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INVARIANT T CELL SUBSETS IN IMMUNE DEFENSE OF ORAL MUCOSA AND SKIN

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Invariant T Cell Subsets in Immune Defense of Oral Mucosa and Skin
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Arcydoktorem cię zwać każdy może śmiele,
Bo ty nie tylko umiesz zleczyć niemoc w ciele,
Ale i na dobrą myśl masz fortelów wiele:
Wino, lutnią, podwikę; to mi to wesele.
Jan Kochanowski, „Do doktora”

This thesis is dedicated to the memory of my late grandfather, Jerzy Gołębiewski

ABSTRACT

Innate-like T lymphocytes such as mucosa-associated invariant T (MAIT) cells and invariant natural killer T (iNKT) cells recognize antigens presented by MR1 and CD1d molecules, respectively. As MR1 presents microbial riboflavin metabolite antigens, and CD1d presents endogenous or microbial glycolipid antigens, MAIT cells and iNKT cells survey the non-protein fraction of the potential antigenic universe. Both these cell types respond in an innate-like fashion without the need for priming and clonal expansion, and are thus rapidly available to help protect the host from viral and bacterial infections. This thesis is focused primarily on the role of the innate-like T cell subsets in immune defense of the human oral mucosa and skin, two tissues that together form a large part of the body barrier to the surrounding world of microbes. In **paper I**, we establish a set of reproducible and adaptable methods to study human MAIT cell activation, cytokine production, proliferation and cytolytic function in response to microbes expressing riboflavin metabolite antigens. These novel and optimized methods establish a framework and open new possibilities to study MAIT cell immunobiology using *Escherichia coli* as a model antigen. In **paper II**, we study the lymphocyte population in healthy human buccal mucosa, and establish the presence of MAIT cells at this site. The buccal mucosal MAIT cell population is located both in the epithelium and in the lamina propria and can be divided into a tissue resident $CD103^+$ subset and a tissue non-resident $CD103^-$ subset. These two subsets differ in their functional profile both between each other and compared to the circulating peripheral blood MAIT cell population. Interestingly, tissue-resident MAIT cells had a specialized polyfunctional response profile with higher IL-17 levels, and were low in the cytolytic effector molecule perforin. In **paper III**, we investigate the involvement of oral mucosal MAIT cells in apical periodontitis (AP). MAIT cells were moderately enriched in AP tissue as compared to the surrounding healthy gingival tissue, but with higher representation of the $CD4^+$ MAIT cell subset. When the abundance of MAIT cell TCR transcript was analyzed in relation to AP microbiome data, we could identify bacterial relative abundances that negatively correlated with Vα7.2-Jα33, Cα, and IL-17A transcript expressions in AP, implying that MAIT cells could play a role in this oral disease setting. In **paper IV**, we investigate the innate immune response to herpes simplex virus 1 (HSV1) in keratinocytes. In response to HSV1 infection keratinocytes rapidly upregulate surface expression of the innate immune activating cytokine IL-15 in a TLR3-dependent manner. Interestingly, the virus actively downregulates the IL-15/IL-15Ra complex in an apparent novel mode of immune evasion. Furthermore, we show that iNKT cells can counteract this viral effect and support the maintenance of IL-15 expression on the surface of infected keratinocytes. In summary, the research covered by this thesis unravels a series of novel aspects of immunity mediated by innate-like T cells at barrier sites of the human body. The findings are of considerable importance for our understanding of immunity against bacterial and viral antigens in the oral mucosa and the skin.

LIST OF SCIENTIFIC PAPERS

I. Joana Dias, **Michał J. Sobkowiak**, Johan K. Sandberg & Edwin Leeansyah (2016) Human MAIT-cell responses to *Escherichia coli*: activation, cytokine production, proliferation, and cytotoxicity. *Journal of Leukocyte Biology* **100**: 233-240.

II. **Michał J. Sobkowiak**, Haleh Davanian, Robert Heymann, Anna Gibbs, Johanna Emgård, Joana Dias, Soo Aleman, Carina Krüger-Weiner, Markus Moll, Annelie Tjernlund, Edwin Leeansyah, Margaret Sällberg Chen & Johan K. Sandberg (2019) Tissue-resident MAIT cell populations in human oral mucosa exhibit an activated profile and produce IL-17. *European Journal of Immunology* **49**: 133-143.

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LIST OF ABBREVIATIONS

αGalCer	Alpha-galactosylceramide
AP	Apical periodontitis
AP-1	Activator protein 1
APC	Antigen-presenting cell
βGlcCer	Beta-D-glucopyranosylceramide
CBP	CREB binding protein
cfu	Colony-forming unit
cGAS	Cyclic GMP-AMP synthase
CTV	CellTrace Violet
DAI	DNA-dependent activator of IRF
DC	Dendritic cell
DCM	Dead cell marker
EI	Expansion index
EGF	Epithelial growth factor
EGFR	EGF receptor
E:T ratio	Effector-to-target cell ratio
FasL	Fas ligand
FLICA	Fluorescent inhibitor of caspases
HAART	Highly active antiretroviral therapy
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA-DR	Human leukocyte antigen – DR isotype
hpi	Hours post infection
HSV1/2	Herpes simplex virus type 1/2
GM-CSF	Granulocyte-macrophage colony-stimulating factor
Grn	Granulysin
Grz	Granzyme
ICAM-1	Intercellular adhesion molecule 1
IEL	Intraepithelial lymphocyte

IFN	Interferon
IL	Interleukin
IL-15Rα	Interleukin 15 receptor alpha
ILC	Innate lymphoid cell
iNKT cell	Invariant natural killer T cell
IRF	Interferon regulatory factor
LAT	Latency associated transcript
LCP	*Lactobacillus*-containing product
MAIT cell	Mucosa-associated invariant T cell
MHC	Major histocompatibility complex
MR1	MHC class I related-molecule 1
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell	Natural killer cell
OPG	Osteoprotegerin
PAA	Phosphonoacetic acid
PD-1	Programmed cell death protein 1
PLZF	Promyelocytic leukemia zinc finger protein
Prf	Perforin
PRR	Pattern-recognition receptor
RANKL	Receptor-activator of NF-κB ligand
sIgA	Secretory immunoglobulin A
STING	Stimulator of interferon genes
TAP	Transporter associated with antigen processing
TBK1	TANK-binding kinase
TCR	T cell receptor
Th cell	T helper cell
TIM-3	T-cell immunoglobulin and mucin-domain containing-3
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand

Treg cell	Regulatory T cell
TRIF	TIR-domain-containing adapter-inducing interferon-β
T_{RM} cell	Tissue resident memory T cell
vhs	Virion host shutoff protein

1 INTRODUCTION

1.1 BARRIER IMMUNOLOGY

1.1.1 Overview

Body surfaces, such as the skin, oral mucosa, gut and lung, face a range of immunological challenges due to the constant contact with microbial and environmental challenges to barrier integrity. Barrier tissues thus need to carry out specific immunological tasks related to three main functions: Maintenance of physical barrier integrity, response to and control of pathogenic microorganisms, and maintenance of equilibrium with the commensal microbiome.

Each body barrier is exposed to a variety of environmental variables, which in turn affect what challenges the barrier tissue faces. Each type of barrier tissue is thus specialized and unique in terms of structure and in the composition of the local immune system. The two tissues which are the focus of this thesis are the oral mucosa and the skin (**Figure 1**).

1.1.2 Tissue resident cells of the immune system

A key immunological feature of barrier tissues is the presence of tissue resident immune cells. This label encompasses a wide variety of immune cells, including T lymphocytes (1), natural killer (NK) cells (2), dendritic cells (DCs) (3), macrophages (4) and innate lymphoid cells (ILCs) (5) – distinguished from their circulating counterparts by the presence of cell surface markers, such as CD69, CD49a and CD103 (1, 6), some of which bind to ligands found on epithelial cells or in the extracellular matrix. Tissue-resident cell populations are present in their tissues for long periods of time, and generally do not recirculate (7).

Tissue resident immune cells provide an early response mechanism at body surfaces and underlying tissues. While antigen-presenting cell (APC) subsets, as well as effector cells of the innate immune system, can provide a general rapid immune response, an important component of the adaptive immune system are resident T lymphocytes. These include conventional tissue resident memory (T_{RM}) cells (7), as well as invariant natural killer T (iNKT) and mucosa-associated invariant T (MAIT) cells (8). T_{RM} cells are antigen-specific $\alpha\beta$ T lymphocytes that migrate into tissues in response to infection, providing the long-lasting ability to mount antigen-specific immune responses (1), and can also play a role in facilitating induction of antigen-nonspecific immune response (9). iNKT and MAIT cells in tissues may complement the conventional T cells and broaden the range of antigens that can be recognized.

Tissue resident lymphocytes perform their local immune effector function through rapid release of effector molecules, including tumor necrosis factor (TNF), interferon-gamma (IFNγ) and in particular a special barrier tissue cytokine known as interleukin 17 (IL-17) (10).

1.1.3 The role of IL-17

The IL-17 family of cytokines consists of IL-17A-F, which share sequence homology (11, 12). This cytokine family plays crucial roles in host defense against microbial organisms and in the development of inflammatory diseases. The prototypic member of the family, IL-17A, is the first discovered and best studied, and is usually referred to simply as "IL-17" (11, 13). IL-17 induces an inflammatory signature in tissues linked to neutrophil recruitment, migration and activation (11, 13, 14). The best described producers of IL-17 are a subset of $CD4^+$ T lymphocytes termed Th17 cells, which accumulate in barrier tissues in response to cytokine release following breach of barrier integrity (15-17). Other significant IL-17 producing T lymphocyte subsets include MAIT cells, iNKT cells (18-20), and γδ T cells, which are considered the main sources of early IL-17 release in response to bacterial infection (14, 21, 22)

IL-17 acts to regulate barrier immunity in three main ways: by stimulating neutrophil migration through induction of chemoattractant release in target cells, by maintaining barrier integrity through induction of expression of tight junction proteins in target cells, and by stimulating the release of antimicrobial peptides (11, 13). IL-17 has been implicated in the immune reactions against *Mycobacterium tuberculosis*, *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*, among others (14, 22-25). Dysregulated IL-17 signaling also plays a role in the development of multifactorial diseases, such as periodontitis and oral lichen planus, where increased IL-17 levels correlate with decreased microbial diversity (26-28).

