patients (Johnson et al. 1999). Another study by Johnson (from 2008), suggested that the raise in 5'-nucleotidase activity in synovial fluid in RA patients could be due to infection with *Mycoplasma fermentas* found to have its own CD73 activity and to be able to increase that of B cells. These previous studies measured 5'-nucleotidase action by

scintillation with the use of [2-^{3}H] AMP, according to the method of (Rowe et al. 1979), while in this study etheno-AMP was utilised.

Alkaline phosphatase (AP) an enzyme capable of metabolising ATP to adenosine, was then also measured in the synovial fluid of patients and plasma of healthy controls, in order to understand whether it could contribute to adenosine generation in the inflamed joint. The levels of AP enzymatic function in healthy child blood were raised compared to those measured in both JIA patient blood and synovial fluid, which contained the lowest with a median of 129U/ml (Figure 5.8). This finding was in contrast with previous results of AP quantification, which previously had been found elevated in patients with autoimmune diseases, including rheumatoid arthritis (Hanna et al. 1997). AP enzyme is known to have lower affinity for its substrates compared to CD73, (CD73 Km of 1-50 μM, (Heuts et al. 2012)) with Km values in the low millimolar range, and to work preferentially in an alkaline environment (Zimmermann 2000). This information, together with the observation of low AP activity in JIA joint, raises the question of whether there is enough AP in the inflamed joint, and whether it is in the right environment, to convert ATP to adenosine independently of CD39 and CD73. Because of the presence of this enzyme, there could be some extent of adenosine generation in the JIA inflamed synovium.

As shown in Figure 5.10, it was not possible to determine clear and separated peaks for adenosine and inosine detected by UV absorbance; therefore, it was not possible to measure ADA activity of either PBMC or SFMC. Another method formerly used to measure the nucleotide/nucleoside-converting ability of ectonucleotidases is the analysis of the metabolism of radiolabeled nucleotides by thin layer chromatography (TLC). This technique, which could have been used to assess ADA activity, has the advantage of using [^{14}C] radiolabeled substrates which are identical to natural substrates and no assumption of identical enzyme specificity needs to be made. However, TLC compared to HPLC is less clean, has lower resolution, sample throughput, and is not quantitative. As the measurement of ADPase activity by TLC in the results of (Longhi et al. 2014) show, there are many nonspecific and unidentifiable bands in the readout, preventing clear identification of metabolites.

Even if it had been possible to quantify ADA activity and know how much inosine was being produced in the synovium, unlike the broad and powerful immune modulator that is

adenosine, not much is known of the function of inosine, which for a long time had been considered simply an inactive derivative of adenosine. This nucleoside has however also been identified to bind to adenosine receptors, for example to the A_3R, leading to mast cell degranulation (Jin et al. 1997). Inosine can also augment extracellular adenosine levels by preventing its uptake inside the cell, as when intracellular build-ups of inosine occur, it gets shunted to the extracellular space by equilibrative nucleoside transporters (ENT) for which adenosine and inosine compete (Haskó et al. 2004). Therefore, the study of enzymes that modify intracellular adenosine concentration is also important. ENT1 allows adenosine to freely cross the cell membrane according to its concentration gradient, so if found elevated inside the cells, adenosine would simply dissipate to the extracellular space. This adenosine transporter is inactivated following A_{2A} receptor occupancy (Montesinos et al. 2003) and ethanol uptake (Krauss et al. 1993), causing elevated extracellular adenosine levels.

Measuring levels of adenosine and ATP and its metabolites in the JIA patient's joint and comparing them to those found in non-inflammatory conditions would have been ideal to determine their role in the regulation of inflammation in arthritis. However, during sampling, tissue extraction and platelet destruction leads to release of ATP and rapid metabolism to adenosine, resulting in levels much higher to those initially present. As the accurate determination of adenosine in physiological and pathological conditions is very difficult to achieve, the literature is replete with incorrect estimates, which cannot be used as reference. This is also the case for the measurement of ATP in human plasma, which in several studies ranged from 28 to 11000 nmol/L (Gorman et al. 2007). Adenosine receptor expression was investigated instead as an alternative, since the availability of receptors to which the ligand can bind, determines the possible formation of ligand-receptor complexes, and the higher the number of receptors available, the more potent and/or efficacious the agonist.

Among adenosine receptors, $A_{2A}R$ is the one with the highest affinity for adenosine and is the most widely distributed on lymphoid cells (Adair 2005); on these grounds it was chosen for analysis in this study. The preliminary investigation of $A_{2A}R$ expression on synovial lymphocytes appeared to suggest that there is no difference between SFMC and PBMC of both healthy controls and patients. These data were again in contrast with findings in RA, were $A_{2A}R$ together with A_3R, by mRNA assay and Western blotting, were found to be upregulated on peripheral blood lymphocytes from RA patients (Varani et al. 2011). At the

same time, an abundance of A_3R was found expressed in the rheumatoid synovium (Stamp et al. 2012).

Other enzymes that would have been interesting to investigate include *S*-adenosyl-homocysteine hydrolase and adenosine kinase, together with other kinases, which contribute to ATP-regeneration such as adenylate kinase. Intracellular adenosine can be formed by the action of cytoplasmic 5'-nucleotidase and that of *S*-adenosyl homocysteine (SAH) hydrolase that converts SAH to adenosine and homocysteine. This pathway however, is believed to contribute only marginally to adenosine production (Adair 2005).

5.3.1 Summary and Conclusion

Data described in this chapter provide a picture of a dysregulated adenosinergic pathway within the JIA joint. The previously published data revealing a raised proportion of CD39^{+} and therefore ATPase^{+} lymphocytes within the JIA joint (Moncrieffe et al. 2010b) reveals potential for an accumulation of pro-inflammatory AMP at the site of inflammation. The data in this study outlining low CD73 and defective AMPase of SFMC maintain this hypothesis of retention of high levels of AMP with limited adenosine generation.

In this study, B cells have been observed to be, among lymphocytes, the only cell type to express both CD39 and CD73. Additionally, B cells are known to be limited in the JIA joint and a lack of complementation was found between CD39^{+} cells and CD73^{+} cells, in that both proteins were required to be expressed on the same cells for the full phosphorylation of ATP to adenosine. This implies that it is unlikely for adenosine to be produced sufficiently to mediate potent anti-inflammatory effects in the inflamed JIA joint.

Analysis of A_{2A} receptor expression by PBMC and SFMC suggested similar levels in either healthy or inflamed conditions. It would have been ideal however, to measure also the expression of $A_{2A}R$ and that of the other adenosine receptors on other cell subsets found in the synovium (such as CD4^{+} T cells), their density, and their affinity for their ligand adenosine.

159

Chapter 6 The synovial milieu and its effects on the expression of ectonucleotidases on leukocytes

6.1 Introduction

The inflamed, hyperplastic synovial joint compartment of patients with juvenile idiopathic arthritis is characterized by high levels of infiltrating T and B lymphocytes, monocytes and neutrophils, found particularly in the sublining layer (Bywaters 1977; Murray et al. 1996). These cells form lymphoid aggregates, in a few cases even with germinal centers, suggesting the process of lymphoid neogenesis may be present and contribute to inflammation (Gregorio et al. 2007). Degradative enzymes, such as metalloproteinases, which can mediate cartilage and bone erosion, are released into the synovial fluid by fibroblast-like synoviocytes (Gattorno et al. 2002a) and are expressed on the synovium of JIA patients (Gattorno et al. 2002b).

The results in previous chapters of this thesis illustrated how among both human peripheral blood and JIA synovial fluid mononuclear cells, CD73 expression is limited to T and B lymphocytes. Synovial lymphocytes were observed to present a lower percentage of CD73 positive cells compared to their blood counterparts with a corresponding reduction in CD73 AMPase activity (Chapter 3 and 5).

Lymphocytes obtained from the fluid of the inflamed joints of JIA patients are known to have an activated phenotype with elevated expression of the activation markers CD69, HLA-DR and CD25 on T cells (Black et al. 2002) and of costimulatory molecules CD80-CD86 and cell surface CD69 on B cells (Corcione et al. 2009; Morbach et al. 2011). In addition, the JIA joint cells show enrichment of CD45RO^{+} memory T cells (Wedderburn et al. 2000) and of CD27^{+} class switched memory B cells (Morbach et al. 2011). It was therefore interesting to investigate whether activating signals for T and B cells (such as stimulation through the TCR for T cells and through BCR, TLR9 and CD40 for B cells) might induce alterations in the surface expression of CD73 protein.

The microenvironment of the JIA synovium is a proinflammatory milieu characterized by the presence of elevated levels of the cytokines IL-1, IL-6 and TNF-α (de Jager et al. 2007), as well as multiple chemokines such as CCL3 in synovial fluid and CCL5 in synovial CD8^{+} T lymphocytes (Pharoah et al. 2006), known to be important mediators of cell recruitment and activation. The effects of such pro-inflammatory cytokines and of other mediators within synovial fluid were investigated for any potential effect on CD73 expression. These cytokines are emerging as critical regulators in the pathogenesis of JIA. Monoclonal antibodies or

biologic agents that target cytokines, such as the anti-TNF-α drugs etanercept and infliximab and the IL-1R antagonist anakinra are now widely used in the treatment of JIA. These inhibitors decrease angiogenesis, suppress other pro-inflammatory cytokines and prevent synovial cellular infiltration (Prakken et al. 2009).

These biologic agents are thought to work by direct blockade of specific cytokines. However, the anti-inflammatory mechanism of action of low dose methotrexate, the mainstay first line treatment option in JIA, has still not been defined. Some of the mechanisms that have been proposed include promotion of adenosine release via CD73 enzymatic activity (Morabito et al. 1998; Montesinos et al. 2007), inhibition of angiogenesis, of neutrophil accumulation and leukotriene B$_4$ production (Cronstein et al. 1995) and modulation of cytokines and their receptors (Seitz et al. 1995). Results presented in Chapter 4 showed that methotrexate therapy did not affect CD73 expression by peripheral blood lymphocytes of JIA patients.

The aims of the experiments presented in this chapter were to:

1) Investigate whether expression of CD73 and other ectonucleotidases CD39 and CD26, is altered by ligation of the T cell receptor and by activation of B cells *in vitro*;

2) Test the effects of inflammatory cytokines and synovial fluid on the expression of CD73 *in vitro*;

3) Determine whether CD73 downregulation on activated T cells *in vitro* is a result of cell cycling;

4) Examine whether reduced CD73 expression after T cell activation *in vitro* is associated with a reduction in AMPase activity;

5) Investigate the effects of the anti-inflammatory drug methotrexate on CD73 and CD39 expression of PBMC *in vitro*.

6.2 Results

6.2.1 Effects of T cell receptor ligation on CD73 expression

T cells in the JIA joint have been established to have an activated phenotype. In this section, the effect of T cell activation was therefore investigated on CD73 expression. T cell activation through the T cell receptor, in the presence of a co-stimulatory signal, was tested for its effect on CD73 expression of PBMC by using anti-CD3 and anti-CD28 monoclonal antibodies (mAb). Anti-CD3 mAb was used to mimic stimulation through the T cell receptor (TCR), whilst in the presence of a costimulatory signal (anti-CD28 mAb).

Representative flow cytometry plots and histograms of healthy adult PBMC cultured for 5 days in either medium alone (left) or in wells coated with anti-CD3 and anti-CD28 mAb (right) are shown in Figure 6.1A. Culture of PBMC in the presence of anti-CD3/CD28 mAb resulted in a decrease in the proportion of T cells positive for CD73 expression (Figure 6.1A). After stimulation, there was a significant decrease in the fraction of $CD8^+CD73^+$ cells among $CD8^+$ T cells, but only a small drop in the proportion of $CD73^+$ cells in the $CD4^+$ population. This small change in $CD4^+$ T cells did not reach significance (Figure 6.1B). In addition to the decrease in the percentage of CD73 positive PBMC after T cell stimulation, there was a significant reduction in the levels of CD73 protein expression on both the CD73 positive $CD4^+$ and $CD8^+$ T cells as assessed by MFI, compared to those cultured in medium alone (Figure 6.1C).

In order to investigate the time course for CD73 downregulation upon activation to occur on healthy T cells, CD73 expression on $CD8^+$ T cells was measured by flow cytometry on day 0, day 1, 3 and 5 of cell culture in the presence or absence of anti-CD3/CD28 mAb. For both PBMC cultured in medium alone or in presence of anti-CD3/CD28 mAb there was an initial small increase in $CD8^+CD73^+$ cells from day 0, which then plateaued at day 3, after which PBMC cultured with anti-CD3/CD28 mAb showed a fall in proportion of $CD8^+CD73^+$ cells, while those in medium alone remained unchanged (Figure 6.1D). This was highly consistent across multiple healthy donors (n=16 tested in this whole project).

To investigate further the mechanism and the requirements for CD73 downregulation upon T cell activation, PBMC were cultured in the presence of anti-CD3 mAb alone or together with anti-CD28 mAb. Of the three different healthy adult PBMC samples that were tested, two had a similar percentage of $CD8^+CD73^+$ cells either after culture with just anti-CD3 or with anti-

CD3 and anti-CD28 mAb together. While one individual showed partial downregulation with anti-CD3 mAb alone. This would appear to suggest in some individuals costimulation was not required for CD73 downregulation to occur (Figure 6.1E).

Gated on CD3$^+$ lymphocytes

A
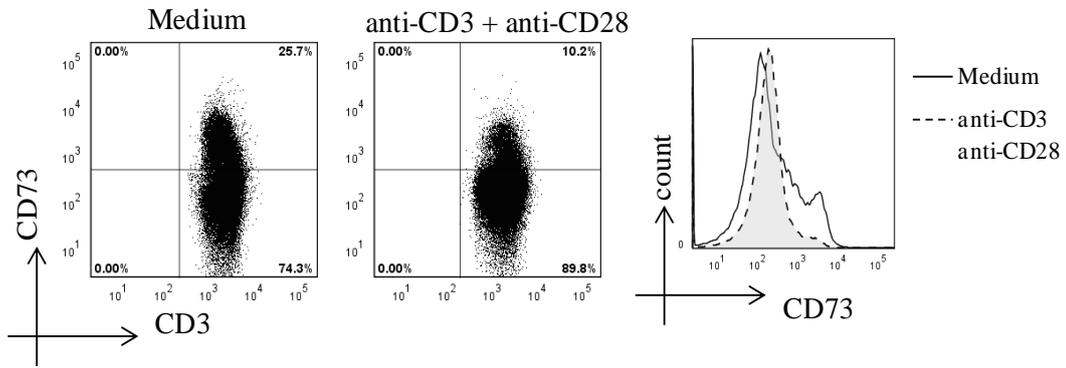
B
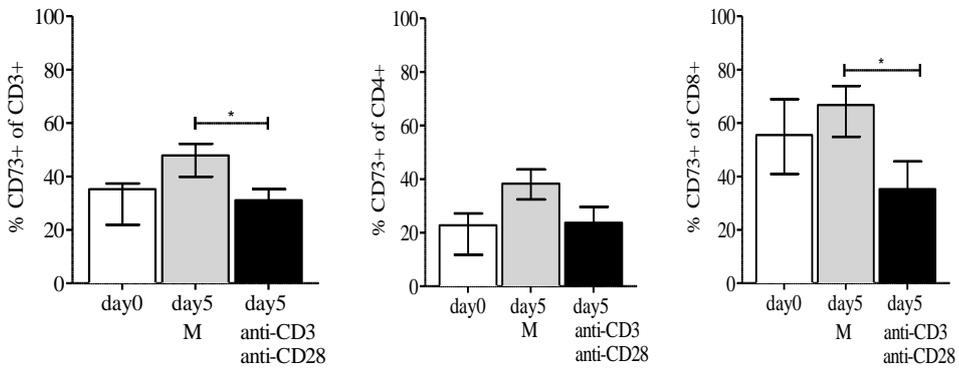
C
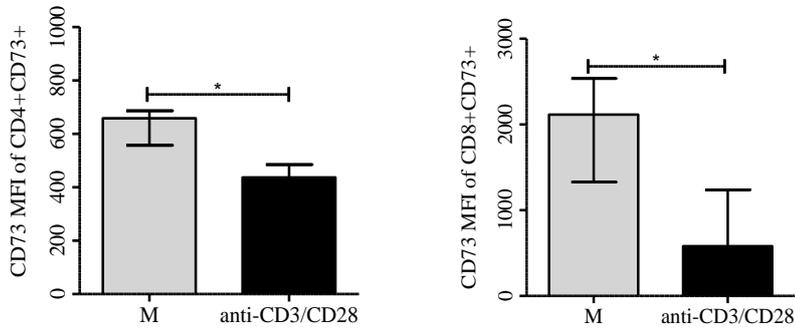
D
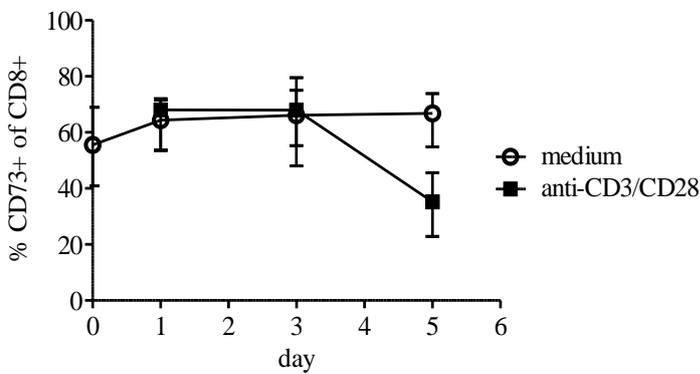
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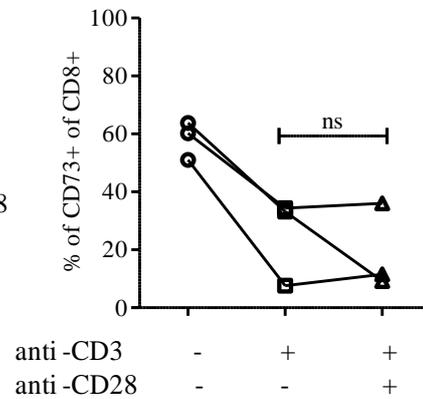
Figure 6-1 CD73 expression is down-regulated on stimulated T cells

PBMC were cultured in medium alone or with plate bound anti-CD3/CD28 mAb for 5 days and analysed by flow cytometry. A) Representative dot plots show CD73 expression on CD3 T cells, gated on $CD3^+$ lymphocytes, from healthy adult peripheral blood of the same individual, cultured in medium alone [left] or following TCR and CD28 stimulation [right] for 5 days. B) Summary bar graphs show proportions of CD73 positive CD3, CD4 and CD8 T cell populations from healthy adult PBMC, before culture and cultured in medium alone (M) or in plates coated with anti-CD3 and anti-CD28 mAb for 5 days (n=6). C) CD73 MFI of $CD4^+CD73^+$ cells [left] and of $CD8^+CD73^+$ cells [right] after 5 days culture of PBMC in medium (M) alone or with anti-CD3/CD28 mAb (n=6). D) Comparison of the percentage of $CD8^+CD73^+$ healthy PBMC analysed by flow cytometry on day 0,1,3 and 5 of cell culture in medium alone or in the presence of anti-CD3/CD28 mAb (n=6). E) Summary data showing CD73 expression on $CD8^+$ T cells after culture in medium, with anti-CD3 or anti-CD3/CD28 mAb for 5 days (n=3). Data are represented as medians with interquartile range (IQR). White bars represent expression prior to culture.

6.2.2 Upregulation of CD39 and CD26 by T cell stimulation

The results in Chapter 3 and previous reports (Moncrieffe et al. 2010b) have demonstrated an upregulation of CD39 positive JIA synovial T cells, together with a decrease in those positive for CD26 expression, compared to peripheral blood T cells. Therefore, it was of interest to determine whether T cell activation contributes to modifications in their expression.

Ligation of the T cell receptor *in vitro* was again achieved with the use of plate-bound anti-CD3/CD28 mAb. Healthy adult PBMC were cultured for 5 days in the presence of TCR stimulus with co-stimulation or control conditions and analysed for their expression of CD39 and CD26. Stimulated T cells showed an increase in the percentage of CD39$^+$ positive cells compared to unstimulated cells as shown in representative flow cytometry plots and summary data in Figure 6.2A and B. The T cell subset on which the most significant increase in CD39 expression occurred was CD8$^+$ T cells (p=0.0001), with a median of 65.4% after stimulation, compared to the 10.8% of the control condition (Figure 6.2B). As with the downregulation of CD73 upon T cell activation, the upregulation of CD39 on T cells was not dependent on the presence of both anti-CD3 and anti-CD28 mAb, as it still occurred with only anti-CD3 without an anti-CD28 signal (data not shown).

The expression of CD26, a surrogate marker for expression of ADA (Kameoka et al. 1993) was also increased after culture with anti-CD3 and anti-CD28 mAb *in vitro*. This can be seen in the representative flow cytometry plots in Figure 6.2C of healthy PBMC cultured for 5 days in medium alone [left] or in the presence of T cell stimuli [right] showing expression of CD26 on CD3$^+$ T cells. Summary data in Figure 6.2D shows the proportion of CD3$^+$, CD4$^+$, and CD8$^+$ T cells expressing CD26 increased after 5 days culture with plate-bound anti-CD3 and anti-CD28 mAb.

A

Gated on CD3+ lymphocytes
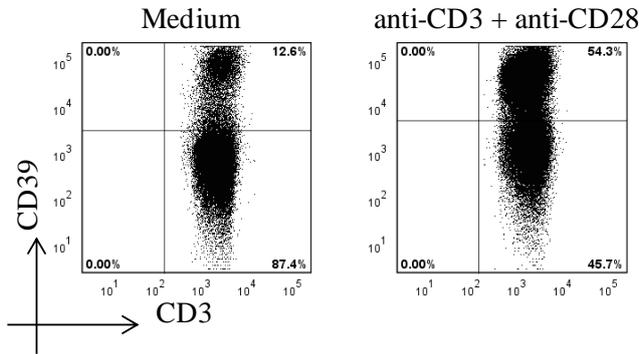
B
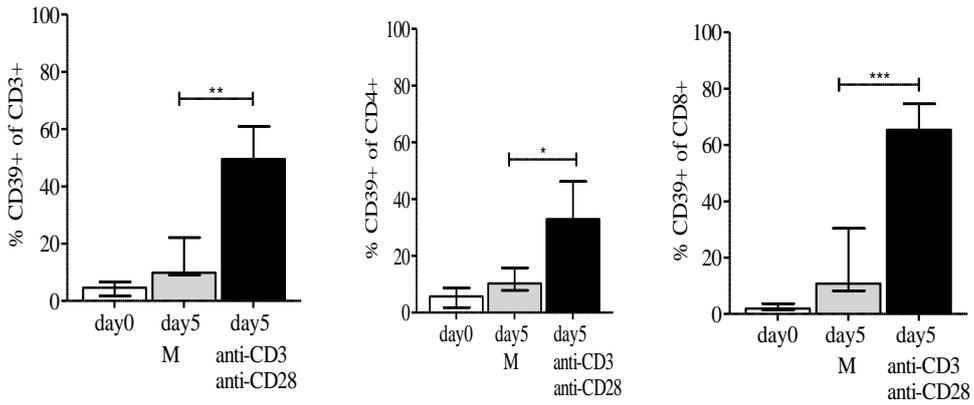
C

Gated on CD3+ lymphocytes
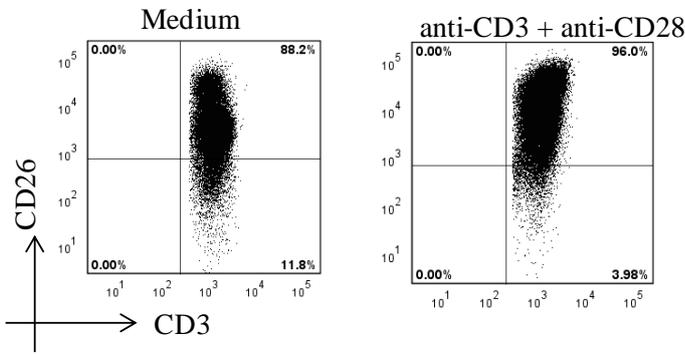
D
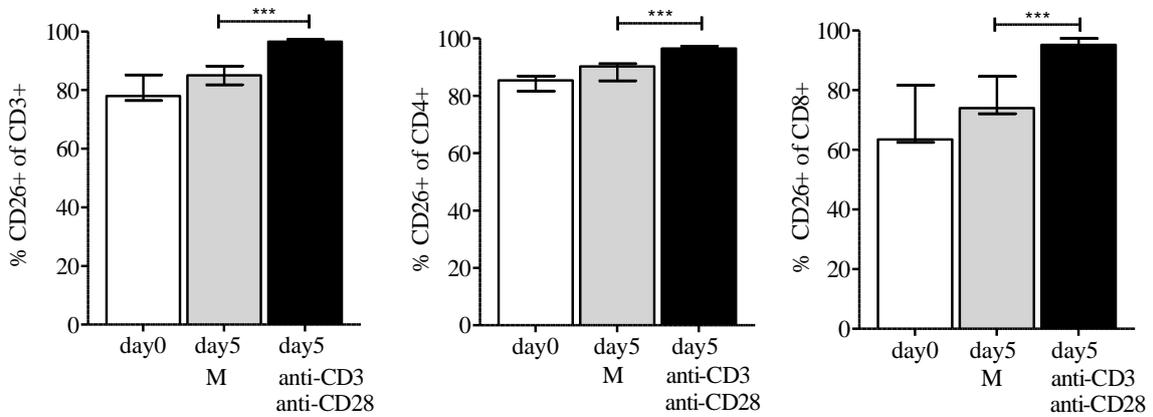
Figure 6-2 Effect of TCR stimulation on CD39 and CD26 expression on T cells *in vitro*

PBMC were cultured in medium alone or with anti-CD3/CD28 mAb for 5 days and analysed by flow cytometry. A) Representative flow cytometry dot plots of CD39 expression of healthy blood CD3$^+$ T cells from the same donor, after 5 days culture in medium alone [left] or with plate-bound anti-CD3/CD28 mAb [right]. B) Summary data showing the proportions of CD3, CD4 and CD8 T cells from healthy adult PBMC expressing CD39, prior to culture and after 5 days culture in medium alone or in the presence of anti-CD3/CD28 mAb (n=6). C) Representative dot plots of CD26 expression by healthy blood T cells gated on CD3 T cells after 5 days culture in medium alone [left] or following stimulation with anti-CD3/CD28 mAb [right] from the same donor. D) Summary data of the percentage of CD3, CD4 and CD8 T cells from healthy adult PBMC expressing CD26, prior to culture and after 5 days of culture in medium alone or in the presence of TCR stimuli anti-CD3/CD28 mAb (n=7). Bars represent medians with IQR. White bars represent expression prior to culture.

In Chapter 3, the SNP rs10748643 in the CD39 gene was found to affect the expression of CD39 by JIA synovial T cells, resulting in those patients with the AA genotype having lower CD39 expression on their T cells from the joint. The elevation in CD39 expression upon activation was therefore tested in genotyped healthy individuals, carrying two copies of the A allele, or two copies of the G allele, or in heterozygous AG individuals. This showed that while all individuals had an increase in CD39 expression after T cell stimulation, the relative increase in expression was dependent on the rs10748643 genotype of the individual with much lower CD39 upregulation upon activation for those individuals with the AA allele of the rs10748643 SNP and an intermediate phenotype in those who were heterozygous (Figure 6.3). When however, this upregulation was calculated as fold change compared to the percentage of $CD3^+CD39^+$ cells on day 0, the increase was highest for individuals with the AA allele of the rs10748643 SNP (Figure 6.3 right).
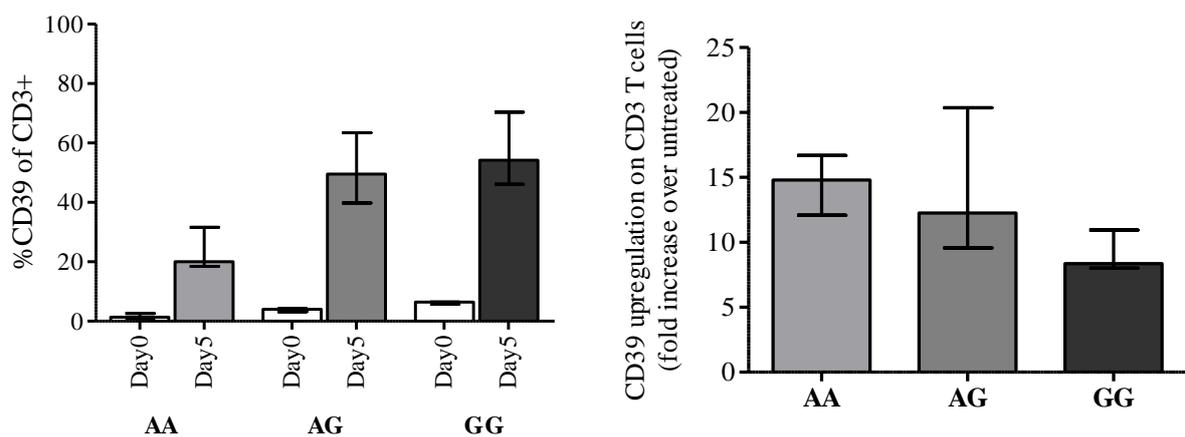
Figure 6-3 Effect of CD39 genotype on CD39 upregulation upon activation

Summary data showing the proportion of CD3 T cells from healthy adult PBMC expressing CD39 on day 0 and after 5 days culture with anti-CD3 and anti-CD28 mAb. Data were grouped according to AA (n=3), AG (n=4), GG (n=3) allele of CD39 SNP rs10748643 (left). Evaluation of fold change increase of the percentage of $CD3^+CD39^+$ cells after 5 days culture with anti-CD3 and anti-CD28 mAb, compared to levels present prior to culture (right). The same individuals were analysed in the two bar graphs (left and right). Sequencing of the rs10748643 SNP was kindly performed by PCR typing by Dr Simona Ursu. White bars represent expression prior to culture. Data are represented as medians with IQR.

6.2.3 The effect of B cell activation on ectonucleotidase expression by B cells

Like T cells, B cells from the joints of patients with JIA, are also known to have an activated phenotype defined by expression of activation markers such as CD69 and co-stimulatory molecules CD80 and CD86 (Morbach et al. 2011). It was therefore investigated whether activation of B cells would lead to downregulation of surface expression of CD73.

Several methods of B cell activation *in vitro* are available: three were used in this study. These include: stimulation using the synthetic oligodeoxynucleotide CpG, a ligand of the Toll-like receptor 9, which induces B cell proliferation and cytokine production (Decker et al. 2000), use of CHO (Chinese hamster ovary) cells transfected with CD40L (also known as CD154, expressed by activated T cells) which binds to CD40 on B cells and mediates B cell proliferation and class switching (Armitage et al. 1993) and use of a $F(ab')_2$ fragment of antibody directed against IgG and IgM surface immunoglobulin on the B cell.

Representative plots in Figure 6.4A show clearly that after 3 days of culture, healthy PBMC cultured with the B cell TLR9 ligand CpG had a loss of $CD19^+CD73^+$ cells (median 62.7%) compared to those who did not receive the stimulus (median 86%). Addition of control un-transfected CHO cells did not affect CD73 expression by B cells, as the proportion of $CD19^+CD73^+$ cells present in this condition after culture was the same as those present in medium alone (Figure 6.4B). In contrast, the culture of PBMC with CHO cells transfected with CD40L led to a reduction of $CD19^+CD73^+$ cells, resulting in a similar percentage of CD73 positive B cells (median 62.5%) as seen when cells were stimulated with CpG. The addition of $F(ab')_2$ fragment against IgG and IgM immunoglobulins resulted in only a small loss in CD73 positive B cells, with no significant difference compared to PBMC in control conditions (Figure 6.4B).

As discussed in Chapter 3, B cells from healthy control PBMC in resting conditions do not express CD26, but after culture of PBMC in the presence of B cell stimulus CpG, 53.6% (median) of B cells expressed CD26, while 34.5% (median) of B cells from PBMC cultured with CHO cells transfected with CD40L acquired CD26 expression (Figure 6.4C-D). This increase in CD26 expression after activation was opposed to the downregulation of CD73 observed after the same treatments. As was observed in T cells after stimulation, B cells showed an increase in CD39 positive cells, but the change was marginal, as around 80% of resting B cells express CD39 before culture (as shown in Figure 3.9B in Chapter 3).

171

A

Gated on CD19$^+$ lymphocytes

Medium   CpG

CD73 / CD19

B

CHO control	-	-	+	-	-	-	-	-
CHO CD40L	-	-	-	+	-	-	-	-
CpG	-	-	-	-	+	-	-	-
F(ab')$_2$	-	-	-	-	-	-	-	+

C

Gated on CD19$^+$ lymphocytes

Medium   CpG

CD26 / CD19

D

day 0   day 3 M   day3 CHO control   day3 CHO CD40L   day 3 CpG
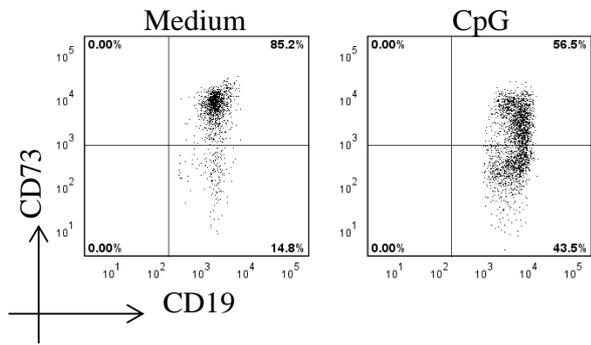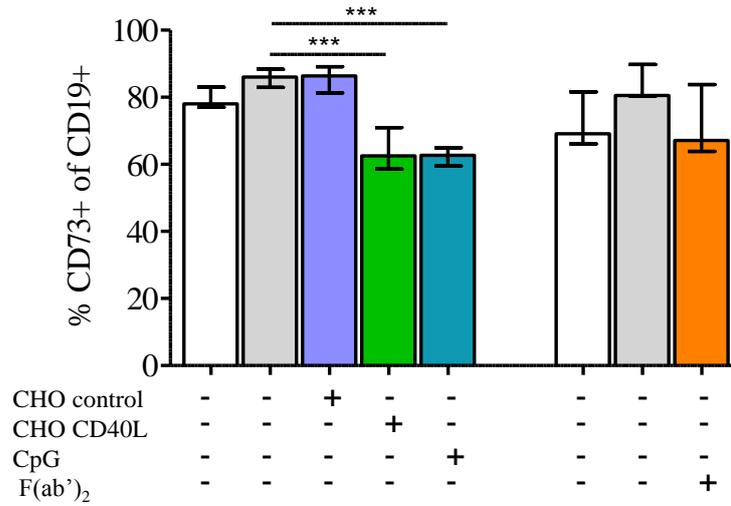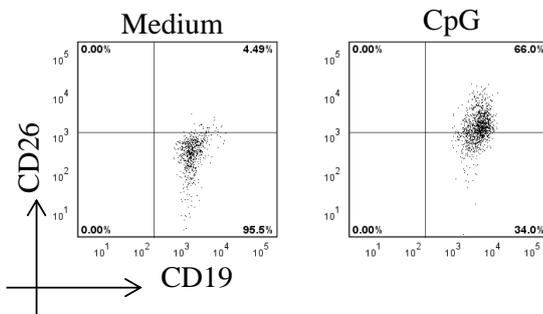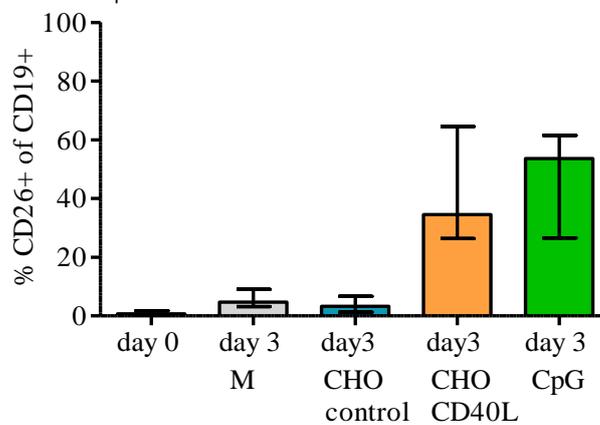
Figure 6-4 Effect of B cell stimulation on CD73 and CD26 expression

Healthy adult PBMC were cultured in medium alone, with TLR9 ligand CpG, CHO control cells, CHO transfected CD40L cells or F (ab') $_2$ fragment against IgG and IgM for 3 days and then analysed for expression of CD73 and CD26 on B cells. A) Representative flow cytometry plots of B cells from healthy adult PBMC cultured for 3 days in medium alone [left] or with CpG [right], representing CD73 expression on B cells. B) Summary data of the proportions of B cells from healthy PBMC expressing CD73, prior to culture, and after different conditions of cell culture and stimulation (n=8) [left part of bar graph] and percent of B cells expressing CD73 after culture in medium or with F (ab') $_2$ (n=3) [right part of bar graph]. C) Representative flow cytometry plots of CD26 expression by B cells from healthy adult PBMC cultured for 3 days in either medium [left] or with CpG [right]. D) Summary data of the proportion of B cells expressing CD26 prior to culture and after culture for 3 days in medium alone, with control CHO cells, CHO CD40L cells and CpG (n=6). Bars represent medians with IQR. White bars represent expression prior to culture.