1.1.4 The oral immune system

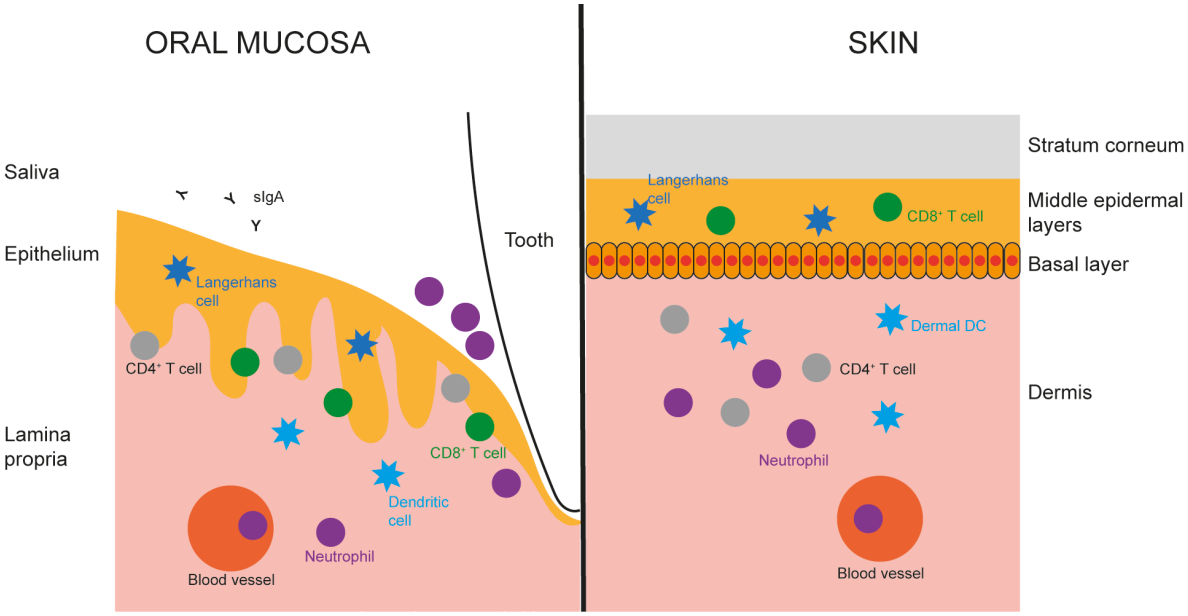

Figure 1. Diagrams of the immune system of the oral mucosa and skin. Based on (29, 30)

1.1.4.1 Overview and structure of the oral mucosa

The oral cavity serves as the point of entry for airborne and foodborne microbes and pathogens, in addition to being home to a rich and diverse commensal microbiota (reviewed in **section 1.3**). Additional risks to barrier integrity at the oral mucosal surface come from damage due to mastication (17), pH variation with food consumption (31), and allergens entering the mouth (32). These challenges impart the need for a robust and tightly-controlled immune network, capable of responding to a wide variety of threats while at the same time avoiding excess inflammation in response to harmless microbes.

The oral cavity is lined with stratified squamous epithelium, which varies in thickness and level of keratinization between locations (29). The epithelial layer at the bucca and floor of the mouth is thin and exhibits little keratinization. In contrast, the palate, tongue and gums are covered in a thick and keratinized epithelial layer (29). Oral epithelium at different sites also varies with respect to permeability, with the junctional epithelium (adjacent to the tooth) considerably more porous than at other sites (33). Below the epithelial layer lies the basement layer and the lamina propria. The region of the oral cavity addressed by most studies of the immune system is the gingival crevice, the region where the gingiva meets the tooth. The local epithelial layer at this site is thinner than in other parts of the oral cavity, and close to the tooth it morphs into junctional epithelium, which is a thin and incompletely differentiated type of epithelial layer (33). This thinness of the barrier and proximity to complex bacterial communities attached to the tooth surface make the gingival crevice a particularly vulnerable site.

1.1.4.2 Immune system of the oral mucosa

The first physical barrier protecting the oral surface is saliva, a watery liquid produced by salivary glands, which covers all surfaces in the oral cavity. Saliva contains secretory immunoglobulin A (sIgA), defensins and antimicrobial peptides, which can either kill or impair the function of pathogens entering the oral cavity (34-36). It also contains epithelial growth factor (EGF), which activates epithelial cells through EGF receptor (EGFR), stimulating release of antimicrobial peptides, as well as epithelial cell growth, thus contributing to maintenance of barrier integrity and wound healing (37). Its function as a lubricant also reduces damage to oral mucosa incurred by mastication (38). Xerostomia (dry mouth), often observed in patients suffering from Sjögren's syndrome and diabetes mellitus, is associated with higher rates of oral candidiasis (39, 40) and higher incidence of dental caries (41), further underscoring the critical role of saliva in protection of the oral cavity.

The second barrier to pathogens entering the oral cavity is the epithelial layer lining the oral mucosa. Cells comprising the stratified squamous epithelium of the mouth, aside from their role as a barrier between the lumen of the oral cavity and the mucosal immune network, act as early detectors of pathogens through pattern-recognition receptors (PRRs) (42) and contribute to the immune reaction by releasing cytokines and antimicrobial peptides (29, 36). On top of those functions, the outermost stratum of the epithelial layer is continually shed,

removing pathogens which attach to the outer epithelium (29). Within the epithelium, populations of intraepithelial lymphocytes (IELs) and Langerhans cells are present. IELs are a subset of predominantly $CD8^+$ T lymphocytes, which respond to infection by releasing IFNγ and IL-10, as well as cytotoxic granules, inducing an inflammatory response (43). Research presented in this thesis suggests that this population may overlap with oral mucosal MAIT cells (**Paper II**). Langerhans cells are a DC subset, taking up antigen present in the epithelium and traveling to peripheral lymph nodes, where they present the antigen on MHC class II molecules to T lymphocytes (44).

Below the epithelial layer and basement membrane, within the lamina propria, the main APC type is the interstitial DC (iDC). These cells do not enter the epithelium directly, but can take up antigen present there by extending dendrites towards the outer layers (45). In the lamina propria, $CD4^+$ T helper (Th) and regulatory (Treg) cells are present, which contribute to the maintenance of barrier integrity and immune homeostasis at the oral mucosal barrier (46). A recent study by Dutzan *et al.* revealed that T lymphocytes are the dominant immune cell type in the oral mucosa, with about 50% of T cells being $CD4^+CD45RO^+$ "memory" lymphocytes, and with populations of $CD8^+$ and γδ T cells also found at the site (47). T helper 17 (Th17) cells accumulate in the mucosa in response to IL-6 released from epithelial cells in response to damage caused by mastication, and not due to the presence of commensal microbial flora, which is the driver of Th17 accumulation at other mucosal sites (17).

Within the gingival crevicular fluid, which is present between the gingival surface and the tooth, the most numerous type of cell is the neutrophil, accounting for the majority of all leukocytes (48). Neutrophils maintain barrier homeostasis through control of bacterial populations, as well as immunoregulation and control of the inflammatory response (29). They continuously migrate from the gingival vasculature across the junctional epithelium into the gingival crevice along an intercellular adhesion molecule (ICAM)-1 expression gradient, regardless of pathogen presence (49).

1.1.5 The skin immune system

1.1.5.1 Overview and structure of the skin

Skin is the largest organ in the human body, and the one most exposed to outside microbial and environmental challenges. In contrast to mucosal surfaces, healthy skin has a largely dry external surface, although various niches across skin surface differ in humidity, along with a number of other environmental variables.

The outermost layer of the skin is the epidermis, which consists mainly of keratinocytes along with a small (about 5% of total cell number) minority of specialized cell types (50). The outer layer of the epidermis, stratum corneum, consists of flattened terminally differentiated keratinocytes and forms the primary physical barrier against outside dangers. This barrier varies in thickness according to specific site, but over most of the body it is about 10 layers thick (50). The basal layer of the epidermis consists of a single layer of keratinocytes. Below

the epidermis, the next skin layer is the dermis, largely composed of a collagen network controlled by fibroblasts and containing several specialized cell types. The dermis also contains blood vessels serving the skin. Below the dermis, the skin meets the subcutaneous fat layer, which contains most of the lipid content of the body (50).

1.1.5.2 Immune system of the skin

In contrast to mucosal surfaces such as the oral mucosa, the skin is not constitutively covered in a layer of mucosal secretions which could act as an antimicrobial barrier. However, skin secretions, sweat and sebum, contain antimicrobial peptides such as lactoferrin, cathelicidin LL-37 and skin-specific dermcidin (51-54) and can be considered the outermost layer of the skin immune system. The stratum corneum is also subject to continuous shedding of its outermost layers, removing pathogens adhered to it.

Within the epidermis, the main specialized innate immune cell type is the Langerhans cell (55). Another DC-like cell type, the melanocyte, is primarily involved in protection of the keratinocytes of the basal layer against ultraviolet radiation, but possesses some immune characteristics, such as upregulation of CD40 in response to IFNγ (56). In terms of adaptive responses, the epidermis is the layer into which pathogen-specific tissue-resident $CD8^+$ T cells migrate and persist following infection (1, 6, 7). Aside from specialized immune cells, a critical role in immune defense at this layer is played by the main structural cells, keratinocytes (57). Keratinocytes play a role as APCs in the skin (58), initiate immune responses by detecting pathogens through pattern-recognition receptors (PRRs) (59), and in response to IL-17 and IFNγ, release proinflammatory cytokines (60). Activated keratinocytes are also major producers of antimicrobial peptides (57), and synergize with dermal fibroblasts in wound healing, thus playing a critical role in maintaining tissue barrier integrity (61).

Within the dermis, the main innate immune cell types are the dermal DCs, histiocytes, macrophages and Mast cells (30). The dermis is the vascularized skin layer, and thus the location where multiple immune cell types can extravasate and migrate into sites of infection and inflammation (62).

In terms of the adaptive immune system, the dermis is the site where $CD4^+$ Th cells are present. The skin shares with other barrier sites the significant presence of Th17 cells, which accumulate in the epithelium driven by IL-1 released in response to commensal flora (16). This IL-1 release induces CD69 expression in $CD4^+$ T lymphocytes, contributing to the establishment of a tissue-resident phenotype (63). Th17 cells produce IL-17 and IL-22, same as at other body sites (12). In the skin, however, a unique T helper cell subset exists which expresses IL-22, but not IL-17; these cells are termed Th22 (64). Th17 and Th22 cells are both implicated in the pathogenesis of the skin autoimmune disease psoriasis, along with Th1 cells (65). Interestingly, recent research on skin commensal-specific T lymphocytes indicates that they can switch their roles from antimicrobial to tissue repair-promoting responses (66).