6.2.4 T cell stimulation *in vitro* affects the percentage of CD19$^+$ CD73$^+$ cells

Given that the above experiments were all performed on total unsorted PBMC, it was hypothesized that stimulation of one cell population may also lead to alterations of CD73 expression on another cell type. This was investigated. During cell culture in the presence of T cell receptor stimulation, it was observed that in addition to the down-regulation of CD73 expression observed on T cells, changes in the proportion of CD73$^+$ cells also occurred in the CD19$^+$ B cell population. In particular, it was found that after culture of healthy adult PBMC in plates coated with anti-CD3 and anti-CD28 mAb, there was a decrease in the percentage of B cells expressing CD73 as shown in representative flow cytometry plots and summary data in Figure 6.5A and B. It is interesting to note that this decrease in the proportion of CD19$^+$CD73$^+$ cells occurred only after 5 days of cell activation (Figure 6.5B), but not after 3 days, unlike the downregulation of CD73 observed with the B cell stimuli tested.

Despite the difference in time point in the downregulation of CD73 expression by B cell stimuli CpG and CHO-CD40L cells (Figure 6.4) and T cell stimuli anti-CD3 and anti-CD28 mAb (Figure 6.5), the resulting percentages of CD19$^+$CD73$^+$ cells after stimulation were similar in the two different experiments. Thus, after stimulation with CpG or CHO-CD40L cells, 62.7% and 62.5% of B cells expressed CD73 compared to the 86% of unstimulated B cells, while after culture in the presence of anti-CD3/CD28 mAb, 63% of B cells were CD73 positive (Figure 6.4B and Figure 6.5B). All the above percentages represent medians of the population.

These results suggested that upon activation and proliferation, changes occur to T cells which lead them to release factors which subsequently alter CD73 expression on B cells.
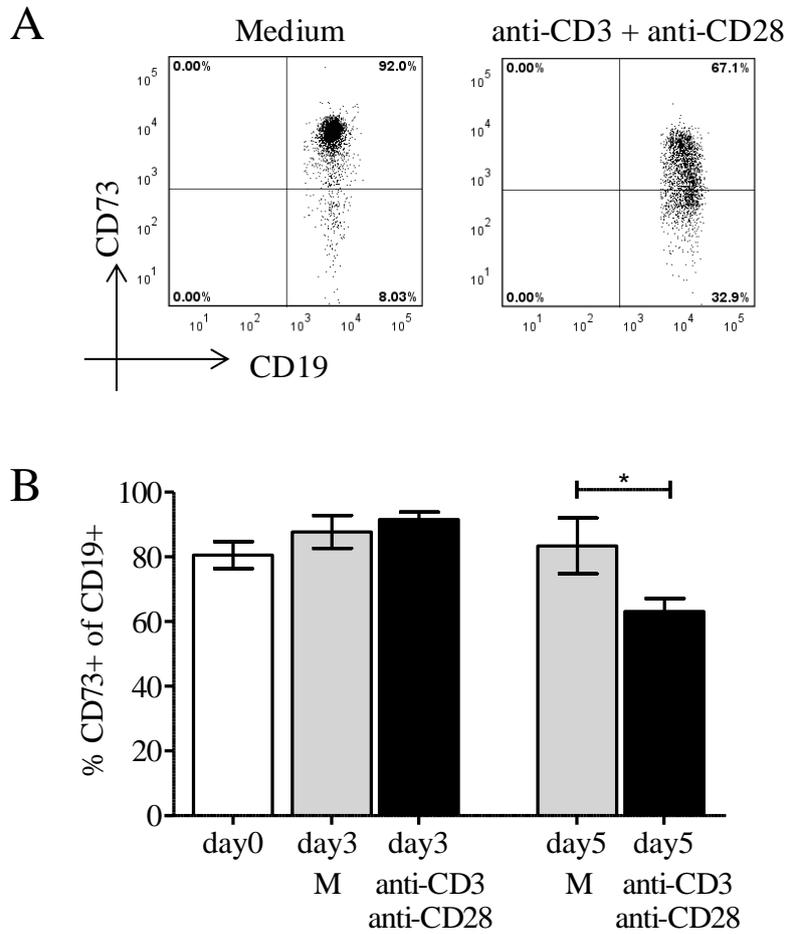
Figure 6-5 Effect of T cell stimulation on CD73 expression by B cells

A) Representative flow cytometry plots of CD73 expression on CD19 B cells of healthy adult PBMC cultured in medium alone [left] and with plate bound anti-CD3/CD28 mAb [right] for 5 days. B) Summary data show percentage of B cells expressing CD73, prior to culture and after 3 or 5 days in medium or with anti-CD3/CD28 mAb (n=3). White bars represent expression prior to culture. Bars represent medians with IQR.

6.2.5 Effects of cytokines on ectonucleotidase expression by T and B cells *in vitro*

In order to understand whether the cytokine milieu present in the JIA joint could modulate CD73 expression of PBMC, the effects of several cytokines, which are known to be elevated in the synovial fluid of patients with JIA (de Jager et al. 2007), were tested on T and B cells *in vitro*.

The pleiotropic cytokines IL-1, IL-6 and TNF-α, known to be key drivers of inflammation, were added to cultures of healthy adult PBMC in either medium alone or together with plate-bound anti-CD3 and anti-CD28 mAb for 5 days and cells were then analysed for CD73 expression by flow cytometry. As expected, the percentage of CD8$^+$CD73$^+$ cells was decreased after culture in the presence of anti-CD3 and anti-CD28 mAb. However, none of the three inflammatory cytokines tested altered CD73 expression of CD8 T cells, whether they were added alone (Figure 6.6A) or when they were tested in combination (data not shown). Moreover, addition of these cytokines to cultures together with anti-CD3 and CD28 mAb did not decrease CD73 expression further compared to the decrease observed with the CD3 and CD28 mAb alone. In addition to having no effect on the percentage of CD73$^+$ T cells, none of the cytokines tested decreased the percentage of CD73$^+$B cells, whereas positive control cultures with CpG still showed a significant decrease (Figure 6.6B).

In addition to high levels of inflammatory cytokines, the JIA joint is characterized by reduced levels of some cytokines such as TGF-β, which may have an anti-inflammatory effect and can promote or oppose the differentiation of immune cell types. This cytokine has been reported to be found at reduced levels in the JIA joint compared to plasma levels of JIA patients, as measured by ELISA (Nistala et al. 2010). TGF-β is also known to prevent activation-induced downregulation of CD73 at both the mRNA and protein expression level of murine CD4$^+$ T cells (Regateiro et al. 2011).

No difference in the proportion of CD73 positive cells healthy PBMC-T cells was found after culture in either medium alone, with anti-CD3 and anti-CD28 mAb in either the presence or absence of TGF-β (Figure 6.6C left). As shown in Figure 6.6C, the addition of TGF-β to cell culture medium or in wells coated with anti-CD3 and anti-CD28 mAb had no ability to prevent the downregulation of CD73 positive CD8$^+$ T cells [right] observed for those PBMC cultured with anti-CD3 and anti-CD28 mAb. Additionally, all the cytokines tested had no effect on the expression of CD39 and CD26 by PBMC (data not shown).
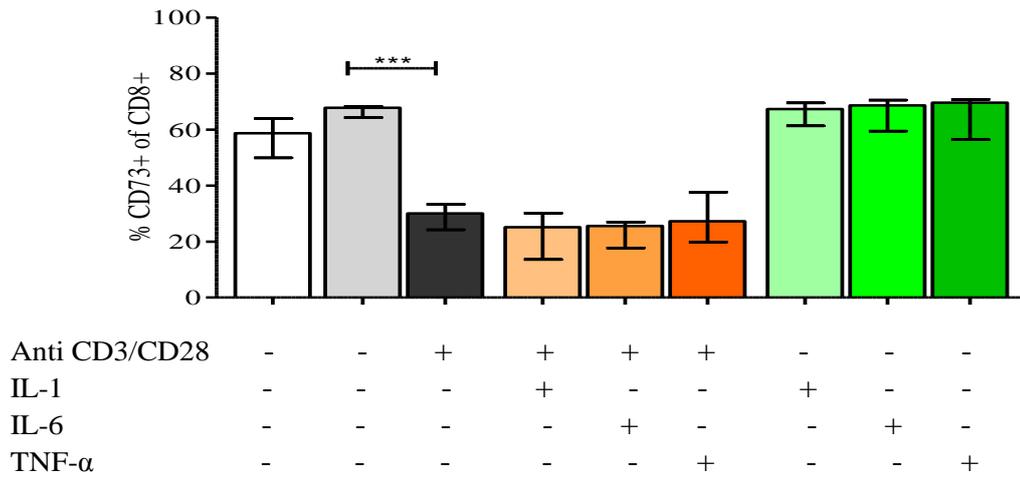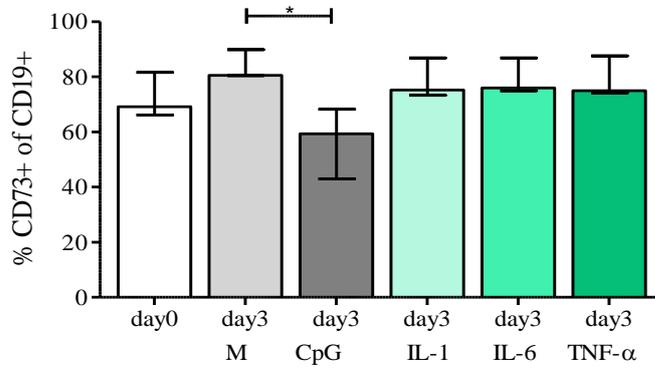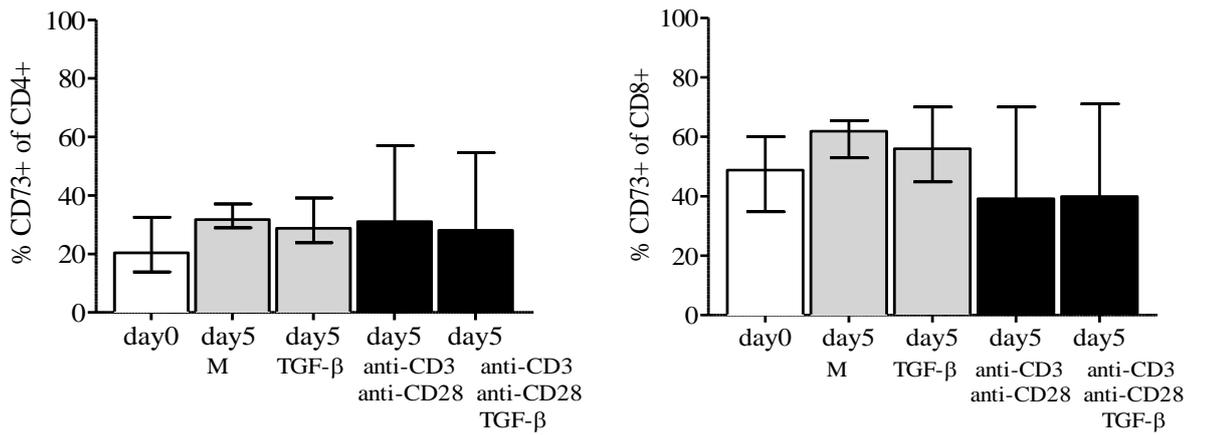
Figure 6-6 Inflammatory cytokines or TGF-β do not alter CD73 expression

A) Bar graph of proportions of CD8$^+$CD73$^+$ cells prior to, and after, culture of healthy adult PBMC for 5 days in medium alone, with cytokines IL-1,IL6,TNF-α, with anti-CD3/CD28 mAb or a combination as shown (n=3). B) Proportion of CD19$^+$CD73$^+$ cells from PBMC prior to and after 3 days culture with CpG or cytokines IL-1,IL-6 or TNF-α (n=3). C) Bar graph of proportions of CD4$^+$ T cells [left] and CD8$^+$ T cells [right] expressing CD73, ahead of culture and cultured for 5 days in medium alone, with anti-CD3/CD28 mAb in the absence or presence of TGF-β (n=6). White bars represent expression prior to culture. Data are represented as medians with IQR.

T-cell activation by ligation of the T cell receptor results in synthesis and release of cytokines such as the cytotoxic IFN-γ and the autocrine-growth factor IL-2 (Badou et al. 2001). In addition, a large proportion of T cells from the JIA joint express CD25, suggesting they may have received an IL-2 signal *in vivo* (Black et al. 2002). The effect of these activation-induced cytokines was therefore tested on the proportion of PBMC T and B cells expressing CD73, as these cytokines are also elevated in JIA synovial fluid (de Jager et al. 2007). As shown in Figure 6.7A, the expression of CD73 by B cells after culture of PBMC with IFN-γ and IL-2 was unaffected. This was also the case for CD8$^+$ T cells and CD4$^+$ T cells (data not shown) suggesting that neither cytokine is responsible for the downregulation of CD73 positive cells seen after T cell stimulation. A small drop in CD8$^+$ CD73$^+$ cells was seen however, after 5 days of culture in IL-2, but this did not reach statistical significance (Figure 6.7B).
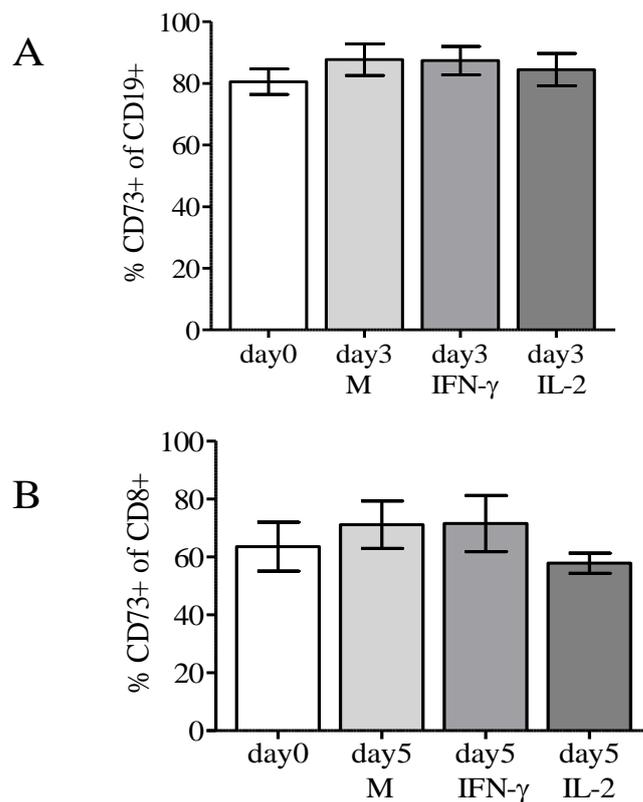
Figure 6-7 Soluble factors released after T cell stimulation *in vitro* do not affect CD73 expression by either B or T lymphocytes

A) Summary data of proportions of B cells expressing CD73 before culture and after culture of PBMC in medium alone, or with soluble IFNγ or IL-2 for 3 days (n=3). B) Summary data of proportions of CD8$^+$ T cells expressing CD73 before and after culture of PBMC in medium alone, or with soluble IFNγ or IL-2 for 5 days (n=3). White bars represent expression prior to culture. Data are represented as medians with IQR.

6.2.6 The *in vitro* effect of synovial fluid on CD73 expression by PBMC

In order to test the effects of an inflammatory environment on ectonucleotidase expression by mononuclear cells, to mimic that present in the synovial environment of JIA patients, the addition of synovial fluid to PBMC in culture was next investigated.

A pool of synovial fluid (SF) which had been collected according to the same protocol as that used to collect venous blood plasma, but otherwise un-treated, was tested and compared to culture in adult venous blood plasma (VBP). All previous culture experiments described so far used cultured medium containing 10% FBS. When culture medium was tested with the pool of 10% SF or VBP, there was no supplementation with FBS, while when 5% SF or VBP were tested, 5% FBS was added to normalise the total protein content in the culture conditions.

A decrease in the proportion of $CD8^+CD73^+$ cells in PBMC cultured with 10% VBP or SF with medians of 54.9 and 54.2% respectively, was observed, compared to control conditions (median 79.1%), but this reduction did not reach statistical significance when tested in three healthy individuals cells (Figure 6.8A). In the case of the proportion of B cells expressing CD73 from healthy adult PBMC cultured for 5 days with either VBP or SF, no difference was found between those in medium alone or PBMC cultured in the presence of SF or VBP (Figure 6.8B).

When the pools of SF and VBP were tested and compared for their cytokine content by luminex, it was found surprisingly, that the healthy plasma pool contained elevated levels of the cytokines TNF-α and IL-1, which are mostly associated with pro-inflammatory roles (Figure 6.8C, data from D.Bending and S.Ursu, unpublished).

A

% CD73+ of CD8+

100

80

60

40

20

0

*

day0 | day5 M | day5 anti-CD3 anti-CD28 | day5 5% VBP | day5 10% VBP | day5 5% SF | day5 10% SF

B

% of CD73+ of CD19+

100

80

60

40

20

0

day0 | day3 M | day3 5% VBP | day3 10% VBP | day3 5% SF | day3 10% SF

C

in pg/ml	TNF-α	IL-6	IL-1	IFN-γ	IL-2	IL-12	IL-17	IL-10
SF	26	2709	10	158	93	133	145	57
VBP	99	2189	17	886	306	229	94	68
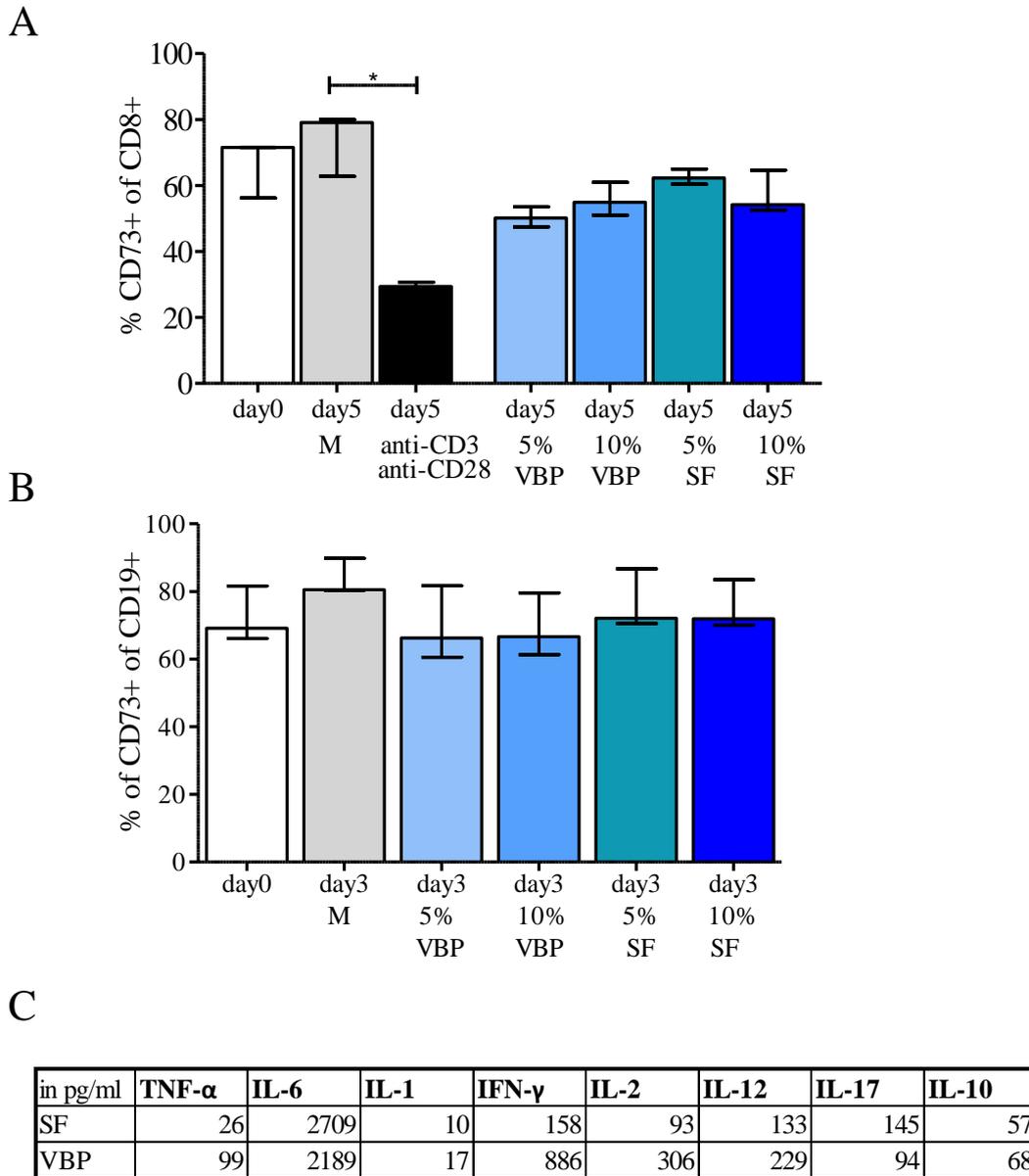
Figure 6-8 Synovial fluid from the joints of patients with JIA does not significantly alter the expression of CD73 by PBMC *in vitro*

Healthy adult PBMC were cultured for 3 and 5 days in medium alone, anti-CD3/anti-CD28 mAb, or in the presence of 5 or 10% VBP or of 5 or 10% SF as shown. A) Summary data of the proportion of CD8^{+} CD73^{+} T cells from PBMC either before culture or cultured for 5 days with either VBP or SF at % as shown (n=3). B) Summary data of the proportion of CD19^{+} CD73^{+} B cells from PBMC before culture and cultured for 5 days with either VBP or SF (n=3). White bars represent expression prior to culture. Data are represented as medians with IQR. C) Cytokine levels in pools of JIA patient synovial fluid (SF) and healthy control venous blood plasma (VBP) as measured by luminex.

6.2.7 CD73 downregulation occurs on proliferating T cells

To test whether the reduction in proportion of CD8$^+$ T cells expressing CD73 after T cell activation was due to down regulation of CD73 expression and not to differential cell death of CD73$^+$ cells, CD8$^+$ T cells were sorted by flow cytometry into CD8$^+$CD73$^+$ and CD8$^+$CD73$^-$ pure populations. They were then cultured either in control conditions or with anti-CD3 and anti-CD28 mAb for 5 days. The mean purity of the sorted populations before culture was 87% for CD8$^+$CD73$^+$ and 98% for CD8$^+$CD73$^-$ cells.

After culture in the presence of anti-CD3 and anti-CD28 mAb, around 50% (median) of cells from the CD8$^+$CD73$^+$ sorted population lost their CD73 expression, becoming CD73 negative CD8$^+$ T cells. In contrast, expression of CD73 on sorted negative CD8$^+$CD73$^-$ cells was unaffected by culture or stimulation as seen in flow cytometry plots and bar graphs in Figure 6.9A.

It was then tested whether CD73 down-regulation was specifically linked to cell proliferation. CD8$^+$CD73$^+$ purified cells were labelled with Carboxyfluorescein diacetate succinimidyl ester (CFSE) and then cultured in the absence or presence of anti-CD3 and anti-CD28 mAb. At the end of the culture period, the cells were stained for CD73 and for the nuclear protein Ki67 to identify actively cycling cells. As shown in Figure 6.9B, at the end of the 5-day culture, CD8$^+$ T cells that retained their CD73 expression [blue box, dot plot 45.1%] were Ki67 negative [blue line, histogram] and had not proliferated (as assessed by lack of CFSE dilution). In contrast, those CD8$^+$ T cells that had become CD73 negative [red box, dot plot 54.9%] were Ki67 positive and had diluted CFSE indicating cell division [red line]. These results indicate that CD8$^+$ T cells downregulate CD73 during cell division. The same cells that decreased in CD73 expression levels during cell division also increased in the proportion of CD39 positive cells (Figure 6.9C).

Expression of the CD45 membrane phosphatase was next analysed to assess the proportion of naïve or memory cells in the cultures, identified by expression of the CD45RA (naïve) or CD45RO (memory) isoforms, respectively. This showed that those CD8$^+$ T cells that lost CD73 expression after culture in the presence of anti-CD3/anti-CD28 mAb were predominantly memory T cells (81%), identified by their expression of CD45RO antigen. In contrast, the CD8$^+$ T cells that remained CD73 positive were predominantly CD45RO negative (14%) (Figure 6.9D).

A
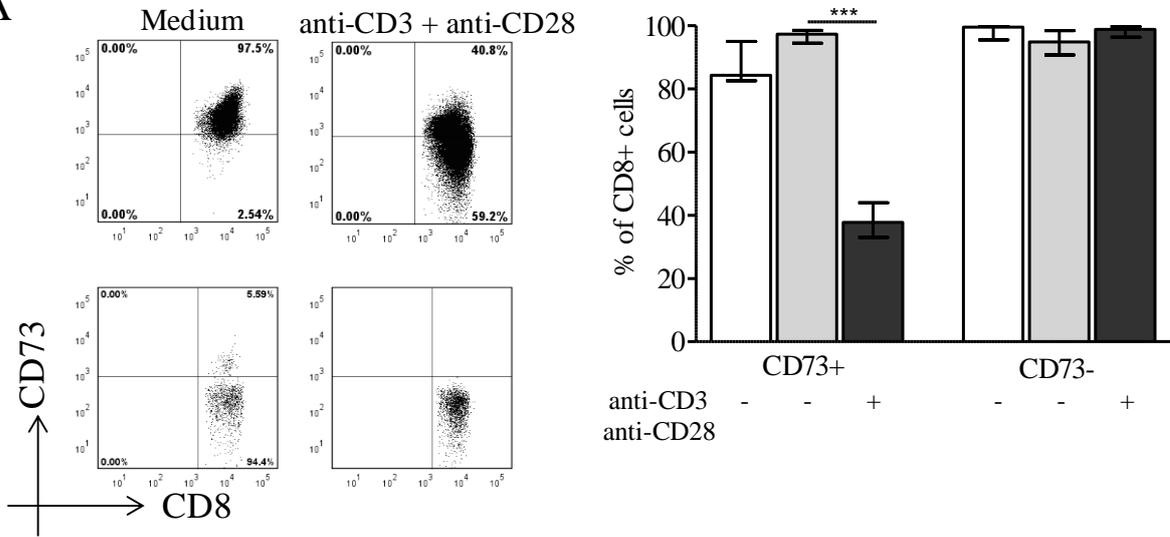
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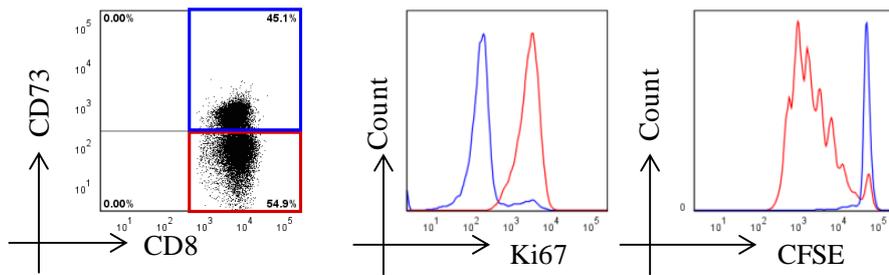
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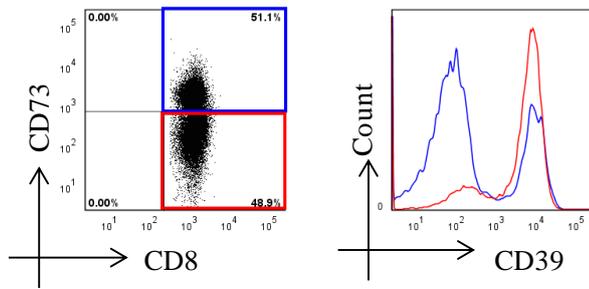
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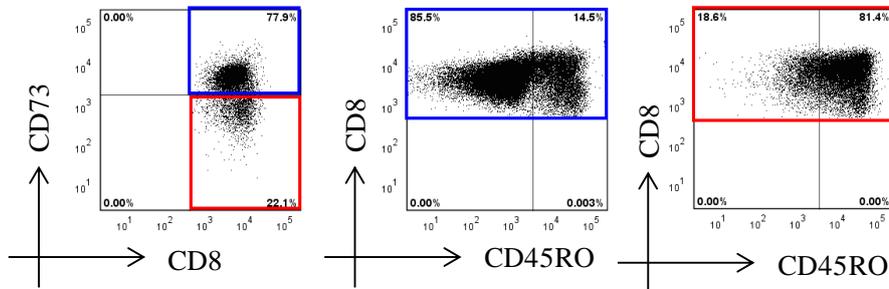
Figure 6-9 Downregulation of CD73 on proliferating cells *in vitro*

A) Flow cytometry plots of sorted CD8$^+$ CD73$^+$ [top] and CD8$^+$ CD73$^-$ [bottom] cells after 5 days of culture in medium alone or stimulated with anti-CD3/CD28 mAb [left]. Bar graph shows proportions of CD8 CD73 positive and negative populations after culture in medium alone (grey) or with anti-CD3/CD28 mAb (black) (n=4) [right]. White bars represent expression prior to culture. B) CD8$^+$ CD73$^+$ were cultured for 5 days with T cell stimuli, then analysed according to CD73 expression. Red box shows cells that had downregulated CD73, while cells in the blue box are those that remained CD73 positive after T cell activation. In the Ki67 and CFSE histograms, the red curves are for CD8 CD73$^-$ cells, while blue is for cells from CD8 CD73$^+$ gate (one experiment representative of three). C) CD73 expression on sorted CD8$^+$CD73$^+$ cells after 5 days culture with anti-CD3/CD28 mAb and boxes to differentiate between those that lost CD73 expression (red) and those that maintained it (blue) [left]. CD39 histogram with red curve for CD8 CD73$^-$ cells and blue curve for CD8$^+$CD73$^+$ cells after T cell activation [right], (one experiment representative of three). D) CD73 expression on sorted CD8$^+$CD73$^+$ cells after 5 days culture with anti-CD3/CD28 mAb. CD45RO expression by CD8 T cells that remained CD73 positive [blue box] and for those that lost their CD73 expression [red box] (one experiment representative of three). Data are represented as medians with IQR.

6.2.8 Synovial CD8$^+$ T cells in cell cycle do not express CD73

To ascertain whether the loss in CD73 positive CD8$^+$ T cells in the joint of JIA patients was also associated with those cells that were actively dividing, synovial T cells extracted from the inflamed synovial fluid were stained for Ki67 and CD73 and compared with peripheral blood T cells from patients and healthy adult controls.

Whilst CD8$^+$ PBMC from either JIA patients or healthy controls were almost entirely Ki67 negative (representative flow cytometry plots in Figure 6.10), Ki67 positive synovial CD8$^+$ T cells were all CD73 negative and vice versa. In addition, even within the small populations of cells in cell cycle (Ki67$^+$) in PBMC from both patients and controls, the majority of cycling CD8$^+$ T cells was CD73$^-$. This result suggests that once T lymphocytes enter cell cycle and proliferate, they lose their CD73 expression, and that this holds true for both healthy and JIA synovial cells.
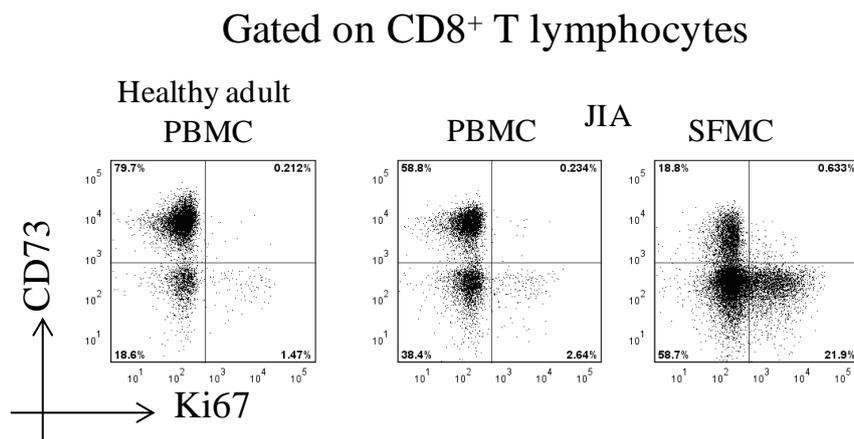
Figure 6-10 No co-expression of proliferation marker Ki67 and CD73 on CD8 T cells from either peripheral blood or the JIA joint

Representative flow cytometry plots from CD8 T cell gate of CD73 and Ki67 expression by healthy adult PBMC, JIA PBMC, and SFMC (one experiment representative of 3).

6.2.9 Soluble CD73 protein detected after T cell activation of PBMC

To investigate whether the loss of CD73 positive cells observed after T cell activation resulted in release of CD73 protein into the cell medium, which might then remain enzymatically active as soluble CD73 protein, culture supernatants were analysed for soluble CD73 protein by ELISA and for AMPase activity by HPLC.

Higher levels of CD73 protein as measured by ELISA were found in the culture supernatants of PBMC cultured with anti-CD3 and anti-CD28 mAb (53.8 ng/ml, median) compared to those cultured in medium alone (29.9 ng/ml, median, p=0.03 (Figure 6.11A left). This would suggest that T cell activation results in shedding of CD73 protein. However, unexpectedly, in the culture supernatant of sorted $CD8^+ CD73^+$ cells, apart from one outlier value (83 ng/ml), no detectable CD73 protein was found for those sorted cells cultured for 5 days in either medium alone or with anti-CD3 and anti-CD28 mAb (Figure 6.11B).