Another T cell subset unique to the skin, and common at this site, are Th9 cells, which release IL-9 and are involved in the control of fungal pathogens. IL-9 released by those cells stimulates local inflammatory responses, contributing to IFNγ and IL-17 release by Th17 cells (67, 68). In light of the subject of this thesis, it is worth noting that invariant T cells are also capable of producing IL-9, IL-17 and IL-22 (18, 69-71) and are found in the skin (72, 73).

1.2 INVARIANT T CELLS

1.2.1 Overview

Traditionally, the immune system has been sub-classified into two arms: innate (rapid, non-specific) and adaptive (slower, antigen-specific). The innate immune system includes cells such as NK cells, monocytes, granulocytes, and other cell types capable of immediate reaction to hazards arising in the body. The adaptive immune system includes T and B cells, reacting by clonal expansion in response to antigen presented by APCs, producing a specific immune response and maintaining immune memory.

This understanding was challenged by the discovery of T lymphocytes presenting NK cell surface receptors in mice (74) and humans (75). These cells were characterized by an invariant TCR recognizing a limited set of antigens not unique to a specific pathogen (76, 77). These were termed NKT cells. Later, another invariant T cell subset was discovered with similar characteristics, termed MAIT cells (78). Both these subsets are rapidly activated in response to infection and have the ability to release inflammatory cytokines and to perform cytolysis (71, 79)

A T cell subset characterized by expression of a γδ TCR instead of an αβ TCR has been known since 1989 (80), and is also sometimes grouped with iNKT and MAIT cells due to similar functionality and non-classical MHC molecule restriction (81). However, the γδ T cells are outside of the scope of this thesis and will not be discussed further.

NKT cells and MAIT cells share the expression of the transcription factor promyelocytic leukemia zinc finger protein (PLZF), which is required for the acquisition of their characteristic "innate-like" functions (19, 82). Another shared characteristic of these T cell subsets is their potential to produce IL-17 in response to stimulation (71, 79), a function related to their expression of CD161 which they share with conventional Th17 cells (83). The activation modes and cytokine production profiles of iNKT and MAIT cells is summarized in **Figure 2**.

1.2.2 Invariant natural killer T cells

1.2.2.1 Biology

A subset of T lymphocytes expressing NK cell surface receptors was discovered in mouse by Ballas and Rasmussen (74) and later termed "NK T cells" by Makino *et al.* (84), a term now rendered as "NKT cells". In 1993 Porcelli *et al.* discovered that certain invariant TCR rearrangements were enriched in $CD4^{-}CD8^{-}$ T cells in human blood (75). Subsequently, it was shown that NKT cells recognize glycolipid antigens presented by the MHC class I-related molecule CD1d (77, 85). While all NKT cells express CD161 and NKG2D, they are divided into two types based on TCR usage. Type I NKT cells are characterized in humans by an invariant TCR comprised of a Vα24-Jα18 chain paired with a Vβ11 chain (76). Type II NKT cells, which express a diverse repertoire of α and β chains in their TCR, recognize

phospholipid antigens (86). Type I NKT cells, also known as invariant NKT (iNKT) cells, are a focus of this thesis.

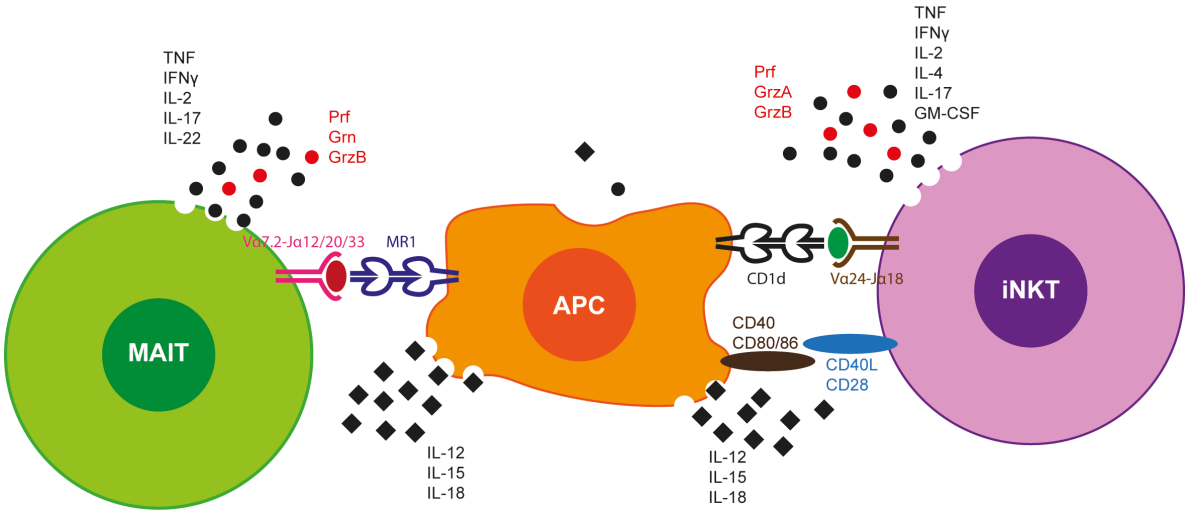

Figure 2. iNKT and MAIT cells activation and cytokine production. GM-CSF: granulocyte-macrophage colony-stimulating factor, Grn: granulysin, Grz: granzyme, Prf: perforin. Based on (79, 87)

The classical model antigen for iNKT cells is alpha-Galactosylceramide (α-GalCer), originally derived from a deep sea sponge *Agelas mauritanius* (88). Since the discovery of α-GalCer, a number of bacterial glycolipid iNKT antigens have been identified (89, 90), but iNKT classification remains defined by the recognition of CD1d-presented α-GalCer. In the context of infection with bacteria or viruses that do not encode CD1d-restricted antigen, CD1d can present endogenous lipid antigens, such as beta-D-glucopyranosylceramide (β-GlcCer), which is enriched in mammalian cells in response to TLR activation (91-93). iNKT cell activation can also be induced and co-stimulated by cytokines such as IL-12 and IL-18, as well as co-stimulatory molecules CD28 and CD40 (94, 95).

In response to activation, iNKT cells rapidly release IFNγ, along with multiple other cytokines, as well as cytotoxic molecules like perforin, TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL) (20, 71, 96). This set of abilities makes iNKT cells a powerful arm of anti-viral defenses.

1.2.2.2 Relevance to disease

Despite the invariant TCR and CD1d molecule restriction, iNKT cells recognize antigens from a broad variety of bacterial pathogens. They have been implicated in the immune response against *Mycobacterium tuberculosis* (97), Lyme disease (90) and staphylococcal infection (98), among others. Thanks to their ability to respond to CD1d-presented endogenous lipid antigens, iNKT cells have also been implicated in the immune response to several viral pathogens (71), and of particular interest for the subject of this thesis, they play an important role in herpes simplex virus (HSV) infection (99). The importance of iNKT cells in the response to infectious disease is evidenced by the fact that numerous bacterial and viral pathogens, possess immune evasion mechanisms targeting the CD1d antigen

presentation pathway (100). In response to infection iNKT cells act in a cytotoxic manner, but also to stimulate NK cell activation (101) and B cell and memory responses (102, 103).

iNKT cells also exhibit cytotoxic activity against tumor cells and can suppress cancer growth *in vivo* (104, 105), and are considered candidates for cell-mediated immunotherapy of cancer (106, 107).

1.2.3 Mucosa-associated invariant T cells

1.2.3.1 Biology

Mucosa-associated invariant T cells were discovered as a distinct T cell subset in 1999 by Tilloy *et al.* (78). They are characterized by expression of a semi-invariant TCR defined by a Vα7.2 gene segment usage coupled with Jα12, 20 or 33, as well as limited β chain diversity (78, 108, 109). MAIT cells recognize microbial vitamin B2 metabolite antigens presented by the MHC class I-related molecule MR1 (110). MR1 is highly conserved among mammals, underscoring its importance in response to infection and control of commensal flora (111, 112).

MAIT cells are activated through TCR engagement with antigen presented by MR1 on APC surface (113), but can also be activated in an MR1-independent manner by cytokines such as IL-12 and IL-18 to mediate some of their functions (114). Upon TCR-mediated activation, MAIT cells release primarily TNF and IFNγ, but they also have the ability to produce other cytokines, particularly IL-17 and IL-22, depending on their tissue localization and the cytokine milieu (115).

1.2.3.2 MAIT cells in peripheral tissues

MAIT cells, as their name suggests, were initially discovered in mucosal tissues. Nevertheless, we now know that MAIT cells can be found in most tissues studied to date. MAIT cells comprise 1-10% of T cells in the blood of healthy donors, while they are most numerous in the liver, where they account for 30-45% of all T cells (19). Substantial MAIT cell populations have also been found at other body surfaces, such as in the intestines (116), lung (117), stomach (118), skin (72), female genital mucosa (18) and, as presented here, in the oral mucosa (**Paper II**).

A feature of MAIT cells present at mucosal surfaces is a bias towards IL-17 production compared to their blood counterparts (18, 19, 72). This is consistent with a role as antimicrobial effector cells in such tissues.

1.2.3.3 Relevance to disease

MAIT cells are a relatively recently described immune cell subset, and their exact role in the development and immune control of various diseases is still being elucidated. They recognize antigen expressed by bacterial and fungal pathogens (110), and are thus mostly associated with the immune response to bacterial/fungal infection. MAIT cell activation has been

reported in bacterial diseases such as cholera (119), tuberculosis (120), *Helicobacter pylori* infection (118), as well as *in vitro* in response to various bacterial and fungal pathogens (121, 122). MAIT cell activation has also been observed in multifactorial diseases with presumed bacterial involvement, such as Crohn's disease and ulcerative colitis (123). In a murine model, MAIT cells are critical for immune responses to bacterial infection in the lungs (117). MAIT cell deficiency may contribute to increased susceptibility to bacterial infection in some cases of cystic fibrosis (124).

MAIT cells are activated in viral infection, such as hepatitis C, influenza and dengue, where they are activated in an MR1-independent manner through cytokine release and can mediate some immune functions (125). Interestingly, recent findings suggest that MAIT cells can confer some protection against influenza virus infection in mice (126).

MAIT cell depletion is associated with systemic immune disorders, such as in human immunodeficiency virus (HIV) infection (127-129) and systemic lupus erythematosus (130), which lends further credence to MAIT cells' central role for antimicrobial defense at body surfaces.

1.3 MICROBIOME OF THE ORAL CAVITY

1.3.1 Overview

The oral cavity is characterized by a rich and unique microbiome, differing from other mucosal sites of the body, with some similarities to the microbiome of the nasal mucosa (131). Microorganisms which colonize the oral cavity can enter with food, through bodily contact with other humans, as well as through the respiratory route. Whether they successfully colonize the oral cavity depends on a variety of factors, including nutrition, local pH, individual immune system characteristics, and microbiota already present at the site. The oral microbiome is a complex ecosystem, characterized by a multifaceted network of multi-actor relationships between commensals, pathogens, and opportunistic pathogens, such as the complex relation between commensal oral fungus *C. albicans* and various members of the oral bacteriome within biofilm communities (132).

While it can be tempting to consider commensal microbiota as largely benign, it is a driver of immune responses (133), although it was not implicated in attracting Th17 lymphocytes to the oral mucosal site, a process which is rather driven by mechanical damage (17). Some commensal microorganisms can become opportunistic pathogens under conditions of immunosuppression or immunodeficiency, such as HIV infection. Examples of such organisms are the fungi *C. albicans* and *Candida glabrata*, which normally cannot form large colonies in the oral cavity of the healthy host (134, 135). The expansion of opportunistic pathogens in the absence of fully competent immune system can be reduced by other members of the oral commensal microbiota, such as *Pichia pastoris*, which was shown by Mukherjee *et al.* to inhibit growth of *Candida, Aspergillus* and *Fusarium* species, with which it competes for nutrients (136).

Pathogenic microorganisms, such as the "red complex" bacteria associated with periodontitis (reviewed in **section 1.3.2**), but also cariogenic *Streptococcus mutans*, *Bifidobacterium* and *Actinomyces* species (137), are normally present within the healthy oral microbiome at low abundance, but expand rapidly under favorable conditions created by factors such as nutrition and host immunosuppression and can lead to the establishment of a chronic dysbiotic state (41).

Dysbiosis in the oral microbiota drives the development of oral diseases, such as dental caries, periodontitis and oral cancers (41). It can also have systemic effects, and disordered oral microbiome has been linked to a range of diseases, ranging from vascular, to neurological, to pancreatic (138-142). An oral disease particularly associated with systemic diseases and linked to an altered oral microbiota is periodontitis.

1.3.2 Periodontitis

Periodontitis is a multifactorial inflammatory disease of the periodontum, the part of the oral tissue surrounding the tooth. The initial phase is recognized as gingivitis, which in the long term leads to the destruction of soft tissue and bone surrounding the root of the tooth and

eventually tooth loss in the final stage. It is the most common cause of tooth loss worldwide (143) and also a major chronic disease which, at various levels of severity, affects the majority of adults (144), while severe periodontitis affects up to 12% of adults worldwide (145). Another common condition is apical periodontitis (AP), a particular form of periodontal disease which is caused by the inflammatory response around the root of the tooth, initiated by an endodontic infection (146).

The role of the host inflammatory response in periodontitis was first shown by Assuma *et al.* (147), who found that blocking IL-1 and TNF responses inhibited bone loss in an experimental mouse model of periodontitis. Levels of IL-1 and TNF, as well as IL-2 and IFNγ were also found to be increased at disease sites in human periodontitis patients compared to healthy controls (148). Further research showed that periodontitis can be induced by overexpression of IL-1α independent of any variance in bacterial load at the mucosal surface (149). Mice lacking IL-10 were also found to suffer from more severe periodontitis, indicating that periodontal disease develops as a result of imbalance between pro- and anti-inflammatory host factors at the mucosal barrier (150). In addition, Niederman *et al.* showed that host immune deficiencies and oral microflora both combine to induce the development of periodontitis (151). These findings reaffirm the status of periodontitis as a disease driven by the host inflammatory response.

The host inflammatory response which leads to periodontitis has been associated with a shift in the species composition of dental plaque at affected sites from Gram-positive to Gram-negative (152). Analysis of microbial samples from disease sites found a strong association between the development of periodontitis and three bacterial species now termed the "red complex": *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* (153). All three of those pathogens can inhibit the release of IL-8 from oral epithelial cells, thus interfering with host neutrophil trafficking and dysregulating the healthy inflammatory response (143). Later research has questioned the importance of the "red complex" as a requirement for development of periodontal disease, and instead suggested changes in a complex polymicrobial community, not driven by any single agent, to be responsible for the process (154). Interestingly, *P. gingivalis*, as well as other bacterial species known to associate with periodontitis including *Streptococcus mutans*, *Actinobacillus actinomycetemcomitans* and *Prevotella intermedia*, are strongly susceptible to growth inhibition by oral *Lactobacillus* species (155), which highlights the importance of preserving healthy commensal microbiota in periodontitis prevention. While the most common driver of inflammation in periodontitis is the bacterial flora, in rare cases periodontitis can also result from other sources of severe inflammation, such as viral infection (156).

Dysregulated IL-17 signaling is implicated in the development of periodontitis, as IL-17 levels are increased at periodontal inflammation sites (28). However, IL-17 mediated recruitment of neutrophils can alleviate periodontal disease progression and prevent disease-associated bone loss (157). It is worth noting that immunodeficiencies which cause dysregulated neutrophil response are also strongly associated with the development of severe

periodontitis (26). As the loss of normal neutrophil function enables the proliferation of multispecies bacterial communities (158), progress of the disease becomes driven primarily by the adaptive immune system, as B cells, T cells and plasma cells are recruited into the site of inflammation (159). An inflammatory cascade contributes further to tissue destruction and bone loss. In tissue, receptor-activator of NF-κB ligand (RANKL) causes alveolar bone degradation through signaling to osteoclast precursors, a process which in healthy tissue is inhibited by osteoprotegerin (OPG), a soluble RANKL ligand (160). RANKL expression is induced by pro-inflammatory cytokines TNFα and IL-1 (161). The resultant imbalance in the RANKL/OPG ratio causes excess alveolar bone loss, which in turn leads to loosening of the tooth root and tooth loss.

1.4 HERPES SIMPLEX VIRUS TYPE 1

1.4.1 Overview and pathology

HSV1 is a member of the alphaherpesvirus family, which comprises large enveloped dsDNA neurotropic viruses. The HSV1 genome has 72 open reading frames, grouped into two regions: the long unique region (U_L) and the short unique region (U_S) (162).

HSV1 establishes primary and recrudescent infection mainly in the skin, although HSV1 primary infections can also be oral, ocular, genital or anal (162). Oral infection is rare, as human saliva has the ability to neutralize HSV1 through soluble IgG and other factors (163). The virus can rapidly penetrate the epithelial layer and basement membrane (164) and upon reaching peripheral neurons, travels through direct cell-cell contact, via formation of a virological synapse (165), to the dorsal root ganglion, where it establishes latency (162). HSV1 spreads within neural tissue, possibly contributing to the development of neurodegenerative disease (166), and it can also spread to other sites, including cartilage and bone (167), although routes of viral spread to these latter sites and the effects of viral presence there on human health are poorly understood. While HSV1 infection is usually benign, in newborns, elderly and immunocompromised, it can cause lethal encephalitis (168). Recurrent ocular HSV1 infection is a significant cause of pathogen-induced blindness (162), and genital HSV1 infection is associated with male infertility (169). HSV1 infection has been associated with development of Alzheimer's disease, although this association is not fully understood (170). In addition, some evidence exists that HSV1 can contribute to the development of periodontitis in immunocompromised patients (156).

The main way by which HSV1 enters the host cell is by membrane fusion between viral envelope and the cell membrane through viral glycoprotein binding to cell surface heparan sulphates (171). However, it can also infect the cell through endocytosis of viral particles (172), or direct cell-to-cell spread via formation of a virological synapse (165). Cell-to-cell spread enables the virus to evade the humoral immune response, and sera from HSV1-infected individuals show minimal capacity to inhibit cell-to-cell spread of the virus *in vitro* (173).

Once the virus has entered the cell, it uses the host cell microtubule network to travel into the nucleus by use of tegument proteins (174). Inside the nucleus, the virus hijacks the host RNA polymerase II to transcribe its DNA (175). Expression of the HSV1 genome is carried out in three steps. First, immediate-early proteins are produced, which shut off host mRNA translation mechanisms and enable the virus to take control of it. Second, early proteins are produced, which replicate viral DNA. Last, early-late and late genes are expressed, which encode structural proteins of the virion (176). Tegument and capsid proteins assemble in the cytoplasm and travel into the nucleus, where they join with newly replicated viral DNA. The complete virion is enveloped at the inner nuclear membrane, and then travels through the outer nuclear membrane into the Golgi apparatus and to the cell membrane where is buds off (177). The complete virion consists of the genome inside a capsid formed of six proteins:

U_L18, U_L19, U_L26, $U_L26.5$, U_L35 and U_L38 (178). The HSV1 genome encodes eleven glycoproteins, of which five – gB, gC, gD, gH and gL – are known to play a role in viral entry into the cell and are thus assumed to be present on the viral envelope (171).

1.4.2 Immune reaction to primary HSV1 infection

1.4.2.1 Pattern recognition receptors

HSV1 is recognized by a number of pattern recognition receptors expressed by host cells. Toll-like receptor 2 (TLR2), expressed on the cell surface, recognizes viral glycoproteins gB and gH/gL (179). Nucleic acid of the virus, on the other hand, is detected by TLR3, which recognizes viral dsRNA, and TLR9, which recognizes unmethylated DNA (180, 181). Viral nucleic acid is also recognized by cytosolic DNA sensors, such as the RIG-I-like receptor MDA5 (182) and DNA-dependent activator of IRF (DAI) (183).

TLR3 is of particular interest, as defects in this receptor and its signaling pathway are associated with the development of herpes simplex encephalitis (168, 184). TLR3 is widely expressed, and from the point of view of HSV1 infection, it is worth noting that it is expressed both by skin keratinocytes (185) and neurons (186), the two cell types where the virus primarily propagates. TLR3 is mainly localized within the early endosome (187), although in some cell types it can be present at the cell surface (188). After binding nucleic acid, TLR3 signals through adaptor protein TIR-domain-containing adapter-inducing interferon-β (TRIF) to activate transcription factors activator protein 1 (AP-1), interferon regulatory factor 3 (IRF3) and nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), leading to the production of type I interferons (particularly IFNβ) and inflammatory cytokines (189). Interferon production and release results in the establishment of an antiviral state in infected cells, as well as activation of NK cells and DC maturation, inducing downstream innate and adaptive immune responses (190).