When the culture supernatants of healthy PBMC cultured for 5 days in either medium alone or anti-CD3 and anti-CD28 mAb were incubated with 25μM E-AMP and assayed for AMPase activity by HPLC assay, no adenosine production was detectable in either condition, despite the presence of soluble CD73 (Figure 6.11B). These results suggest that at least in total unsorted PBMC culture, CD73 protein may be shed into the medium during CD73 downregulation, but that this protein is then no longer enzymatically active, at least *in vitro*.
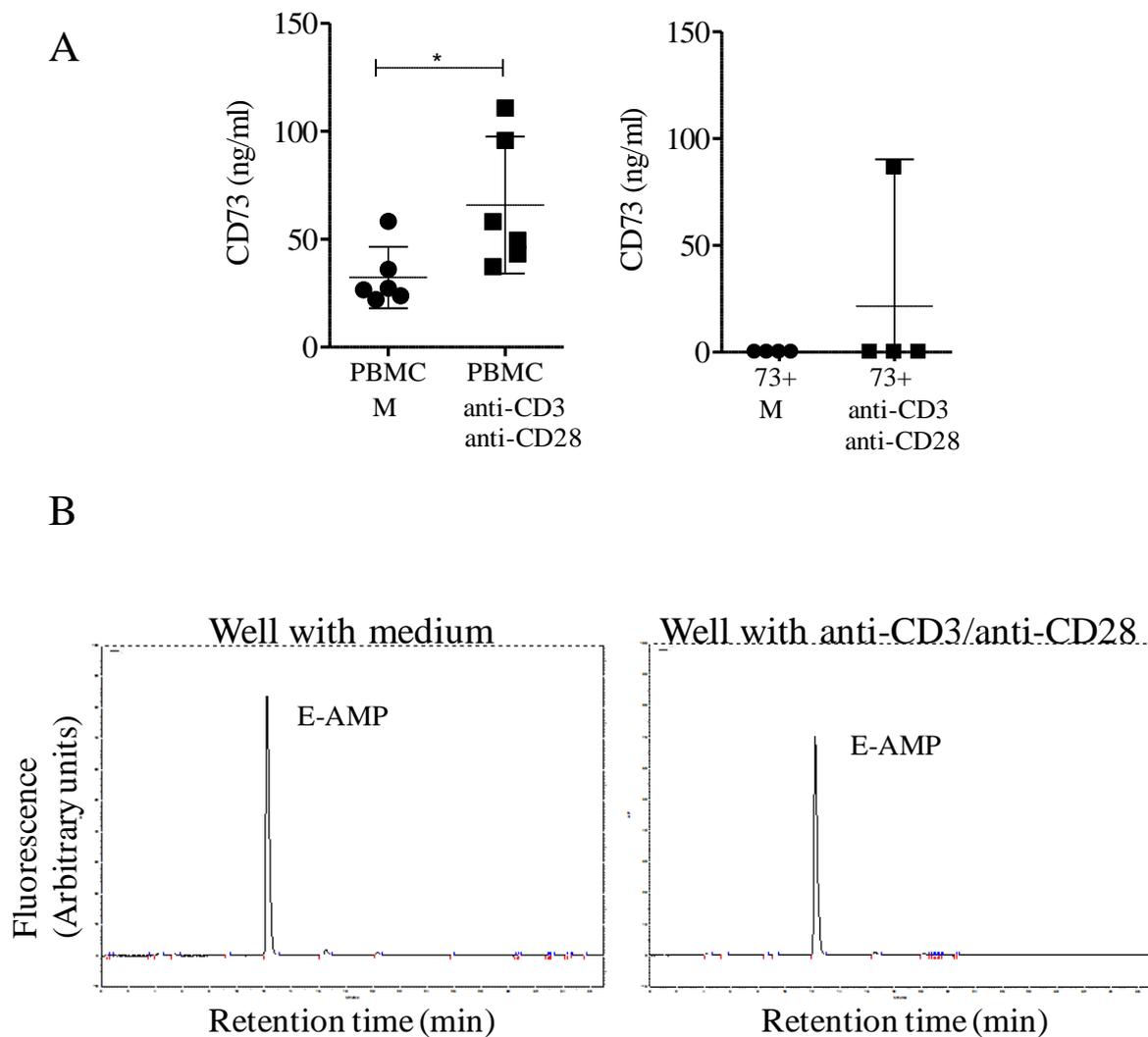
Figure 6-11 Presence of CD73 protein in culture supernatant of T cell activated cells but no correspondent AMPase activity

A) Culture supernatant of PBMC [left] or CD8$^+$CD73$^+$ cells sorted by flow cytometry [right] cultured for 5 days in either medium alone or with plate-bound anti-CD3 and anti-CD28 mAb and measured for CD73 protein content by ELISA (n=6,4 with medium and anti-CD3/anti-CD28 mAb respectively). Data are represented as medians with IQR. B) Representative HPLC chromatograms of culture supernatants of healthy PBMC cultured for 5 days in either medium alone or with plate-bound anti-CD3 and anti-CD28 mAb and following incubation with 25μM E-AMP for 45min (one experiment representative of 5).

6.2.10 Decreased adenosine synthesis by PBMC following TCR stimulation

To confirm that the loss of CD73 positive cells observed after T cell activation of PBMC resulted in reduced AMPase activity of these cells, healthy control PBMC harvested after culture in control conditions or cultured with anti-CD3/CD28 mAb were incubated with E-AMP and assayed for their AMPase activity as before. Representative HPLC chromatograms and summary data in Figure 6.12A-B show that activated PBMC did indeed produce less E-adenosine compared to unstimulated PBMC, after incubation of the cells with E-AMP.
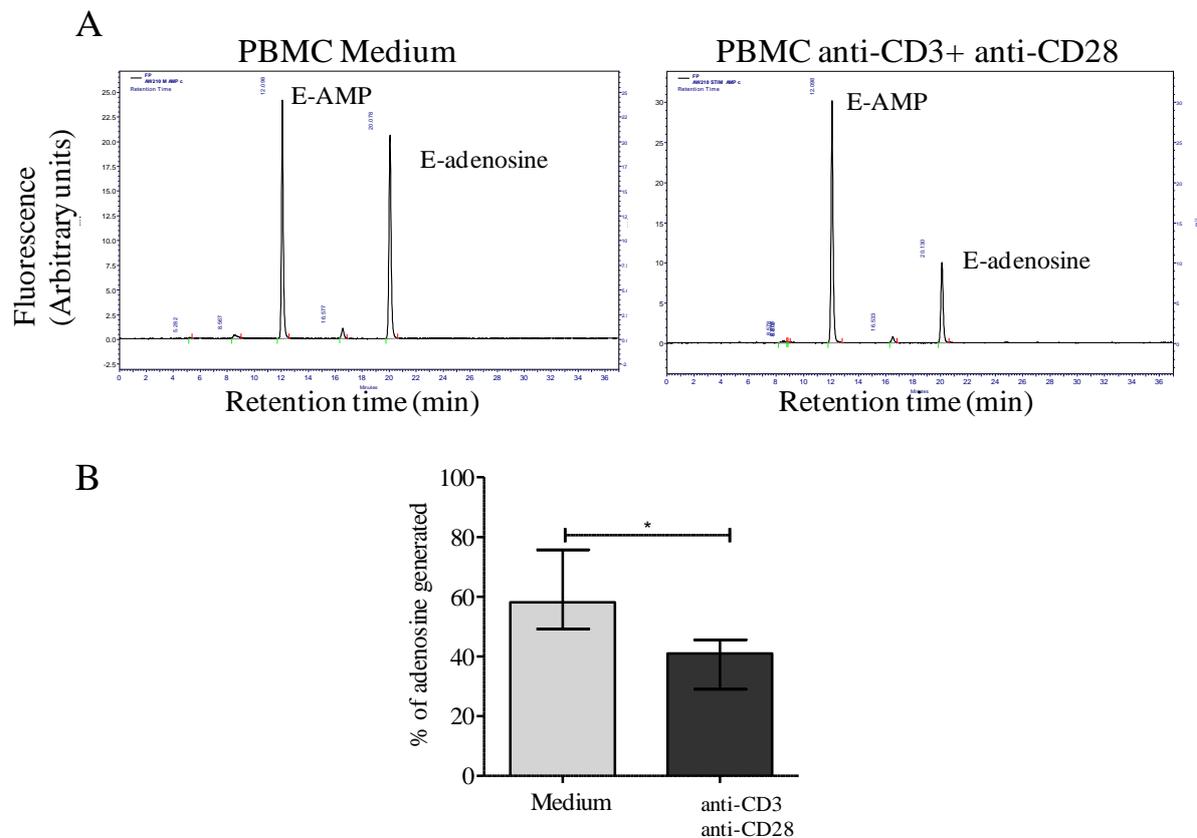
Figure 6-12 Reduction in adenosine production by PBMC after T cell activation

A) Representative HPLC chromatograms of PBMC incubated with 25µM E-AMP for 45min, after 5 days of culture in medium (left) or with plate-bound anti-CD3 and anti-CD28 mAb (right). B) Summary bar graph of percentage of adenosine generated by PBMC after 5 days of culture in medium or with plate-bound anti-CD3 and anti-CD28 mAb (n=4). Bars represent medians with IQR.

6.2.11 Effect of anti-inflammatory methotrexate on CD73 and CD39 expression by T cells from PBMC in cell culture

It has been suggested that methotrexate (MTX) a drug widely used as a first line disease modifying agent in JIA, may have its effect through the generation of anti-inflammatory adenosine via the action of CD73 (Morabito et al. 1998; Montesinos et al. 2007). It is possible therefore, that MTX may affect CD73 protein levels on immune cells, thereby altering their potential to generate adenosine. The effect of the drug on expression of CD73 and CD39 by T cells was tested by its addition to healthy control PBMC in culture for 5 days (Figure 6.13). Methotrexate is known to enter the cell and have glutamatic acid residues added to its structure by the action of folylpolyglutamate synthase (FPGS) (Becker 2012). The resulting methotrexate polyglutamates are retained inside the cell, where they are metabolically and clinically active (Chan et al. 2002). Different MTX polyglutamates (MTXGlu$_{3,4,5}$) were therefore added to PBMC cultures at concentrations similar to those found in red blood cells (van Haandel et al. 2009).

Of the three different MTX polyglutamates tested, only MTXGlu$_4$ had a significant effect on CD73 expression by CD8$^+$ T cells in culture, with a median drop of CD73$^+$ cells from 54.7% (control culture) to 30.9% after 5 days of culture in MTXGlu$_4$ (Figure 6.13A). Although the addition of MTXGlu$_5$ to PBMC in culture also led to a detectable decrease in the proportion of CD8$^+$ T cells expressing CD73, from a median value of 54.7% of those in medium alone to 39.5%, this decrease was not statistically significant in a group of 3 healthy individuals. These results may need repeating in a larger group of individuals to confirm whether MTX has a direct effect on CD73 expression i*n vitro.* The drug had no significant effect on CD73 expression by CD8$^+$ T cells cultured in wells with plate-bound anti-CD3 and anti-CD28 mAb (Figure 6.13A).

When the effects of MTX on CD39 expression by CD8$^+$ T cells were investigated, MTXGlu$_4$ was again found to be the only form of polyglutamated methotrexate to have a significant effect. In this case, MTXGlu$_4$ resulted in an increase in the percentage of CD8$^+$ T cells expressing CD39 (22%, median) compared to those in control conditions (5.2%, median). MTXGlu$_4$ or any other MTX polyglutamate had no effect however on CD39 expression when added to PBMC cultured with plate-bound anti-CD3 and anti-CD28 mAb (Figure 6.13B).

The finding described in Figure 6.13 for the effect of MTXGlu$_4$ on CD73 and CD39 expression was unexpected as the resulting decrease in CD73 expression could have a net

pro-inflammatory effect, whereas the upregulation of CD39 expression could have an anti-inflammatory consequence. It is interesting however that the effects of MTXGlu$_4$ *in vitro*, leading to a fall in CD73 and an increase in CD39 expression, appeared to parallel the effects of T cell activation itself. In contrast the MTXGlu$_3$ form appeared to prevent, to some extent, the down regulation of CD73 or upregulation of CD39 seen upon T cell proliferation, although this effect in a group of n=3 did not reach statistical significance (Figure 6.13).
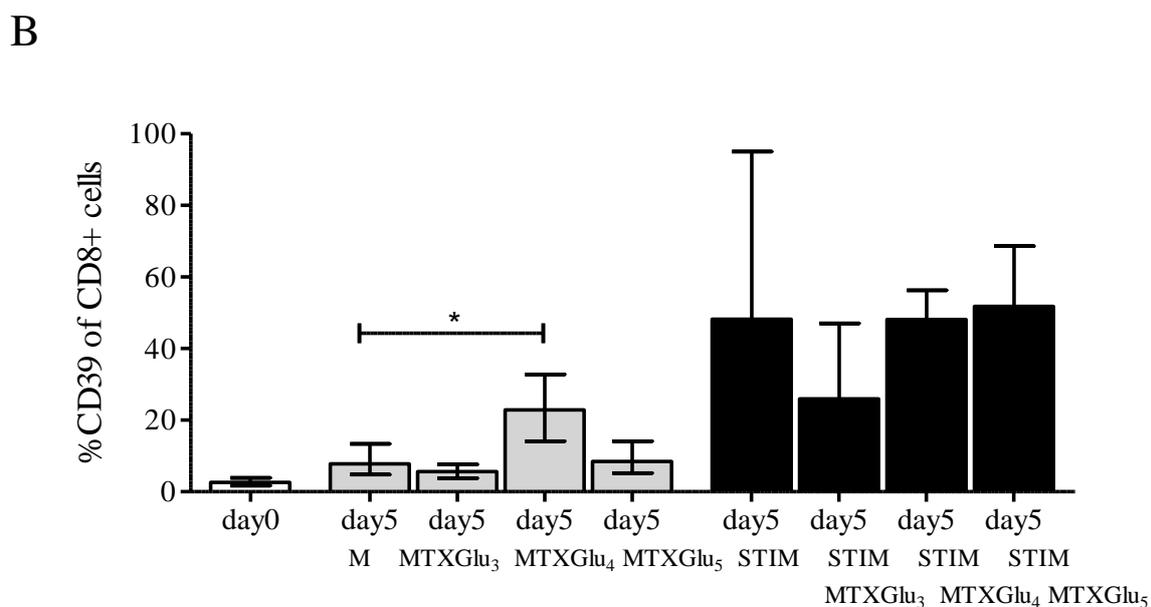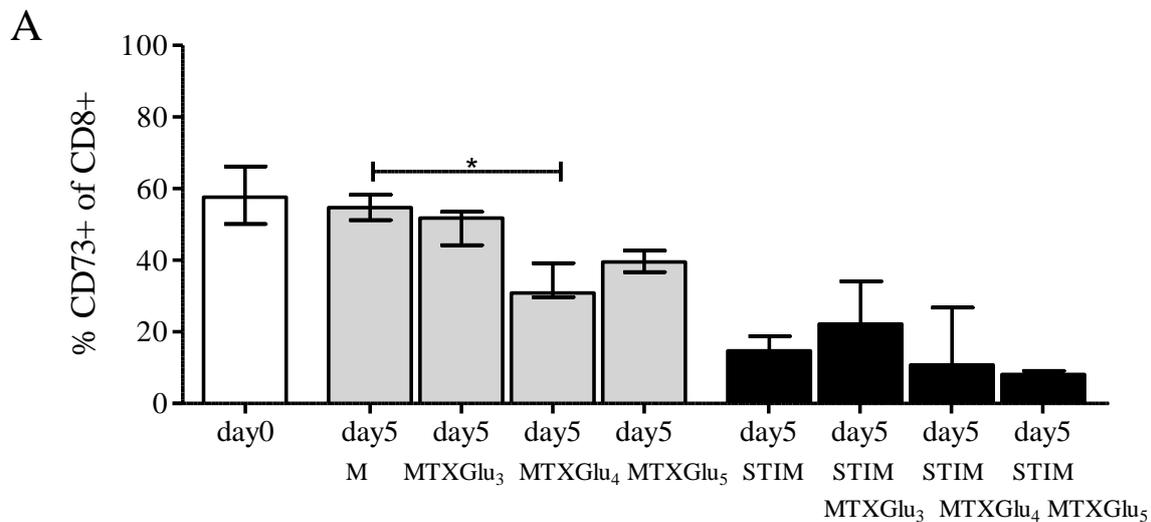
Figure 6-13 Effect of MTX in cell culture on CD73 and CD39 expression by CD8 T cells

A) Proportions of CD8$^+$ T cells from healthy adult PBMC expressing CD73, prior to culture and after 5 days culture in medium alone, with MTXGlu$_{3,4,5}$, with plate-bound anti-CD3 and anti-CD28 mAb alone or together with the MTX polyglutamates (n=3). B) Proportions of CD8$^+$ T cells expressing CD39, prior to culture and after 5 days culture in medium alone, with MTXGlu$_{3,4,5}$, with plate-bound anti-CD3 and anti-CD28 mAb alone or together with the MTX polyglutamates (n=3). Bars represent medians with IQR.

192

6.3 Discussion

The results in the preceding chapters and in previous reports of JIA demonstrated that lymphocytes derived from the joints of children with JIA had a particular phenotype, distinct from that of PBMC. Several studies have found a major decrease of CD19^{+} B lymphocytes and an accumulation of T lymphocytes, particularly of CD8^{+} T cells in synovial fluid compared to blood (Silverman et al. 1993; Hunter et al. 2010). JIA synovial fluid T cells were mainly of the Th1 phenotype, had an elevated ability to produce the macrophage-stimulator IFN-γ (Gattorno et al. 1997) and expressed high levels of the memory marker CD45RO and of the early and late activation markers CD69, CD25 and HLA-DR (Wedderburn et al. 2000; Black et al. 2002).

Although the presence of B cells is known to be limited in the inflamed joint and there is a lack of clinical data linking these cells to the pathogenesis of JIA (Wiegering et al. 2010), B cells could be contributing to inflammation. B cells could be acting as activated class switched memory-effector cells, recognised by their elevated expression of CD27, CD69 and of the co-stimulatory molecules CD80-CD86 (Corcione et al. 2009; Morbach et al. 2011), which interact with CD28 expressed by T cells, providing them with signals for proliferation.