1.4.2.2 The role of iNKT cells

Mice lacking either CD1d or iNKT cells suffer from a more severe HSV1 infection and higher morbidity than wild-type when infected with HSV1 strain SC16 (99). These results were challenged by Cornish *et al.*, who found no effect of iNKT cells on infection severity in mice infected with HSV1 strain KOS, and therefore suggested that the need for iNKT cells to control HSV1 infection might be dependent on viral strain (191). While the ability of iNKT cells to contribute to control HSV1 infection in mice has been further demonstrated using strain SC16 again (192), the question of whether iNKT cells are necessary to control HSV1 infection in humans has so far not been resolved. Data obtained from *in vivo* studies of HSV1 infection in mice do not necessarily mirror the course of infection in humans, as for instance viral immune evasion mechanisms have evolved to target the human immune system, and may be inefficient within a mouse model, as seen with CD1d downregulation (193).

A number of lines of evidence point to an importance of the CD1d-iNKT axis in the response to HSV1 infection. HSV1 possesses several immune evasion mechanisms specifically

targeting CD1d (reviewed in **section 1.4.3.2**). Bosnjak *et al.* have also described a mechanism of HSV1 evasion of iNKT cell activation which is independent of CD1d targeting, but instead acts through direct cell-to-cell contact between the iNKT cell and the infected cell, possibly via galectin-9 signaling through T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), or viral protein transfer (194). Myeloid DCs have been found to upregulate CD1d mRNA in response to HSV1 infection (195). Additional support comes from HSV1's close relative, HSV2. Mice lacking iNKT cells failed to control genital HSV2 infection (196), and α-GalCer was shown to be a powerful adjuvant enhancing the protective effect of mucosal vaccination against HSV2 (197). All of those lines of evidence point to an important role of iNKT cells in protection against and response to infection with HSV1 and HSV2.

The exact function of iNKT cells in HSV1 infection remains to be determined. iNKT cells are known to be capable of activating NK cells via IFNγ release, induce DC maturation through CD40-CD40L signaling, and stimulate the induction of antibody response and memory establishment via IFNγ and IL-4 signaling to B cells (71), all of which contribute to the immediate immune response to acute viral infection, as well as to the control of latent infection. iNKT cells are not productively infected by HSV1 (194), in contrast to activated conventional T cells (198). Research presented in this thesis suggests that iNKT cells contribute to the immune response against primary HSV1 infection in keratinocytes by slowing down viral replication and maintaining interleukin 15 (IL-15) production in response to infection, via their production of IFNγ (**Paper IV**).

1.4.2.3 Function and significance of IL-15

IL-15 is a 14 kDa cytokine expressed by a variety of cells, including DCs and keratinocytes (199). It is structurally and biologically similar to IL-2, and signals through the IL-2 receptor β subunit (200). In contrast to IL-2 however, very little of IL-15 is secreted and the majority of IL-15 produced by the cell is retained on the cell surface, where it acts via *trans*-presentation in direct cell-to-cell contact (201). In the context of inflammatory reactions, *cis*-presented IL-15 produced by NK cells themselves can contribute to their activation, but is not necessary (202). IL-15 is presented bound to its receptor, the IL-15 receptor α (IL-15Rα), which serves as its chaperone protein within the Golgi apparatus and the two proteins remain bound to each other both when presented on cell surface and when secreted (203, 204). IL-15 has a variety of functions, but in terms of the immune response its three main mechanisms of action are: activation of NK cells, promotion of survival of T lymphocytes in the absence of antigen, and induction of cytotoxic T cell proliferation upon infection (205). In the specific context of invariant T lymphocytes, IL-15 promotes long-term survival of peripheral iNKT cells (206), but it can also activate both iNKT cells and MAIT cells (207).

Fawaz *et al.* found that human monocytes upregulate IL-15 in response to HSV1 infection and that this upregulation results in enhancement of NK cell cytotoxicity (208). This upregulation was later shown to be dependent on TLR2 activation (209). Research presented in this thesis indicates that in human keratinocytes, HSV1 infection induces IL-15 expression

via TLR3 (**Paper IV**). The exact mode by which IL-15 contributes to the immune defense against HSV1 has not been experimentally defined, but it is required for immune clearance of the closely related HSV2 (196). In this context, IL-15 can act independently of NK and iNKT cell activation by stimulating TNF and IFNβ expression in infected cells (210). IL-15/IL-15Rα upregulation on cell surface and *trans*-presentation to NK cells is an important mechanism of NK cell activation in virus infection, as shown in hantavirus-infected endothelial cells, where IL-15 blocking inhibits NK activation (211). IL-15 may also play a role in establishing the adaptive immune response, as $CD8^+$ T lymphocytes are activated and proliferate rapidly after HSV1 infection (212), and in the long term are involved in control of latent HSV1 and response to new primary infection over the lifetime of the host (7, 213, 214). In an experimental mouse HSV1 infection model, IL-15 was required for the development and maturation of virus-specific tissue-resident memory $CD8^+$ T cells in the skin (1).

1.4.3 Immune evasion mechanisms

1.4.3.1 Overview

HSV1 is a large dsDNA virus, and possesses a large number of mechanisms countering the host antiviral immune response (215). The first HSV1 immune evasion mechanism was described by York *et al.* (216), who discovered that HSV1 ICP47 protein inhibits antigen presentation by MHC class I. This is accomplished by the viral protein binding to transporter associated with antigen processing (TAP) complex and blocking MHC class I transport from the endoplasmic reticulum to cell surface (217). The advantage for the virus is the inhibition of the cytotoxic T cell response, although reduced levels of MHC class I on cell surface render the infected cells vulnerable to killing by NK cells (218).

Some immune evasion mechanisms do not target specific steps of the immune response, but are more generalized. For instance, HSV1 depends on its ability to co-opt the translational mechanism of the host cell for reproduction. To this end, the virion host shutoff (vhs) protein combines with tegument protein U_L47 to degrade host mRNA (219). This has an additional effect of inhibiting the host immune response by blocking cytokine production. The vhs is early acting and its levels decrease rapidly, which protects viral mRNA from being degraded by it (220). It also inhibits the host protein tetherin, which acts to prevent mature HSV1 virions from budding off the host cell membrane (221). In the general context of infection, the virus can inhibit type I interferon production through the action of viral latency associated transcript (LAT), thus preventing apoptosis of the infected cell (222).

Up to now, a wide variety of immune evasion mechanisms possessed by HSV1 has been described, and highlighted here are mechanisms targeting the TLR3 signaling pathway and CD1d antigen presentation, both of which are relevant to research described in **paper IV**.

1.4.3.2 TLR3 pathway-targeting mechanisms

As TLR3-mediated recognition of viral nucleic acid is crucial to the maintenance of effective cellular immunity against HSV1 (180), the virus has developed a number of immune evasion mechanisms targeting this pathway (215).

The viral serine/threonine protein kinase U_S3 has been found to downregulate TLR3 expression in HSV1 infected monocytes, resulting in downstream inhibition of IRF3 activation and downregulation of type I interferon production (223). U_S3 can also act directly on IRF3, as shown by Wang *et al.* (224), who showed that U_S3-mediated hyperphosphorylation of IRF3 blocks production of IFNβ in virus-infected cells. Another viral protein affecting IRF3 function is VP16, which associates with CREB binding protein (CBP), inhibiting the formation of IRF3-CBP complex required for transcriptional activation of type I interferon genes (225). VP16 also affect another TLR3 downstream effector, NF-κB, by associating with the p65 subunit and inhibiting activation (225). The same NF-κB p65 subunit is another target of U_S3 hyperphosphorylation, blocking its translocation into the nucleus (226). Nuclear translocation of p65 is also blocked by viral E3 ubiquitin ligase ICP0, which also degrades the NF-κB subunit, p50 (227).

It should be noted that IRF3 is key to antiviral immunity, and can be activated by pathways independent of TLR3 signaling, such as the cyclic GMP-AMP synthase-stimulator of interferon genes-TANK-binding kinase 1 (cGAS-STING-TBK1) pathway. In human macrophages, HSV1 protein ICP27 blocks this activation pathway at the TBK1-IRF3 step, associating with activated STING and TBK1 and preventing phosphorylation of IRF3 (228).

1.4.3.3 CD1d-targeting mechanisms

As iNKT cells recognizing antigen presented by CD1d serve an important role in the immune response to HSV1 (99), CD1d expression is a prime target for viral immune evasion in infected cells. In fact, immune evasion mechanisms targeting CD1d antigen presentation are common among herpesviruses (229).

CD1d molecules synthesize in the endoplasmic reticulum, where they form a complex with β_2-microglobulin and associate with endogenous glycolipid. The successfully folded complex travels through the Golgi apparatus to cell surface, from where they are internalized into endosomes. Pathogen-derived glycolipid antigen, or endogenous glycolipid antigen, binds to CD1d in the maturing endosome. CD1d then recycles back to the cell surface, where it can be recognized by the iNKT TCR (230). The process consists of several steps which can be specifically targeted by specialized immune evasion mechanisms possessed by viral and bacterial pathogens (100).

The ability of HSV1 to evade iNKT cell activation by targeting CD1d was first described by Yuan *et al.* in 2006 (231), who discovered that HSV1 infection reduced CD1d cell surface expression in HeLa cells by inhibiting CD1d recycling. It was later established that the process depends on two viral proteins, glycoprotein gB and protein kinase U_S3 (232). gB

binds to CD1d in the endosome, and U_S3 can inhibit CD1d recycling by several mechanisms: through phosphorylating gB, promoting gB internalization and colocalization with CD1d (232), through direct phosphorylation of CD1d, thus targeting it for lysosomal degradation (233), or through phosphorylation of kinesin motor protein KIF3A, inhibiting CD1d transfer to the cell surface (234). Later studies also identified HSV1 tegument protein VP22 as necessary for inhibition of CD1d antigen presentation, although whether this is accomplished by VP22 directly affecting CD1d trafficking or interaction with other virus-encoded proteins is not clear (235). It should be pointed out that HSV1-induced downregulation of cell surface CD1d is not observed in some cell types, such as keratinocytes (194), suggesting it is cell type-dependent. Importantly, HSV1's ability to disrupt CD1d trafficking specifically targets human, not mouse CD1d, which underscores the importance of iNKT cells in control of HSV1 infection in humans, despite iNKT cells being a rather small T cell subset in humans compared to mice (193).