Due to this particular phenotype of synovial lymphocytes, for many decades, investigation in the field of childhood arthritis pathogenesis has been trying to answer the question of whether these activated cells are specifically selected to migrate to the joints for example by specific chemokines or other factors, or whether they become activated *in situ*. Unfortunately, this question remains unanswered, and there is evidence supporting both theories.

One study analysing JIA synovial fluid demonstrated an enrichment of the protein levels of the chemokines CCL3 (MIP-1α) and CXCL10 (IP-10) compared to both that found in healthy control and patient plasma, as measured by ELISA, and an elevated proportion of synovial CD8^{+} T cells containing intracellular CCL5 (RANTES) (Pharoah et al. 2006). Simultaneous to the high levels of chemokines in the JIA joint, an enhancement in the proportion of JIA synovial of all T cell subsets expressing the receptors CCR5 and CXCR3 for these chemokines has also been observed (Wedderburn et al. 2000; Gattorno et al. 2005). This was not the case for JIA synovial fluid class switched memory B cells, which expressed less CCR5 compared to their PBMC counterparts, while still having elevated CXCR3 expression (Corcione et al. 2009). The lack of CCR5 on synovial B cells could be in part an explanation

for why they do not home to the synovial microenvironment and could explain their low proportion in the JIA joint. The presence of chemokines in JIA synovial fluid and the expression of correspondent chemokine receptors on synovial lymphocytes would suggest that there is ongoing preferential migration and homing of specific cells to the target tissue, possibly activated in a secondary lymph node, or activated through the process of entry into the inflamed site.

On the other hand, evidence for activation *in situ* of synovial lymphocytes derives from studies determining clonal expansion of T cells in the joint, with instances of Vbeta8 and Vbeta20 TCRs expanded in the joint rather than peripheral blood (Thompson et al. 1998). This could result from activation of T cells in the joint by a yet unidentified antigen. Other studies which found differences in oligoclonality of T cells between SFMC and PBMC are that by (Wedderburn et al. 1999) and (Wedderburn et al. 2001) which also demonstrated that the dominant T cell clone present in the joint was affected by the JIA disease subtype.

There is also the third possibility however, that both theories are correct and that there is concurrent selective homing of cells to the joint and activation *in situ*.

6.3.1 The effect of T cell activation on ectonucleotidase expression and activity

To clarify the role of CD73 in an inflammatory setting, healthy PBMC were stimulated by using anti-CD3 and anti-CD28 monoclonal antibodies (mAb) in this study, to replicate the effect of T cell receptor activation in the presence of co-stimulation. This led to a clear reduction of the proportion of $CD3^+$ T cells, particularly $CD8^+$ T cells, expressing CD73, after 5 days of this stimulation. There was only a trend for a fall in proportion of $CD4^+CD73^+$ cells after culture with plate-bound anti-CD3 and anti-CD28 mAb. After T cell stimulation however, there was a significant decrease in CD73 protein expression per cell of $CD4^+CD73^+$ cells compared to control conditions (Figure 6.1C). This was also the case for $CD8^+CD73^+$ cells.

In order to understand whether the signal relayed through ligation of the T-cell receptor, by anti-CD3 mAb was sufficient to cause CD73 downregulation, or whether co-stimulation with anti-CD28 mAb was also required, PBMC were cultured with both anti-CD3 mAb and anti-CD28 mAb. This was important to determine as ligation of CD28, which mediates co-stimulation, can drive and control T cell numbers and fates by enhancing TCR-induced proliferation via activation of the transcription factors NFκB, nuclear factor of activated T

cells (NFAT) and activator protein 1 (AP1) (Acuto et al. 2003). Ligation of CD28 together with that of the T cell receptor is required for a productive immune response to occur, as in its absence, a TCR signal could lead naïve T cells to become inactivated and enter a state of anergy. In this condition, following TCR ligation even with co-stimulation, T cells are unable to produce IL-2 or proliferate (Alegre et al. 2001). The molecule CD28 has also been observed to have an essential role in the upsurge of glucose uptake and glycolysis required to meet the extensive metabolic demands of cell activation and proliferation (Frauwirth et al. 2002).

The requirement of co-stimulation for CD73 downregulation is particularly important in the biology of JIA, since PB T lymphocytes, particular CD8$^+$ T cells, frequently show a CD28$^-$ phenotype (Wedderburn et al. 1999). This lack of CD28, characteristic of CD8$^+$ T cells in the inflamed joint, is considered to be a consequence of T cell division and clonal senescence (Effros 1997), as distinguished by the lack of correlation of TREC numbers, relative telomere length and Ki67 expression with chronological age of JIA patients (Prelog et al. 2009; Dvergsten et al. 2013). Replicative senescence of CD8$^+$ T cells is defined not only by the lack of CD28, but also by the expression of CD57 (Brenchley et al. 2003), linked to chronic immune activation. JIA SF CD8$^+$ T cells are indeed mostly CD57^{high} (Wedderburn et al. 1999). The result found in this study that CD73 downregulation subsequent to T cell activation could indeed still occur without co-stimulation suggests that the same could occur in the JIA joint, without the presence of a CD28 signal.

Given the data in Chapter 5, showing the significant correlation between MFI of CD73 positive healthy CD8$^+$ T cells and the amount of E-adenosine generated, it was not unexpected that CD73 downregulation following T cell activation also resulted in a decreased ability of PBMC to generate E-adenosine after incubation of cells with E-AMP (Figure 6.12).

When the other two ectonucleotidases CD39 and CD26 that have an important role in the adenosinergic pathway were analysed, to understand how they are influenced by T cell activation, an increase in both CD39$^+$ and CD26$^+$ T cells was observed. In the case of CD39, this upregulation was correlated with genotype at the SNP rs10748643, which lies close to the CD39 promoter. These results correlate well with the literature since both CD26 and CD39 proteins have been demonstrated to be lymphoid cell activation markers (Kameoka et al. 1993; Maliszewski et al. 1994). The upregulation of CD26 on T cells following TCR

ligation *in vitro* was however in contrast with the result presented in Chapter 3, of the decrease of SF CD26$^+$ T cells, since these are known to have an activated phenotype (Black et al. 2002; Dvergsten et al. 2013). A possible explanation for this divergence could be the chronically activated status of SF T cells, compared to the short-term activation during the *in vitro* culture systems.

To further characterise the phenotype of those cells that lost CD73 expression and the mechanism behind this loss, PBMC were sorted into CD8$^+$CD73$^+$ and CD73$^-$ populations and cultured with anti-CD3 and anti-CD28 mAb. This experiment showed that loss of CD73 was not simply associated with increased cell death of CD73$^+$ cells and that after separate culture CD8$^+$CD73$^-$ cells were almost unaltered, while CD8$^+$CD73$^+$ cultured alone with anti-CD3/CD28 mAb lost CD73 expression.

Proliferative activity of these sorted cells was then assessed by analysing the nuclear protein Ki67, which plays a role in the regulation of cell division and is expressed during all active phases of cell division, but is absent in quiescent cells and during DNA repair (Soares et al. 2010). This assessment of Ki67 expression together with the use of the division tracking dye CFSE (Hawkins et al. 2007), found a very interesting relationship between CD73 and cell proliferation. As shown in Figure 6.9B, only those CD8$^+$ T cells that expressed Ki67 and had gone through multiple cell cycles as demonstrated by the several CFSE peaks had become CD73$^-$, while those that remained CD73$^+$ were Ki67$^-$. Additionally those CD8$^+$ T cells that had become CD73$^-$ cells were mostly positive for the memory marker CD45RO, while the remaining CD73$^+$ cells were CD45RO$^-$ (Figure 6.9D).

To understand whether this relationship was also found in the inflamed joint, SFMC, together with PBMC, were stained for CD73 and Ki67 (Figure 6.10). Among CD8$^+$ T cells, this indeed was the case, as there was almost no extent of CD73 and Ki67 co-expression, especially for SFMC, which expressed the highest levels of Ki67.

However, results from previous studies analysing the cell cycle of SFMC are contradictory. One study found very few (<1%) SFMC in the S phase (actively synthesizing DNA), with no difference compared to PBMC (Black et al. 2002), while another observed a much higher mean of T cell SFMC in S phase compared to PBMC (Brunner et al. 2010), suggestive of rapid cell turnover. The difference in results between the two studies could be because the first performed cell cycle analysis with propidium iodide on all mononuclear cells, while the

latter analysed only those in the CD3$^+$ T cell gate. When (Dvergsten et al. 2013) measured the proliferative doubling capacity of T cells from blood and synovial fluid, a much lower proliferative capacity was observed for SFMC compared to PBMC. This could indicate cell exhaustion, which is known to be found in JIA (Prelog et al. 2008) and fits with the CD28$^-$ CD57^{high} phenotype of SF T cells (Wedderburn et al. 1999). The previously observed loss of CD73 expression in the joint (Chapter 3) could therefore be a consequence of persistent cell activation and exhaustion. This result is in accordance with the inverse correlation found between expression of CD73 and markers of cell activation and exhaustion (Tóth et al. 2013) for CD8$^+$ T cells from HIV-infected patients. Other known effects of T cell activation on the adenosinergic pathway include the rapid upregulation of adenosine receptor expression, particularly A$_{2A}$R (Lappas et al. 2005).

6.3.2 Expression of ectonucleotidases by B cells and their stimulation

As JIA synovial B cells, like T cells, are also known to have an activated phenotype, (Corcione et al. 2009; Morbach et al. 2011), B cell activation *in vitro* obtained by a range of stimuli, was tested for effects on CD73 expression of PBMC.

B cells express the pattern recognition Toll-like receptor 9 (TLR) which recognises certain CpG-oligodeoxynucleotide (ODN) sequences of microbial DNA, leading to their activation. Exposure of B cells to CpG-ODN 2006 therefore leads to up-regulation of activation markers, proliferation, cytokine secretion (Decker et al. 2000; Gantner et al. 2003) and class switch recombination (He et al. 2004) of B cells.

There is no published study that analyses TLR9 expression in the JIA joint. However, exposure to the cytokine IL-17 increased expression of TLR-9 in a CIA model of arthritis (Lee et al. 2009), and in the JIA joint there is an enrichment of IL-17 producing cells (Nistala et al. 2008) and therefore of IL-17 itself (Agarwal et al. 2008). Therefore, it could be suggested that there may be induction of TLR9 expression on B cells in the JIA joint. Culture of PBMC with CpG led to a decrease in the proportion of CD19$^+$CD73$^+$ cells, while increasing that of CD19$^+$CD26$^+$ cells. CD26, which is lacking on resting B cells, was known to be induced on these cells after their stimulation (De Meester et al. 1999). It is important to note also that in human naive B cells, TLR9 expression is almost undetectable and is increased only after B cell receptor triggering; so unless CpG is added together with a BCR ligand, only memory B cells are able to proliferate in response to CpG (Bernasconi et al.

2003). Therefore, in this study the only B cells that lost their CD73 expression after culture with CpG were memory B cells.

It is known that CD40L (CD154), which is transiently expressed by activated T cells, allows them to interact with cells bearing CD40. The ligation of CD40 on B cells leads to their proliferation and class switching (Armitage et al. 1993). A soluble form of CD40L (CD154) has been found elevated in the sera of JIA patients (Prahalad et al. 2008), potentially released by activated T cells in synovial fluid, where it is likely that sCD154 levels are also elevated. Because of the stimulatory role of CD40L on B cells, PBMC in this study were co-cultured with CHO cells transfected with CD40L. This experiment brought a decrease in CD73$^+$ B cells and an increase of CD26$^+$ B cells, similar in number to that observed after culture with CpG.

The use of an F (ab')$_2$ fragment of antibody directed against IgG and IgM on B cells lead to a decrease in the proportion of CD19$^+$CD73$^+$ cells compared to control conditions, which did not however reach statistical significance. It is possible that with a higher sample number, this decrease could have reached significance.

It was interesting to note that when PBMC were cultured, T cell activation also resulted in a decrease in the proportion of CD19$^+$CD73$^+$ B cells. However when compared to "classical" B cell stimuli, CD73 downregulation on B cells after T cell stimulation took 5 days instead of 3. A possible explanation for this decrease in CD73 expression and divergence in time points is that T cell stimulation resulted in the upregulation of the T cell activation marker CD40L. This process occurs shortly after TCR activation, but a second phase of expression, after which CD40L expression reaches its maximum, only occurs after 2 days and is CD28-dependent (Snyder et al. 2007). CD73 downregulation was therefore likely to be linked to CD40L upregulation on T cells, leading in turn to stimulation of B cells, and there was a longer kinetic for the change on B cells because it takes several days for this upregulation on T cells to occur.

The downregulation of CD73 expression on B cells following T cell activation could also explain the differences found between the cell culture supernatant of PBMC and sorted CD8$^+$ CD73$^+$ cells. While some soluble CD73 was detected in the supernatant of PBMC and was found elevated in that of PBMC cultured with anti-CD3 mAb and anti-CD28 mAb (Figure 6.11A) compared to those in control conditions, none was found in that of sorted CD8$^+$

CD73$^+$ cells in either condition. This could be explained by the fact that in cultures of total PBMC, CD73 lost by B cells was being shed into the supernatant, while in cultures of CD8 cells alone, CD73 protein lost by CD8 T cells met another fate such as internalisation. Thus it is possible that T and B cells down-regulate their CD73 through divergent mechanisms. If time had allowed I could have tested this, for example by the use of Imagestream which consists of a flow cytometer linked to a confocal microscope, allowing for the visualisation of fluorescent labelled cells on a cell by cell basis. Therefore, T cells prelabelled with a fluorescent anti-CD73 mAb could have been stimulated with anti-CD3/anti-CD28 and then analysed for CD73 protein internalisation by Imagestream.

6.3.3 Effect of cytokines and soluble mediators

Since the synovial environment is enriched in cytokines, particularly the inflammatory cytokines TNF-α, IL-1 and IL-6 (de Jager et al. 2007), known to have important activities in the context of JIA pathogenesis, these together with the activation-induced cytokines IFN-γ and IL-2 were tested for potential effects on CD73 expression by PBMC *in vitro*. As represented in Figures 6.6 and 6.7, none of these cytokines affected significantly the proportion of CD73$^+$ cells among either CD8$^+$T cells or CD19$^+$ B cells.

This result disputes the findings of previous studies which observed CD73 induction and loss by the cytokines IL-1 and TNF-α (Savic et al. 1990; Kalsi et al. 2002). These studies were however on non-leukocyte cells, such as glomerular mesangial cells and when (Niemelä et al. 2004) observed a long-term upregulation of CD73 in response to IFN-α, this was limited to endothelial cells and did not affect lymphocytes. Christensen et al.(in 1992) measured the effects of pro-inflammatory cytokines on CD73 nucleotidase activity of human blood lymphocytes. This study found TNF-α to increase CD73 enzymatic activity, IFN-γ to decrease it and IL-1 and IL-6 to have no effect. However since in this study, nucleotidase activity was measured by quantifying the formation of [^{14}C]-adenosine from [^{14}C]-AMP and no adenosine deaminase inhibitor was used, adenosine could have been broken down to inosine, resulting in unreliable measurements of CD73 activity.

Another cytokine tested in this present study was TGF-β, which is produced by activated T cells and inhibits their proliferation, suggestive of a negative feedback role (Rubtsov et al. 2007). As mentioned in the introduction, levels of this cytokine are decreased in the synovial fluid of patients with JIA compared to levels found in their plasma (Nistala et al. 2010) as

measured by ELISA. However since the ELISA test measured latent TGF-β which may not bind to TGF-β receptors and therefore may not have biological activity, before the occurrence of an activation step, a bioassay test would have been more physiological (Oida et al. 2010).

The expression of CD73 after culture of PBMC with active TGF-β was particularly interesting to analyse as this cytokine has been found to control adenosine generation by preventing the down-regulation of CD73 on activated murine CD4^{+} T cells (Regateiro et al. 2011). It was therefore considered that the lack of CD73 on JIA synovial lymphocytes might be in part due to a lack of TGF-β preventing the loss after T cell activation. The addition of TGF-β to PBMC *in vitro* in this study had however no effect; neither preventing downregulation on activated cells nor inducing CD73 expression in control conditions.

To replicate *in vitro,* the conditions as close as possible to those present in the synovial environment, PBMC were cultured in the presence of synovial fluid pools, in parallel to the addition of control plasma (VBP) pools. In order to define what was being added to PBMC, these pools were analysed for their cytokine content by Luminex. This determined unexpectedly, control plasma pools to contain higher levels of TNF-α and IL-1 compared to SF, while both contained similar levels of the cytokine IL-6.

Despite the lack of statistically significant differences in the proportion of CD73^{+} cells among B cells and CD8^{+} T cells after culture with SF or VBP, there did appear to be some decrease after culture in these conditions. Thus, a small fall in CD73^{+} cells was observed after culture in both blood plasma and synovial fluid. There is the therefore the possibility that with a higher number of samples tested, the difference could have been significantly different.

6.3.4 MTX and its effect on CD39 and CD73 expression in culture

So far, little is known of the mechanism of action of MTX, particularly at the low dosages used for the treatment of JIA and RA. At the higher doses employed for the treatment of malignancies such as cancer, MTX is known to act as a cytotoxic agent (Wosikowski et al. 2003). One of the immunomodulatory effects of MTX appears to be mediated by the release of immunosuppressive adenosine dependent on the action of CD73 (Montesinos et al. 2007). MTX can also alter cytokine release and receptor binding, such as is the case for IL-1 (Connolly et al. 1988; Brody et al. 1993) and for IL-6 and TNF-α, potentially through the increase in adenosine levels (Cronstein 1997).