2 AIMS

The overall aim of the project was to characterize the involvement of invariant T cell subsets, MAIT cells and iNKT cells, in the immune defense at two barrier sites, the oral mucosa and the skin. This included *ex vivo* studies of human blood and tissue samples, as well as cell culture experiments for specific infection studies. Specific goals were:

1. To establish and optimize methods to study MAIT cell responses to bacteria in human samples derived from both blood and peripheral tissues.

2. To characterize the presence, phenotype and function of MAIT cells in the human oral mucosal tissue, in comparison to the circulating MAIT cell population in blood.

3. To investigate possible relationships between oral mucosal MAIT cell characteristics and health and environmental factors which could affect the compartment across the general population.

4. To define oral mucosal MAIT cell involvement in apical periodontitis in relation to the tissue-associated bacterial profile.

5. To characterize the expression of IL-15 in human keratinocytes in response to HSV1 infection, and investigate possible viral countermeasures to this response.

6. To determine if iNKT cells can act to inhibit HSV1 immune evasion mechanisms.

3 STUDY DESIGN

All experimental methods are described in detail in in the prospective papers included in the thesis. This section contains information about the selection of specific methods and reagents.

3.1 ETHICAL CONSIDERATIONS

All four papers included in the thesis involved the use of material obtained from human donors. This included peripheral blood for all four papers, as well as tissues for **papers II and III**. Ethical approval was obtained from the Ethical Review Board and written informed consent was obtained from all donors. Samples analyzed for **papers II and III** were de-identified for purposes of the analysis. Specific information about donor selection criteria and sample collection is listed in the methods section of each paper.

3.2 CHOICE OF CELL LINES AND VIRAL STRAIN

For the cytotoxicity assay described in **paper I**, we used human kidney cell line 293T stably transfected with human MR1 gene (293T-hMR1) (112). This cell line was chosen as it is relatively resistant to bacteria-induced cell death (**Paper I suppl. figure 3G**).

For **paper IV**, we decided to use HaCaT cells, a spontaneously immortalized human keratinocyte cell line (236). An immortalized cell line was used rather than a primary cell line like for example NTERT-1, because it does not feature a cell density-dependent terminal differentiation switch, which could confound results obtained over long-term timecourse experiments.

Viral strain used in **paper IV** was HSV1 strain F, isolated in 1967 from a facial lesion at the University of Chicago Hospital (237) and commonly used since then in laboratory studies, as a viral strain with its infectivity and replication mechanisms fully intact.

3.3 SELECTION OF ANALYTICAL METHODS

3.3.1 Flow cytometry

The main analytical method used in **papers I, II and IV**, as well as for part of the data presented in **paper III**, was flow cytometry. Single-cell suspension samples were acquired on an LSRFortessa flow cytometer equipped with 355, 405, 488, 561, and 639 nm lasers (BD Biosciences). Data collected by flow cytometry were analyzed using FlowJo 10 software (FlowJo LLC). The advantage of flow cytometry over other analytical methods is the ability to rapidly create large datasets containing single-cell phenotypic data, making it well-suited to analysis of immune cell responses. Gating strategy used for identifying T cell subsets in the oral tissue samples (used in **papers II and III**) is shown in **Figure 3**.

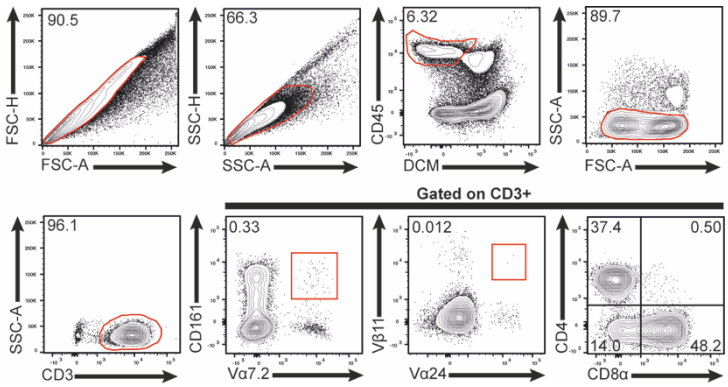

Figure 3. Gating strategy for detection of T cell subsets in the oral mucosa by flow cytometry. Overall T cells are defined as $CD45^{+}CD3^{+}$. MAIT cells: $V\alpha7.2^{+}CD161^{hi}$. iNKT cells: $V\alpha24^{+}V\beta11^{+}$. Conventional T cells are assumed to be all $CD45^{+}CD3^{+}$ cells outside the MAIT and iNKT gates.

For all MAIT cell experiments, we defined MAIT cells in flow cytometric analysis as $CD45^{+}CD3^{+}V\alpha7.2^{+}CD161^{hi}$ cells. During the time of the project, after most of the data had already been gathered, human MR1:5-OP-RU tetramer became available for researchers from the NIH Tetramer Core Facility (238). While it was too late in the process to change established experimental methods, we ensured that cells we identified as MAIT using our definition were positive for MR1:5-OP-RU tetramer staining (**Paper II figure 1B** and **paper III figure 4**).

3.3.2 Microscopy

To obtain microscopic data featured in **paper II**, we used *in situ* antibody staining for either immunohistochemical or fluorescent imaging. As CD161 is an unreliable marker for *in situ* staining, we defined MAIT cells as $V\alpha7.2^{+}$IL-18Rα^{+}, similar to a previous study which examined MAIT cells in the female genital mucosa (18). After the MR1:5-OP-RU tetramer became available (238), we considered using it for *in situ* MAIT cell staining, but pilot stains were unsuccessful and we decided to continue using the previously established staining method.

4 RESULTS AND DISCUSSION

4.1 ESTABLISHING METHODS FOR ANALYSIS OF MAIT CELL RESPONSES

MAIT cells are involved in the immune response to a range of microbes (79) and have been studied in infectious diseases and conditions of inflammation and immunodeficiency (120, 128, 239-241). Experimental approaches to study the involvement of MAIT cells in diseases can be affected by variability in MAIT cell extrinsic factors such as APC type and availability, antigen type and concentration, and cytokine milieu. To that end, we sought to develop a reasonably standardized and adaptable MAIT cell response assay which could produce consistent results and be easily modified when necessary. We chose the *E. coli* strain D21, which expresses the riboflavin synthesis pathway (110), as the model microbial antigen in this project.

4.1.1 Development of an *E. coli* activation assay for MACS-purified MAIT cells

4.1.1.1 MACS purification does not affect MAIT cell phenotype

In order to control MAIT:APC ratio in the assay, we isolated Vα7.2$^+$ cells from healthy donor blood using magnetic cell selection (MACS) purification, the quality of which was subsequently confirmed using flow cytometry (**Paper I suppl. figure 1A**). To determine whether this purification method affects the phenotype of MAIT cells, we measured CD4/CD8 expression, as well as surface activation marker CD69, CD25, CD38, Human leukocyte antigen – DR isotype (HLA-DR) and TIM-3 expression, in purified MAIT cells (**Paper I suppl. figure 1B-C**). The data showed that the CD4/CD8 phenotype of purified MAIT cells does not differ from that of their parent population in the blood, and that the process does not activate the cells.

4.1.1.2 Optimization of the MAIT cell activation assay

We next sought to optimize a MAIT cell bacterial stimulation and activation assay with regards to several variables: *E. coli* fixation time, microbial dose, co-activation by anti-CD28 antibody, Vα7.2$^+$ cell:monocyte ratio, and co-culture time (**Paper I figure 1**). As readout for MAIT cell activation, CD69/IFNγ co-expression was used, measured by flow cytometry (**Paper I figure 1A**).

To avoid bacterial overgrowth in the medium during co-culture incubation as part of the assay, *E. coli* was fixed with 1% formaldehyde prior to addition to the monocytes. However, this fixation procedure carries the risk of destroying the MAIT cell-activating riboflavin metabolite antigen, and therefore the MAIT cell activation capacity depending on the length of *E. coli* fixation was assessed (**Paper I figure 1B**). CD69/IFNγ expression was roughly unchanged with *E. coli* fixation times of 1-10 minutes, but dropped sharply at fixation times of 32 and 64 minutes. The alternative approach of preventing bacterial overgrowth by heat-killing of *E. coli* led to loss of MAIT cell activation, indicating that the antigen is heat-

sensitive. Further, comparison of MAIT cell activation in response to live and mildly fixed *E. coli* showed similar activation levels (**Paper I figure 1C**). MAIT cell activation also increased with higher microbial dose up to 100 colony-forming units (cfu) per monocyte, dropping at a higher dose, indicating the dose of 100 cfu/monocyte as optimal to provoke strong responses (**Paper I figure 1D**) in this assay. The addition of anti-CD28 antibody to the assay enhanced *E. coli*-induced MAIT cell activation at anti-CD28 levels of 1.25 μg/ml and higher, whereas the antibody did not activate MAIT cells on its own (**Paper I figure 1E**). Further, adjusting the number of purified Vα7.2$^+$ cells in the assay revealed the optimal Vα7.2$^+$ cell-to-monocyte ratio at between 1 and 2 (**Paper I figure 1F**), with decreased MAIT cell activation at ratios lower than 1 or higher than 4. Optimization of assay duration showed that CD69/IFNγ co-expression increases with longer co-culture times, reaching a plateau at about 16 hours, although in cells from one of the donors, MAIT cell activation kept increasing up to 24 hours of co-culture (**Paper I figure 1G**). **Paper I figure 1H** shows an optimized assay timeline established following optimization.

4.1.2 Identification of CD25 expression as readout for MR1-dependent MAIT cell activation

Screening surface markers of activation in MAIT cells exposed to *E. coli*-pulsed monocytes revealed that while CD38, HLA-DR and TIM-3 expression remained little changed, CD69 and CD25 were both robustly upregulated (**Paper I figure 2A**). This co-expression of CD69 and CD25 was inhibited by blocking MR1 (**Paper I figure 2B-C**), a pattern mirroring that seen with CD69/IFNγ co-expression (**Paper I figure 2D-E**). Together, these results establish CD69/CD25 co-expression as an alternative readout for measuring MR1-dependent MAIT cell activation. Usage of this readout presents an additional advantage in not requiring intracellular staining.