After injection of MTX, the drug is poly-glutamated *in vivo* to various polyglutamate forms. After culture of PBMC with the different MTX polyglutamates, a significant difference in the proportion of $CD73^+$ or $CD39^+$ cells among $CD8^+$ T cells was observed in the culture of PBMC with $MTXGlu_4$, compared to control conditions (Figure 6.13), with a decrease of $CD73^+$ and an increase of $CD39^+$ cells. In contrast, the $MTXGlu_3$ form of the drug appears to counteract the effects of cell stimulation, at least partially. Because of the anti-inflammatory effects of MTX, it had been hypothesised that its addition would have resulted in the increase of expression of both ectonucleotidases, leading to a net regulatory and suppressive effect. Additionally, this result was not in the accordance with the data presented in Chapter 4, where PBMC of JIA patients obtained after 6 months of treatment with MTX, and tested directly *ex vivo*, presented no changes in $CD73^+$ proportions prior to culture and the frequency of $CD39^+$ T cells decreased after treatment.

6.3.5 Conclusion and Summary

The main findings presented in this chapter are that:

- T and B cell activation result in a downregulation of CD73 protein expression on both T and B cells and that this decrease is associated with cell cycling and proliferation.

- The activation of T cells can in turn lead to interactions with B cells which then downregulate CD73 on their cell surface

- At the same time as this CD73 downregulation, the other main ectonucleotidases of the adenosinergic pathway CD39 and CD26, are upregulated

- A range of both pro- and anti-inflammatory soluble mediators do not affect CD73± cell proportions *in vitro*

- After T cell activation, an unknown cell subset, other than CD8 T cells, most likely B cells, can shed their CD73 protein into the extracellular space. The resultant culture supernatant does not however have enzymatic AMPase activity.

Chapter 7 Final Discussion

The principal objective behind the research presented in this thesis was to determine whether purinergic pathways may be abnormal at the site of inflammation in human chronic arthritis in children, and whether these alterations can contribute to the pathogenesis of JIA. This work was performed particularly to address the question whether a reduction in adenosine generation could be part of the abnormalities seen in the chronic inflammation in JIA.

The rationale behind this question arises from the fact that adenosine is a crucial regulator and modulator of the immune system that can dampen inflammation. Adenosine can attenuate the inflammatory events ensuing from cell damage (Ohta et al. 2001) and/or cell activation (Huang et al. 1997) to contribute towards the restoration of immune homeostasis. Mechanisms by which adenosine has been suggested to reinstate normal immune function include the inhibition of T cell activation, function and expansion (Huang et al. 1997; Csóka et al. 2008; Himer et al. 2010), together with the promotion of induction of regulatory T cells (Zarek et al. 2008). In contrast ATP, known to be produced by activated lymphocytes (Schenk et al. 2008; Schena et al. 2013), at sites of inflammation (Bodin et al. 1998) and following cell damage (Yegutkin et al. 2000) contributes to the promotion of inflammation (de Oliveira et al. 2014) following its release from the intracellular compartment.

Investigation of adenosine generation is therefore relevant to JIA, since both lymphocytes aspirated from the arthritic joint and the synovial layer itself have an activated phenotype (Black et al. 2002; Gattorno et al. 2007) and are therefore believed to potentially result in release of ATP within the inflamed joint.

7.1 Characterisation of ectonucleotidase expression by leukocytes in healthy and inflammatory environments

To address the question of capacity to generate adenosine at the inflamed site in JIA, the initial work of this thesis focused upon the protein expression and enzymatic activity of the main ecto-nucleotidases that affect nucleotide and nucleoside extracellular availability; that is CD39, CD73, and ADA enzymes.

A previous study in the Wedderburn group of Moncrieffe et al. (2010b) had revealed an increase in the proportion of CD39^{+} synovial T cells together with increased CD39 protein level per CD39^{+} cell aspirated from the joint, which resulted in a higher ability to hydrolyse ATP. This result concurs well with other studies which have shown CD39 to be expressed on

activated and memory T cells (Maliszewski et al. 1994; Zhou et al. 2009; Dwyer et al. 2010). Because of the pro-inflammatory effect of ATP, it would appear that increased expression of CD39 could have a potential immunomodulatory effect by decreasing the availability of ATP in the extracellular environment. The work presented in this thesis confirmed the high proportion of JIA CD39$^+$ synovial T cells and extended this observation to an upregulation of CD39$^+$ B cells in the inflamed joint.

Because CD39 and CD73 work in tandem to hydrolyse ATP to adenosine, it was also relevant to ask whether the expression of CD73 is also increased on synovial lymphocytes of JIA patients. No previous studies with a focus on CD73 expression in human arthritis were published at the time this work was initiated.

A decrease in CD73$^+$ cells among JIA SFMC (Chapter 3) was however revealed. This decrease in the proportion of CD73$^+$ cells from the JIA joint was observed among T cells (both CD4$^+$ and CD8$^+$) and B cells and also specifically Foxp3$^+$ Treg. Interestingly, a similar result to JIA Treg has recently been found for synovial Treg from adult RA patients which were observed to present many CD39$^+$ cells, but few CD73$^+$ cells (Herrath et al. 2014). This would suggest that ongoing events in the inflamed joint of patients with arthritis affect CD73 expression and that these mechanisms may be common to both JIA and adult RA.

Expression of CD26 is often used as a marker for ADA (adenosine deaminase), since it allows ADA to anchor to the outside of the cell membrane (Kameoka et al. 1993). ADA is important to investigate in purinergic studies since it deaminates adenosine to inosine, therefore terminating the effects of adenosine. As CD26 has been suggested to be an activation marker (Dong et al. 1996) and synovial T cells have an activated phenotype (Black et al. 2002), it was interesting that the work presented here also found reduced a proportion of CD26$^+$ cells among synovial T lymphocytes compared to their blood counterparts. The finding is in contrast with previous findings in adult RA, which observed increased percentages of CD3$^+$ CD26$^+$ cells in SF compared to blood T cells (Mizokami et al. 1996).

Given that both extracellular ATP and adenosine can modulate inflammation such as that seen in chronic arthritis, measurement of their concentration in plasma and synovial fluid could help determine to what extent these molecules respectively stimulate and restrain the inflammatory processes in JIA. However, an accurate measurement of their concentrations is difficult to obtain due to release of ATP and ADP from platelets and erythrocytes during

measurement resulting in fluctuations of nucleotide levels (Gorman et al. 2007), together with other technical limitations.

A mouse model of arthritis such as CIA cannot provide a better understanding of JIA because many differences have been found between human and mice, regarding ectonucleotidase distribution, particularly that of CD73, on leukocytes. An example of these differences include the lack of CD73 expression on both healthy blood and synovial fluid granulocytes or monocytes observed in this study (Chapter 3), whereas in mice, CD73 protein was instead upregulated on these cell subsets aspirated from the knee joint of CIA mice (Flögel et al. 2012). Human and mice Treg also differ in their proportion of $CD73^+$ cells. In this and in previous studies (Dwyer et al. 2010), only a small proportion of healthy human Treg were found to be $CD73^+$, with a median of 11% (IQR 7.5-15.0%) of Treg expressing CD73 protein (Chapter 3). On the other hand, typically more than 60% of healthy murine Treg are $CD73^+$ (Mandapathil et al. 2010).

7.2 Reduction in $CD73^+$ cells in JIA and other immune arthritis

As discussed above, this thesis demonstrated a reduction in the proportion of JIA $CD73^+$ synovial lymphocytes as compared to both healthy control and JIA patient peripheral blood lymphocytes. This reduction affected both B and T synovial fluid lymphocytes, particularly $CD8^+$ lymphocytes. Together with the diminished percentage of $CD73^+$ SFMC, median expression level of the CD73 protein per cell was also found reduced on $CD73^+$ SFMC compared to equivalent cell populations in the blood of both patients and controls (Chapter 3).

CD73 protein has been previously found to continuously cycle between the cell surface membrane and the cytoplasmic membranes of rat hepatoma cell lines (Widnell et al. 1982; van den Bosch et al. 1988). The reduction of CD73 surface expression on JIA SFMC was hypothesized to be due to intracellular accumulation of the protein. However, no difference was found between the proportion of $CD8^+$ SFMC or any other synovial fluid lymphocyte subset (data not shown) that expressed CD73 on the cell surface or in total (surface + intracellular expression), as assessed by flow cytometry suggesting that this hypothesis was not correct. The total CD73 protein levels in $CD8^+$ cells from blood and joint were also assessed by the Western blot experiment (see Figure 3.4), which also suggested a lack of detectable CD73 protein in JIA SFMC. However, this experiment was performed on only two

replicates and does not distinguish the membrane from intracellular protein fractions in the cells analysed.

Interestingly genetic studies of familial disease presenting with calcification of the arteries and the small joint capsules, has been shown to be associated with mutations in CD73 (St Hilaire et al. 2011; Fausther et al. 2014). The Fausther et al. study determined that nonsense or missense mutations in the CD73 (NT5E) gene cause significantly reduced subcellular trafficking, but that total protein levels in cells transfected with these mutations was not decreased. That study did not assess CD73 in immune cells and no immune phenotype was described in these families. In the St Hilaire study, *in vitro* genetic rescue experiments that normalized CD73 activity in patient's cells restored normal calcification (St Hilaire et al. 2011). If time had allowed, confocal microscopy could have been potentially used in my JIA synovial cell study to rule out a role for altered trafficking of CD73 protein in activated cells.

In order to investigate whether the observed downregulation of CD73^{+} synovial T cells might relate to disease severity, the proportion of CD8^{+}CD73^{+} synovial cells was investigated in different groups of patients. Specifically both polyarticular JIA (who have 5 or more joints involved early in disease), and extended oligoarticular JIA (whose disease extends to 5 or more joints after 6 months or longer) are considered to be severe clinically (defined by number of joints involved as well as degree of inflammation). This severity is higher than that seen in patients with the subtype known as persistent oligoarticular JIA, in whom disease remains mild and involves 4 or less joints throughout. The decrease in the proportion of CD73^{+} JIA synovial lymphocytes was more marked for those patients suffering from the more severe extended oligoarticular JIA and polyarticular JIA, compared to those with the more benign persistent oligoarticular JIA (Chapters 3 and 4).

This finding corroborates and extends results of a previous publication of Hunter et al. (2010). That study had the objective of identifying potential markers for the prediction of extended oligoarticular JIA, prior to the extension to this more severe disease. The Hunter study included a microarray analysis of mRNA prepared from total SFMC in persistent and so-called "extended-to-be" oligoarticular JIA, i.e. those who had mild early disease but went on to more severe disease. Among the genes differently expressed between patients with persistent and extended-to-be oligoarticular JIA, the Hunter study demonstrated a 2.02 fold decrease in mRNA expression level of NT5E (encoding CD73) for patients with extended-to-

be oligoarticular JIA compared to those who then continued to have persistent oligoarthritis. Thus, it is possible that in the "extended-to-be" patients the synovial inflammation is already more severe before extension occurs, and that one biological correlate of this severe inflammation is more marked reduction of CD73 expression. In this thesis, mRNA levels for CD73 were not measured in the samples studied, making it difficult to compare the two sets of data.

Given the differences observed between the different clinical subtypes of JIA, it was hypothesised that there might be genetic polymorphic variations in the NT5E locus between these two subtypes. Several other genetic regions have been shown to differ between the two subtypes of oligoarticular JIA such as IL-10 and CD25 (Crawley et al. 2001; Hinks et al. 2009; Omoyinmi et al. 2012). To determine whether these two types of JIA had genetic differences in the NT5E gene locus, SNP genotyping was undertaken as part of this thesis in collaboration with Prof W Thomson et al from Manchester University. No significant genetic differences in the NT5E gene region were however revealed in this genetic association analysis (Chapter 4), either between persistent and extended oligoarticular patients, or between all JIA cases and healthy controls. This therefore provided no evidence to support a genetic predisposition to low CD73. However, a caveat of this analysis is that it may not have included all possible genetic variants (since both GWAS and Immunochip data do not provide whole sequence data). It is also still difficult to determine the causality of a SNP and to verify its effect on the expression of a particular gene, particularly as about 90% of variations occur outside of the definite protein-coding gene sequences (Dunham et al. 2012). Thus, genotyping strategies may not fully include all regulatory regions (or SNPs) that influence gene expression. Recent increasing understanding of gene regulation shows that many genetic SNPs associated with disease in GWAS studies localise to genetic regions in non-coding DNA, acting as enhancer or so-called 'super enhancer' regions that affect gene expression (Farh et al. 2014), and that these regions may have different effects in different cell types. The Farh et al. study determined, for example, that most casual SNPs are located in the T and B cell enhancer regions. Furthermore, some genetic variation only alters expression in activated cells.

In addition to reduced CD73 expression, other alterations in the co-expression of CD73 with other proteins on JIA SFMC were observed in this study. Whilst for both healthy and patient blood, production of the pro-inflammatory cytokine IFN-γ by CD8^{+} lymphocytes was mostly

limited to CD73$^-$ cells (enriched in the memory T cell CD45RO$^+$ population), in JIA synovial CD8$^+$ T cells the distinction between CD73$^+$ IFN-γ$^+$ and CD73$^-$ IFN-γ$^+$ cells was less clear (Chapter 3). This would appear to be further evidence of dysregulated immunoregulatory mechanisms at the site of chronic inflammation. In healthy blood samples, a similar result was found for a component of cytolytic granules known as perforin, where in healthy blood CD73$^+$ cells did not express perforin, whilst the majority of perforin$^+$ CD8$^+$ T cells were CD73$^-$. Perforin not only has an effector function (Smyth et al. 1995), but also has also been suggested to have a role in regulating and turning off T cell responses (Bi et al. 2009). However, interestingly in SF CD8$^+$ T cells, perforin expression was rare in both the CD73$^+$ and the CD73$^-$ populations. Thus, JIA synovial lymphocytes were characterised as a whole by decreased levels of perforin production. This finding is of particular interest given mounting evidence that suggests that perforin expressing CD8$^+$ T cells may themselves be immunoregulatory.

A disease typically presenting in young children, in which perforin expression is frequently dramatically reduced and which has striking activation of the immune system, is hemophagocytic lymphohistiocytosis (HLH) (Henter et al. 1998). This condition may be either primary HLH (some cases now understood to be due to genetic mutations) or secondary HLH, typically associated with other diseases (e.g. systemic JIA (sJIA), SLE, and sometimes infection). As well as profound immune activation, patients with HLH also display a severe impairment of the cytotoxic function of NK and CD8$^+$ T cells (Egeler et al. 1996). Some of these patients have been shown to have associated loss-of function mutation of the gene encoding perforin (Stepp et al. 1999). One form of secondary HLH, (sometimes called macrophage activation syndrome MAS), can also occur in the most severe form of JIA, sJIA. These sJIA patients have a perforin defect in blood CD8$^+$ T cells, which recovers once the disease is treated (Wulffraat et al. 2003).

The data presented here and the HLH data are interesting, because both JIA and HLH patients may have high serum levels of IFN-γ (especially SF for JIA) (Akashi et al. 1994; de Jager et al. 2007). Furthermore, murine models of HLH which were perforin-deficient (Jordan et al. 2004; Chen et al. 2012) also presented very high levels of IFN-γ. Therefore, it appears that a loss of perforin cytotoxic function can be correlated with an imbalance in the immune system, possibly promoting an imbalance of T-cell derived cytokines such as IFN-γ. A recent study has suggested that the mechanism of this imbalance may be that CD8$^+$ perforin$^+$ T cells

might control a subset of pro inflammatory DC (possibly presenting self-antigens), and that a lack of these cells allows unchecked DC driven T cell activation (Terrell et al. 2013).

Another form of chronic arthritis that presents a low proportion of CD73$^+$ SFMC is adult RA. As mentioned previously, the Herrath et al study (2014) also observed a decrease in the proportion of CD73$^+$ synovial fluid Treg and CD4$^+$ T cells. However, it remains to be determined whether CD73 downregulation in RA SFMC also affects B cells and CD8$^+$ T cells and whether it results in reduced AMPase activity, as is the case in JIA.

7.3 Reduced CD73 expression in other immune disorders

While few studies have considered CD73 expression on mononuclear cells from the inflamed joint, several other immune conditions have been described in which CD73 expression on lymphocytes, and their AMPase activity is altered. For example, there are several published reports which observed deficiencies or total lack of CD73 AMPase of lymphocytes as a whole, or for T and B cells separately, in patients with hypogammaglobulinemia (Johnson et al. 1977; Rowe et al. 1979; Edwards et al. 1980), with Omenn's syndrome (an autosomal recessive form of SCID) (Gelfand et al. 1984), and with acquired immune deficiency syndrome (Salazar-Gonzalez et al. 1985). It was only possible to study CD73 protein expression after the study of Airas et al. (of 1993), which introduced the first anti-CD73 antibody, after which patients with inflammatory common variable immunodeficiency (CVID) (Marashi et al. 2011) and HIV patients (Tóth et al. 2013) were also found, as in JIA synovial cells, to have a reduced percentage of CD8$^+$CD73$^+$ T lymphocytes. Both naïve and switch memory B cells from CVID patients also presented a significant downregulation in their proportion of CD73$^+$ cells (Schena et al. 2013).

Many of these types of immunodeficiency are characterised by low levels of most immunoglobulins, reduced percentage of B cells and defects in B cell maturation. It is therefore difficult to establish whether the observed reduction of CD73$^+$ B cells in these conditions is a contributor to the pathogenic process or is simply a reflection of defective cell maturation. In addition, there is evidence that extracellular adenosine is required for immunoglobulin class switch recombination in B cells, and lack of CD73 prevents the generation of class-switched B cells (Schena et al. 2013). CD73 is also considered a B cell maturation marker, due to its low levels on cord blood B cells and elevated levels on adult

peripheral blood B cells (Thompson et al. 1986). Therefore, deficiency of CD73$^+$ B lymphocytes could be a result of defective B cell maturation.

One possible explanation for the observed downregulation of CD8$^+$CD73$^+$ T cells in the JIA joint is cell activation, as observed for HIV patients (Tóth et al. 2013). This study described an inverse relationship between the proportion of CD8$^+$CD73$^+$ cells in blood from HIV infected patients with the cell activation and exhaustion status, as indicated by the reduced CD73$^+$ cells among the subset of CD8$^+$ cells which expressed the highest levels of HLA-DR and the exhaustion maker programmed death (PD)-1. In contrast, CD8$^+$CD73$^+$ cells had higher proliferative capacity and an increased ability to produce IL-2 upon stimulation.

Intererestingly, a subset of untreated infected HIV patients able to spontanously control HIV replication, known as HIV controllers (HICs), posses a subset of CD8$^+$ T cells which express high levels of CD73 (Carrière et al. 2014). HICs are able to preserve these CD8$^+$CD73$^+$ cells despite residual cell activation. The authors suggested that CD73 plays a key role in the establishement of a strong anti-HIV CD8$^+$ T cell response. Other than the CD73 downregulation, T cells from the peripheral blood of HIV patients also presented an upregulation of CD39$^+$ cells (Schulze Zur Wiesch et al. 2011) as seen in JIA. Mechanisms of CD73 downregulation in JIA are discussed in section 7.5.

7.4 Decreased ability to generate adenosine and implications in arthritis

Following the observed reduction of the proportion of CD73$^+$ JIA SFMC, the ability of these cells to produce E-adenosine from E-AMP was measured in an HPLC assay and compared to that of patient and healthy control PBMC. As shown in Figure 5.3, JIA synovial lymphocytes were less able to hydrolyse E-AMP compared to both patient and healthy control PBMC, consistent with their reduced CD73. The downregulation of CD73$^+$ PBMC that occurred *in vitro* following cell activation (discussed in section 7.5) also resulted in reduced production of adenosine, compared to those PBMC cultured in control conditions (Chapter 6).

As well as the reduced capacity to produce adenosine as measured by HPLC, other observations include the inability of JIA synovial fluid to generate E-adenosine from E-AMP despite detecting CD73 protein in the fluid (Chapter 5). Furthermore, the demonstration that co-expression of CD39 and CD73 (limited to B-lymphocytes) is needed for these proteins to hydrolyse ATP to adenosine (Chapter 5) suggests that in the synovial environment the overall

capacity for adenosine production will be reduced. In addition, the enzymatic activity of alkaline phosphatase, which can generate adenosine directly from ATP, was also reduced in JIA synovial fluid, compared to both patient and healthy control plasma (Chapter 5). Together these findings suggest a picture of disturbed adenosine generation within the JIA joint.

It is therefore important to consider the potential consequences of the lack of immunosuppressive adenosine, and the supposed elevated AMP levels due to the high proportion of CD39$^+$ synovial lymphocytes within the JIA inflamed joint. Knowledge of the immunological effects of AMP is very limited and AMP is considered almost inactive (Meghji et al. 1995). It has only been determined recently that AMP can activate the adenosine A$_1$ receptor (Rittiner et al. 2012). As this receptor is coupled to the G$_i$ (inhibitory) protein, the activation of which results in inhibition of adenylate cyclase and thus suppression of accumulation of immunosuppressive cAMP (Fredholm et al. 2001), ligation of AMP to the A$_1$R, could be suggested to have pro-inflammatory effects.

A recent study by Saze et al. (of 2013) suggested that AMP could be immunosuppressive. This is because they observed that activated B cells, which were more CD39$^+$ and less CD73$^+$ compared to resting B cells (as was also found in this thesis) had an elevated ability to produce AMP from ATP, and an augmented ability to inhibit T cell proliferation. It must be kept in mind however, that AMP concentration within the cell medium was not measured in this study and that their suggestion was therefore speculative. The suppression of T cell proliferation by activated B cells could be not associated at all with purines, and be dependent on the release of anti-inflammatory cytokines IL-10 and TGF-β, which can be increased after B cell activation (Parekh et al. 2003; Griffin et al. 2012).

In the co-culture experiments of CD8$^+$ CD73$^+$ cells with CD14$^+$ CD39$^+$ in this thesis, the potential role of adenosine production by exosomes was not investigated. Exosomes are 30-100nm membrane vesicles which contain a multitude of both cytosolic and membrane proteins and are released by most cells, including immune cells, and particularly by tumour cells. Exosomes have been revealed to express both CD39 and CD73 and to have potent ATPase and AMPase activity (Clayton et al. 2011), which could potentially explain the immunosuppressive levels of adenosine present in the extracellular fluid of tumours (Blay et al. 1997). A study by Schuler et al. (of 2014) revealed that healthy human Treg, which are

highy $CD39^+$, but also almost entirely $CD73^-$ were able to generate adenosine after co-incubation of with exosomes. This result suggests the importance of investigating the presence of exosomes in JIA patient blood and synovial fluid. A recent study has suggested that T-cell derived exosomes (microparticles) are present in the synovial fluid of arthritis patients (György et al. 2012), but it did not investigate CD73 expression on these particles.

In addition to lymphocytes, other cell types that may be present with the JIA joint could contribute to production and release of regulatory adenosine. An immunoregulatory subset of cells that are being infused as part of therapy to control autoimmune diseases in children (Swart et al. 2012) and mice models of arthritis (Chen et al. 2013), are mesenchymal stem cells (MSC). These highly proliferative cells are capable of generating skeletal tissues, including bone and cartilage. MSC have been found distributed in the synovial membrane of both healthy donors and osteoarthritis (OA) patients (Hermida-Gómez et al. 2011), in gelatinous Heberden's nodes of OA patients (Baboolal et al. 2014) and in the synovial fluid of patients with osteochondral lesions of the talus (Kim et al. 2015). Interestingly, a classical marker of MSC is CD73 expression. In fact, CD73 was expressed by all the MSC in these reports of MSC detection. It would therefore be of interest to further investigate whether MSC are also expressed in the JIA joint (a preliminary report of MSC in the JIA joint was made by Lazić et al. 2012) and whether they can contribute to adenosine generation, particularly as ATP has been found to strongly enhance the metabolism and immunomodulatory effects of MSC (Abstract 275, Hang et al. 2013).

Endothelial cells (EC) can also modulate JIA pathology and are considered to have a central role in the inflammatory process. This is because of their location in blood vessels in the subintima where they interact with blood cells, and faciliate migration of leukocytes into the joints or plasma extravasation leading to swelling of the joints, and contribute to pannus formation through the angiogenic process. At the same time however, EC can catabolize extracellular nucleotides (Pearson et al. 1985) since they express CD39 and CD73 (Robson et al. 2005; Hart et al. 2011) and therefore can also have an immunosuppressive role. The ability of EC in and around the JIA joint to produce adenosine is therefore also worth exploring.

The generation of adenosine through CD73 is particularly important in understanding JIA pathogenesis since the first line DMARD to control arthritis, methotrexate, exerts is anti-inflammatory mechanism in part through adenosine production by CD73 (Morabito et al.

1998; Montesinos et al. 2007). Understanding of this protective process has instigated research to try to target CD73 for therapeutic intervention. For example, Flögel et al. (2012) have indeed synthesized an $A_{2A}R$ agonist that requires for its activation the upregulated presence of CD73, as found on synovial leukocytes of CIA mice. This prodrug would allow separation of the desired immunosuppressive effects of adenosine from the unwanted vasodilatory side effects. However, due to the lack of this CD73 upregulation in human arthritis at the site of inflammation, this drug could not be exploited in humans.

7.5 Potential mechanisms that lead to CD73 downregulation

So far, this discussion has focused on the reduction of CD73$^+$ JIA synovial lymphocytes and of their AMPase enzymatic capacity, together with the functional implications of these observations. Data within this thesis also reported a downregulation of CD73$^+$ and of purified CD8$^+$CD73$^+$ PBMC following stimulation of T and B cells *in vitro* (Chapter 6). To address the possible mechanisms for this CD73 downregulation, several routes were explored within this study.

There has been some evidence that pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-1, IL-2, and IL-6 can alter the expression and activity of CD73 (Savic et al. 1990; Christensen et al. 1992; Kalsi et al. 2002). Since most of these cytokines are released by cell activation and are known to be elevated in the synovial fluid of JIA patients (de Jager et al. 2007), the effect of the addition of all these cytokines to PBMC in culture was investigated (Chapter 6). None of the cytokines tested led to significant alterations in the proportion of CD73$^+$ PBMC. This was also the case for the addition of synovial fluid from patients with JIA, tested in comparison with healthy control venous blood plasma (VBP). In these experiments (see Figure 6.8), both SF and VBP caused some reduction of the proportion of CD8$^+$CD73$^+$ PBMC, which was not statistically significant. Unexpectedly, VBP was found to contain higher levels of classical pro-inflammatory cytokines including TNF-α, IFN-γ, and IL-2 compared to SF (Figure 6.8). This result would not however explain why with increasing concentration of VBP there was less of a reduction of CD73$^+$ PBMC, whilst with higher concentrations of SF there was a higher decrease of CD73$^+$ PBMC. It therefore appears that soluble mediators in the JIA extracellular medium could not explain the downregulation of CD73$^+$ JIA synovial lymphocytes. However, as there was some extent of alteration of the proportion of CD73$^+$ PBMC after incubation with VBP and SF, this route would be worth

exploring further. It is likely that the cytokines present at the microenvironment in tissues may be different to those measureable in extracted free fluid form the joints in arthritis, making it difficult to model the effect of this environment *in vitro*.

Data obtained from the culture of purified CD8$^+$CD73$^+$, stained for Ki67 and CFSE, helped determine that there is no increased cell death among CD73$^+$ cells which could explain their loss, and that only those CD8$^+$ T cells in cell cycle had lost their CD73 expression (Chapter 6). As PBMC cultured with B cell stimuli (CpG, CD40L) were not tested for Ki67 antigen expression, it cannot be said whether the loss of CD19$^+$CD73$^+$ B cells is also associated with cell proliferation. However, since the B cell stimuli used here mediate B cell proliferation (Johnson-Léger et al. 1997; Decker et al. 2000) this could be hypothesized to be likely to be the case.

It is still not clear what causes the loss of CD73$^+$ protein expression on T and B cells. This loss would not appear to be associated with internalisation of the protein, as the same proportion of CD73$^+$ PBMC was found after cell activation whether they were stained for surface or total CD73 expression. A more likely explanation for this loss is shedding of CD73 protein. This is because CD73 protein was detected in the culture supernatant of PBMC stimulated with anti-CD3/anti-CD28 and the proteins levels were higher than those of PBMC cultured in medium alone (Figure 6.11). A potential explanation of the increased extent of CD73 shedding after cell activation could be associated with the CD73-GPI anchor.

Like over 100 other proteins, such as alkaline phosphatase, CD73 is anchored to the outer leaflet of the cell membrane by a glycosyl phosphatidlyinositol group at its C-terminus (Lehto et al. 1998), and can be released from its anchor by the action of the phosphodiesterase phosphatidylinositol specific phospholipase C (PI-PLC) (Strohmeier et al. 1997). PI-PLC isoenzymes are activated downstream of many receptors, and are essential for the activation of the downstream transcription factors through PKC stimulation (Petro et al. 2000), and for the production of the second messenger diacylglycerol in response to BCR cross-linking (Antony et al. 2003). Activation of the T cell receptor results in PI-PLC stimulation within seconds (Turka et al. 1992), as is also the case for B cells through stimulation of CD40 (Ren et al. 1994) and CpG (He et al. 2008; Zhu et al. 2009). Since downstream signalling pathways of antigen-mediated stimulation of T and B cells share the activation of PI-PLC that can cleave the CD73-GPI anchor, cell activation of PI-PLC could explain the reduction of CD73$^+$ cells.

214

Airas et al. (in 1997) also observed shedding of CD73 protein by lymphocytes a few hours after incubation of the cells with anti-CD73 antibody. An explanation provided by the authors for this observation was that engagement of CD73 is involved with promoting binding of lymphocytes to endothelial cells (Airas et al. 2000), and the that shedding of this protein can regulate these cell-cell interactions. This mechanism would not however appear to explain the findings described in this thesis, since loss of CD73$^+$ cells occurred both among PBMC, which had not been previously stained with anti-CD73 antibody, and with sorted CD8$^+$CD73$^+$ that had been stained with anti-CD73, as it had been used to sort them.

However, it is difficult however to explain why the culture supernatant of whole stimulated PBMC contained much more soluble CD73 protein compared to that of sorted CD8$^+$CD73$^+$ that had been stimulated (Figure 6.11).

It is unclear whether methotrexate can affect expression of CD73 by PBMC since addition of MTX polyglutamates, particularly MTXGlu$_4$, had an effect *in vitro* (Chapter 6), whilst treatment of JIA patients with MTX for 6 months did not change the proportion of CD73$^+$ PBMC (Chapter 4).

Alterations in CD73 protein expression could have also been occurring through changes in either CD73 mRNA levels or its turnover or stability. This would have been worth exploring by comparing mRNA of stimulated and unstimulated PBMC. The potential changes in CD73 mRNA could occur following stimulation of transcription factors NFAT (nuclear factor of activated T cells), NF-κB, and AP-1 (activator protein-1), which bind to the promoter and enhancer region of a variety of genes. This would appear relevant to this study since T and B cell receptors have in common the stimulation of these three transcription factors (Rincón et al. 1994; Petro et al. 2000; Herndon et al. 2001; Antony et al. 2003; Antony et al. 2004; Yin et al. 2008). This could therefore explain why both T and B cell stimulation resulted in CD73 downregulation. Interestingly, in the DECODE database of binding sites of transcription factors (http://www.sabiosciences.com/chipqpcrsearch.php?app=TFBS) the ± 10kb region upstream of the CD73 gene, NT5E, did indeed contain a binding site for AP-1. Of note, this database also revealed that the gene-encoding region CD39 (ENTDP1) did not have any binding sites for transcription factors in common with NT5E. This could explain in part why the expression of CD39 and CD73 were altered differently, on stimulated cells, both *in vitro* and *in vivo*.

If the decline of CD73^{+} JIA synovial lymphocytes is linked to antigen-stimulation of these cells then the natural question to ask is, what causes this stimulation? It could be speculated that enrichment of activated APC, such as DC in the JIA synovial environment could be involved (Varsani et al. 2003). These cells express high levels of MHC class II proteins, as well as costimulatory molecules CD80 and CD86. Interestingly in RA, plasmacytoid DC (pDC) expressed high levels of HLA-DR and were efficient APC, since they were able to activate allogeneic PB T cells *in vitro* and induce secretion of TNF-α (Cavanagh et al. 2005). The chemokine CXCL10 would appear to allow recruitment of blood-derived pDC to the RA joint as it is elevated in RA SF (Lande et al. 2004). As there also are high levels of CXCL10 in JIA SF (de Jager et al. 2007), a similar inflammatory process could also be occurring in JIA. Other than the cells in the joint, the JIA synovial membrane itself is activated (Gattorno et al. 2007) and contact of lymphocytes with this structure could result in their stimulation and loss of CD73.

As mentioned in the introduction, the antigen-triggers of JIA (if they are any) are still unknown, but determination of their nature could help determine the reason for CD73 loss.

7.6 Future studies and Conclusion

The findings shown and discussed in this thesis raise many more questions and therefore many ideas for future projects. As discussed so far, the two main points that could be explored further to understand the effects of purines in JIA pathogenesis are investigation of the release and generation of adenosine from ATP via CD39 and CD73 by cells other than lymphocytes, and of the mechanisms that lead to CD73 downregulation within the JIA synovial environment. Many suggestions of how this could be explored have been already made, but other two molecules that could affect CD73 expression have not been mentioned yet: these are zinc and cAMP.

A potential explanation for the low CD73^{+} JIA synovial lymphocytes could be deficiency of the key regulatory cofactor zinc. This is because zinc deficiency has previously been shown to create an imbalance of Th1 and Th2 cells (Solomons 1998), a characteristic of the JIA joint, where there is an accumulation of type 1 SF CD4^{+} T cells (Wedderburn et al. 2000). At the same time, the serum of JIA patients contains lower concentrations of zinc compared to those of healthy controls (Honkanen et al. 1989; Haugen et al. 1992). Interestingly, patients suffering from sickle cell anaemia (SCA) had cellular zinc deficiency associated with a

significant decrease in the proportion of CD8$^+$CD73$^+$ cells (Beck et al. 1997) and normal levels of CD8$^+$CD73$^+$ cells were restored only after daily supplementation with zinc. In order to test whether this is also the case in JIA, it would be interesting to investigate any effects of the addition of zinc to cell culture- or treatment of children.

Evidence that cAMP can also regulate CD73 derives from a study of Savic et al. (from 1991) showing that cAMP-stimulating agents (which stimulated either adenylate cyclase or repressed phosphodiesterase) induced CD73 activity. This study did not answer the question of whether CD73 protein expression was altered, while a study by Narravula et al. (from 2000) revealed an increase in surface expression of CD73 by the endothelium through transcriptional induction following exposure to adenosine. A possible explanation for this phenomenon could be linked to the presence of cAMP response element (CRE) sequences in the CD73 gene promoter (Hansen et al. 1995). From these findings it could hypothesized that NT5E (encoding CD73) is a cAMP-responsive gene and that cAMP is required for CD73 transcriptional induction.

This thesis provides evidence that CD73 has a role in juvenile arthritis, which is unable to restore the "status quo" prior to inflammation as it presents defective adenosine generation. The marked decrease in the proportion of CD73$^+$ synovial lymphocytes for patients with the more severe subtype of disease suggests that reduced adenosine production could change the synovial microenvironment preventing suppression of chronic inflammation. Furthermore, CD73 downregulation may arise from proliferating cells that are shedding their CD73.

Future studies are needed to define exactly how this altered expression of an immune-regulatory molecule fits into the overall picture of pathogenesis of arthritis. It is likely that modulation of the adenosine-generating pathway will become a novel target for drugs in the future and if so, inflammatory arthritis may be a group of conditions where such drugs could be tested for a beneficial role (Jacobson et al. 2006; Gessi et al. 2011).

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233

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