4.1.3 Development of an *E. coli*-induced MAIT cell proliferation assay

We next sought to optimize an assay for measuring MAIT cell proliferation in response to *E. coli* (**Paper I figure 3, suppl. figure 2**). To that end, purified Vα7.2$^+$ cells were labeled with CellTrace Violet (CTV) prior to being co-cultured with *E. coli*-pulsed monocytes for 3, 5 or 7 days. In select assay conditions, anti-CD28 and anti-MR1 antibodies (or relevant isotype control) were added to the cells, and IL-2 was added at days 1 and 3 of culture, with anti-MR1 also added again after 3 days (**Paper I figure 3C**). Blocking of MR1 inhibited MAIT cell proliferation at all assessed time points, regardless of microbial dose (**Paper I figure 3A-B**). MAIT cells showed little proliferation regardless of microbial dose after 3 days, whereas after 5 days there was a clear difference in expansion index (EI) between microbial doses, with the dose of 30 cfu/monocyte resulting in EI of around 2, and the dose of 100 in EI of up to 8 (**Paper I figure 3B**). The longest assay time of 7 days produced the highest EI values of up to 45 at the microbial dose of 100 cfu/monocyte (**Paper I figure 3B**), but also rendered CTV peaks difficult to discern (**Paper I figure 3A**), and thus was deemed suboptimal. We observed a slight downregulation of CD161 in proliferating MAIT cells, which however rebounded back to baseline levels with more cell divisions (**Paper I suppl. figure 2A-B**).

4.1.4 Development of a MAIT cell cytotoxicity assay

We next set out to develop and optimize an assay to measure MAIT cell cytotoxicity against *E. coli*-exposed 293T-hMR1 cells. First, the efficiency of bacterial internalization by 293T-hMR1 target cells was assessed by incubating them with pHrodo Red dye-labelled *E. coli* for 3 hours at different microbial doses and measuring fluorescence with flow cytometry (**Paper I figure 4A-B**). pHrodo Red increases in fluorescence in an acidic environment such as the lysosomal compartment, and thus fluorescence intensity can be used to assess *E. coli* internalization. The majority of 293T-hMR1 cells internalized *E. coli* at microbial doses of 30 cfu/target cell and higher. This microbial dose was selected for use in the following experiments.

MACS-purified Vα7.2$^+$ cells were pre-treated with IL-7 for 72 hours to prime MAIT cells for cytolytic response (242). Afterwards, they were incubated with *E. coli*-pulsed 293T-hMR1 cells at varying effector-to-target cell (E:T) ratios for 24 hours (**Paper I figure 4C-D**) to assess the optimal E:T ratio for cytotoxicity. As readout for target cell death, we used a combination of the fluorescent inhibitor of caspases (FLICA) reagent (apoptosis indicator) and dead cell marker (DCM). At E:T ratios of 10 and higher, FLICA$^+$DCM$^+$ cells reached a plateau of between 70-80% of total target cell number (**Paper I figure 4D**). To assess the involvement of MR1 in MAIT cytotoxicity, we measured target cell death and MAIT cell degranulation (assessed by CD107a expression) in the presence or absence of anti-MR1 blocking antibody at different co-incubation durations (**Paper I figure 4E-F**). The majority of target cells became apoptotic (FLICA$^+$) already after 1 hour of incubation, and the entire target cell population was apoptotic by 6 hours of incubation. However, target cell death progressed slower, and total target cell death (defined as the whole population being FLICA$^+$DCM$^+$) only happened after 24 hours of incubation. MAIT cell degranulation showed a similar pattern over time as target cell death, with essentially all MAIT cells becoming CD107a$^+$ after 24 hours. Blocking of MR1 partially inhibited the cytotoxic process, with both cell death and MAIT cell degranulation occurring at substantially at lower levels than with no anti-MR1 blocking antibody added; however, the majority of target cells still became apoptotic by the end of the assay duration. The efficiency of blocking of cytotoxicity by anti-MR1 antibody was not improved by increasing antibody concentration (**Paper I suppl. figure 3F**). This may be caused by the continuous cycling of MR1 between cell surface and the endosome (and, possibly, other compartments) (243), or by high MR1 expression on the cell surface of 293T-hMR1 cells.

4.2 MAIT CELLS OF THE ORAL MUCOSA

MAIT cells can be identified in a variety of mucosal barrier tissues and are implicated in functional responses to bacterial infections at those sites (reviewed in **section 1.2.3**). The oral mucosa, a site hosting a rich and unique bacterial microbiome (reviewed in **section 1.3**), is a potential locale for robust MAIT cell activity. To determine the involvement of MAIT cells in immune defense of the oral mucosa, we sought to characterize this cell population within the

healthy oral mucosal tissue (**paper II**), and to examine their involvement in a common and clinically important disease condition, apical periodontitis (**paper III**).

4.2.1 MAIT cells in the healthy buccal mucosa

4.2.1.1 MAIT cells reside in the healthy buccal mucosa

We gathered punch biopsies from the buccal mucosa of healthy, 20-50 years old, non-smoking and non-HIV positive donors. In addition, clinicians collecting the samples examined the overall health of the donors' oral cavities at the time of sample collection (**appendix 7.2**) to avoid sampling of donors with abnormalities stemming from active inflammation or disease at the site.

We were able to identify MAIT cells within the buccal mucosa of all 94 donors evaluated, whether by flow cytometry, microscopy, or qPCR analysis (**Paper II figures 1A, 1D and 2E**). MAIT cells were usually found close to the basement membrane, although they were also present in the epithelium and lamina propria (**Paper II figure 1D**), and importantly, in the same regions where MR1-expressing APCs could be identified (**Paper II figure 1E**). Moreover, $CD3^+$ T lymphocytes also clustered in the same regions (**Paper II figure 1F**), although we did not identify specific subsets. Importantly, the $V\alpha7.2^+CD161^{hi}$ definition used to identify oral mucosal MAIT cells via flow cytometry defined the same subset as staining with an MR1 tetramer loaded with the 5-OP-RU antigen (**Paper II figure 1B**).

To determine the relative abundance of MAIT cells in the oral mucosa compared to blood, we stained single cell suspensions obtained from processing buccal biopsies for MAIT cells, iNKT cells and conventional T cells (**Figure 4, paper II figure 1C**). Overall, there was no significant pattern of differential MAIT cell levels in the oral mucosa compared to blood across the cohort, although there was heterogeneity among individual donors where many showed different levels in oral mucosal MAIT cells as percentage of $CD3^+$ cells compared to blood. For other T cell subsets analyzed, we found buccal mucosal biopsies to be enriched in conventional $CD8^+$ T cells and $CD4^-CD8^-$ T cells and with lower percentage of $CD4^+$ T cells, with no difference in iNKT cell content compared to peripheral blood (**Figure 4B, paper II figure 1C**).

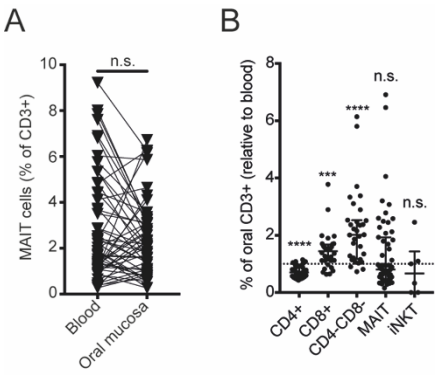

Figure 4. (A) Frequency of MAIT cells in the blood and oral mucosa. (B) Frequency of various T cell subsets in the oral mucosa in proportion to peripheral blood. ***p<0.01 ****p<0.001

4.2.1.2 The oral mucosal MAIT cell population is phenotypically distinct from blood

Within the oral MAIT cell population, we enumerated $CD4^+$, $CD8^+$ and $CD4^-CD8^-$ subsets (**Paper II figure 2A**), and identified enrichment of $CD4^+$ and $CD4^-CD8^-$ subsets and lower representation of $CD8^+$ MAIT cells in the oral mucosa compared to blood. Further, we analyzed the size of the CD8αα and CD8αβ subpopulations within the $CD8^+$ populations of MAIT cells and conventional T cells (**Paper II figure 2B-D**). In both peripheral blood and oral mucosa, MAIT cells contained a significantly higher proportion of $CD8\alpha\alpha^+$ cells than did conventional T cells, consistent with earlier results (244). However, there was no significant difference in the proportion of $CD8\alpha\alpha^+$ cells between the blood and oral mucosal MAIT cell populations. MAIT cells formed, on average, 50% of cells in the $CD8\alpha\alpha^+$ T cell subset within the oral mucosa (**Paper II figure 2D**) with significant donor variation, although intriguingly, data points appeared to cluster into two separate subpopulations. We were unable to identify a specific factor which could explain this phenomenon.

Furthermore, we used qPCR analysis to determine Jα TCR segment usage in buccal MAIT cells, including the three commonly used MAIT cell Jαs, the Jα12, Jα20 and Jα33, in six donors (**Paper II figure 2E**). While the pattern of Jα usage in the blood MAIT cells was consistent, with dominant involvement of Jα33, lower usage of Jα20, and lowest proportion of Jα12, the pattern in oral mucosal MAIT cells was much more varied, with dominant Jα inconsistent across the donors. Of particular interest were donors A44 and A60, who exhibited either undetectable or low usage of the blood-dominant Jα33 segment and more frequent usage of segment Jα12, usually low in the blood.

4.2.1.3 Oral mucosal MAIT cells exhibit a perforin^{low} and activated PD-1$^+$ phenotype

To further characterize the phenotype of the oral mucosal MAIT cell population, we stained them for activation markers HLA-DR, CD38 and programmed cell death protein 1 (PD-1) (**Paper II figure 3A-C**). An activated $HLA\text{-}DR^+CD38^+$ subpopulation was readily identified within the oral MAIT cell compartment (**Paper II figure 3B**). Overall, oral mucosal MAIT cells expressed higher levels of HLA-DR as well as the checkpoint inhibition receptor PD-1, but lower levels of CD38 than their blood counterparts (**Paper II figure 3C**), indicating an activated phenotype.

We also measured expression levels of the cytolytic proteins perforin and granulysin, as well as the innate-like T lymphocyte master transcription factor PLZF, in MAIT cells from the oral mucosa (**Paper II figure 3D-E**). Oral Mucosal MAIT cells showed no difference compared to their blood counterparts in PLZF and granulysin expression, but the expression of perforin was significantly lower (**Paper II figure 3E**). These results suggest that oral mucosal MAIT cells exhibit an activated phenotype, with relatively low cytolytic potential.

4.2.2 MAIT cells of the oral mucosa can be divided into resident and non-resident subsets by CD103 expression

Having identified that MAIT cells are present within the oral mucosal tissue (**Paper II figure 1D,F**), we sought to determine whether they express a tissue-resident phenotype. To that end, we stained single cell buccal mucosal suspensions with antibodies against tissue residency markers CD69 and CD103 for analysis by flow cytometry (**Paper II figure 4A-B**). We identified a substantial $CD69^+CD103^+$ subpopulation, along with smaller $CD69^-CD103^+$, $CD69^+CD103^-$ and $CD69^-CD103^-$ subpopulations (**Paper II figure 4A**). CD69 expression in oral mucosal MAIT cells differed quite a lot between donors (ranging from 10% up to 90%), but in all donors tested CD69 was higher in oral mucosal MAIT cells than in their blood counterparts. CD103 expression ranged from 20% up to 80% of oral mucosal MAIT cells with a mean around 50%, compared to less than 5% of blood MAIT cells. Within the $CD103^+$ oral mucosal MAIT subset, the majority were also $CD69^+$ (**Paper II figure 4B**). $CD69^+CD103^+$ and $CD69^-CD103^+$ subpopulations shared a similar CD4/CD8 expression pattern, different than the one shared by $CD69^+CD103^-$ and $CD69^-CD103^-$ subpopulations (**Paper II figure 4C**). We therefore focused the analysis on the differences between the total $CD103^-$ and total $CD103^+$ subpopulations (**Paper II figure 4D-F**). $CD103^+$ oral MAIT cells included a significant enrichment of $CD8^+$ cells and lower abundance of $CD4^-CD8^-$ cells, with no difference in $CD4^+$ cells. Within the $CD8^+$ subset, there was no difference in the proportion of $CD8\alpha\alpha^+$ cells (**Paper II figure 4D**). $CD103^+$ mucosal MAIT cells exhibited a more activated profile than the $CD103^-$ MAIT cells, with higher expression of HLA-DR and CD38 (**Paper II figure 4E**). Finally, the $CD103^+$ oral mucosal MAIT cells expressed lower levels of perforin than their $CD103^-$ counterparts, whereas PLZF levels showed no difference between the two subpopulations (**Paper II figure 4F**).

4.2.3 MAIT cells of the oral mucosa are biased towards IL-17 release and produce less IFNγ than their blood counterparts

There are several recent observations supporting the notion that MAIT cells located at body surfaces have a superior capacity to produce IL-17 when activated (18, 19, 72). To determine if oral mucosal MAIT cells express a similar functional profile to those previously described, and whether a difference in functional profile between $CD103^+$ and $CD103^-$ oral MAIT cell subsets exists, we stained single cell suspensions of PMA/ionomycin-activated blood and oral mucosal cells for TNF, IFNγ, IL-2, IL-17 and granzyme B (GrzB) for analysis by flow cytometry (**Paper II figure 5**). In oral mucosa, as well as in the blood, the majority of MAIT cells responded to PMA/ionomycin activation by producing at least one of these proteins (**Paper II figure 5C**). All three populations analyzed (blood, oral $CD103^-$ and oral $CD103^+$) produced IL-2 upon activation at a similar rate. Interestingly, while it was shown by Gherardin *et al.* that IL-2 production by MAIT cells in the blood is associated with the $CD4^+$ subset (244), the enrichment of $CD4^+$ MAIT cells which we observed in the oral mucosa (**Paper II figure 2A**) does not correspond to higher IL-2 production. All three populations also produced large amounts of TNF, although the numbers of TNF^+ MAIT cells in the blood were higher than in both $CD103^+$ and $CD103^-$ oral mucosal populations. More blood MAIT

cells produced IFNγ on activation than in either of the oral mucosal populations, and in fact $CD103^+$ oral mucosal MAIT cells did not show any significant increase in IFNγ production in response to activation, although they did exhibit a low baseline positive IFNγ expression level even when not treated with PMA/ionomycin. Blood MAIT cells, with the exception of one outlier donor, produced little to no IL-17 upon activation, consistent with previous observations (79). Meanwhile, $CD103^+$ buccal MAIT cells produced IL-17 at a higher level than either their blood, or $CD103^-$ mucosal counterparts. Finally, GrzB was released by a small proportion of blood MAIT cells upon activation and while neither of the oral mucosal MAIT cell subsets showed a significant increase in this marker, activated $CD103^+$ MAIT cells released it at a higher level than their activated $CD103^-$ counterparts.

Analysis of the MAIT cell polyfunctionality profile using SPICE 5 software (245) suggested a slight preference towards 4- or 5-functionality in $CD103^+$ resident oral mucosal MAIT cells, although differences between the analyzed populations were not significant (**Paper II figure 5D**). In terms of specific polyfunctional response profiles, blood MAIT cells mainly expressed TNF, alone or in conjunction with other cytokines, whereas $CD103^+$ resident oral mucosal MAIT cells mainly expressed IL-17 alone or with other cytokines, and $CD103^-$ non-resident oral mucosal MAIT cells occupied the space in between the other two in terms of cytokine preference (**Paper II figure 5E**). Particularly interesting polyfunctional subsets are $TNF^+IL\text{-}2^+IL\text{-}17^+$, $TNF^+IL\text{-}17^+$, $IL\text{-}2^+IL\text{-}17^+$ and $IL\text{-}17^+$, all of which are absent or near-absent from the blood MAIT cell population, but comprising a major part of $CD103^+$ resident oral mucosal MAIT cells, and also markedly present in the $CD103^-$ non-resident oral mucosal MAIT cell population. Worth noting are also $IFN\gamma^+$ polyfunctional subsets, only one of which ($TNF^+IFN\gamma^+IL\text{-}2^+$) is present in $CD103^+$ oral MAIT cell population at a level above 5% of total activated cells. This analysis, even more than the single-parameter analysis (**Paper II figure 5B**), highlights the bias of tissue-resident oral mucosal MAIT cells towards IL-17 production.

4.2.4 Possible involvement of MAIT cells in protection against oral candidiasis

IL-17 is critical in the control of oral candidiasis (23, 25), which is a common opportunistic infection in HIV-1 infected patients (134). MAIT cells can mount effector responses against the most common culprit, *C. albicans* (113), and circulating MAIT cells are depleted in the blood and lymph nodes of HIV-infected individuals and do not recover due to highly active antiretroviral therapy (HAART), in contrast to conventional $CD4^+$ T cells (127-129). Notably, while HAART leads to a decrease in active oral candidiasis, it does not significantly reduce *Candida* colonization in HIV-1 infected patients (246). These previous findings, along with our identification of oral mucosal MAIT cells as significant local producers of IL-17 (**Paper II figure 5**), suggest that MAIT cells may be an important antifungal component of the local mucosal immune system in the oral cavity, contributing to control of opportunistic pathogens along with elements of the adaptive immune system such as Th9 and Th17 cells (23).

4.2.5 MAIT cells in apical periodontal lesions

To determine whether oral mucosal MAIT cells are present in periodontal tissues, we measured MAIT cell content in surgically resected gingiva and apical periodontal (AP) lesions using PCR for MAIT cell TCR rearrangements (**Paper III figure 1**). The measurements revealed the presence of the three common MAIT TCR rearrangements Vα7.2-Jα12, Vα7.2-Jα20 and Vα7.2-Jα33, in AP lesions, at levels significantly higher than in adjacent gingival tissue. The Vα7.2-Jα33 rearrangement was the most abundant in AP lesions, followed by Vα7.2-Jα20 and Vα7.2-Jα12, similarly to what is normally observed in blood (**Paper III figure 2**). To confirm the presence and identity of MAIT cells in these periodontal tissues, we used flow cytometry, following tissue processing and staining protocols developed for **paper II** (**Paper III figure 4**). Flow cytometric analysis confirmed the presence of $V\alpha7.2^{+}CD161^{hi}$ cells in the $CD3^{+}$ population in both AP lesions and healthy gingival tissue, and staining with MR1 tetramer loaded with 5-OP-RU antigen confirmed their identity as MAIT cells. Further analysis of CD4 and CD8 expression showed an enrichment of $CD4^{+}$ and reduction of $CD4^{-}CD8^{-}$ MAIT cells within the AP lesions compared to healthy gingival tissue (**Paper III figure 5**).

A PCR analysis also revealed upregulation of TNF, IFNγ and IL-17 gene expression within the AP lesions (**Paper III figure 3a**). We did not determine whether those cytokines were predominantly produced by MAIT cells present in the lesions, as the minute tissue amount permitted no functional analysis; however, as shown in **paper II figure 5**, oral mucosal MAIT cells readily express TNF and IL-17 upon activation, but not IFNγ.

Further analysis of the MAIT cell population in the lesions could potentially elucidate the contribution of these cells to the cytokine release in periodontitis. Measuring the baseline and post-activation expression of TNF, IFNγ and IL-17, as well as other cytokines, such as IL-22, which is associated with chronic periodontitis (247), and which tissue-resident MAIT cells can produce (18), in MAIT cells, but also other cell types such as Th17, within the AP lesions, would provide valuable information about MAIT cell involvement in disease conditions in the oral cavity. Another intriguing question is about the source of MAIT cells in AP lesions, or whether they infiltrate from neighboring tissue or from the blood. Measurement of the expression of tissue residency markers CD69 and CD103 in MAIT cells detected in AP lesions, and their relation to the cytokine expression profile, would provide data about the mechanism of MAIT cell recruitment to infection sites at barrier tissues.

4.2.6 Factors influencing the MAIT cell population in the healthy oral mucosa

As part of the research project into oral mucosal MAIT cells, donors were asked to fill out a questionnaire about health and lifestyle parameters which could possibly be linked with the oral mucosal T cell compartment (**Appendix 7.1**). For most of the parameters in this questionnaire, we found no association or correlation with the frequency, phenotype or

function of the oral MAIT cell compartment. However, some association were found, as described in the following paragraphs.

4.2.6.1 Higher percentage of MAIT cells in the oral mucosa of men than in women

While there was no difference in the frequency of MAIT cells as percentage of T lymphocytes in PBMC between men and women (median for both sexes close to 2%), men exhibited a higher frequency of MAIT cells among the oral mucosal T cells than women (median around 3% for men, around 2% for women) (**Figure 5A**). However, the difference between frequency of oral and blood MAIT cells in men was below the threshold of significance. This suggests a local immune difference between men and women. At the same time, oral MAIT cells in men expressed significantly lower levels of CD38 than both their blood counterparts and oral MAIT cells of women (**Figure 5B**). In women there was no difference in MAIT cell CD38 expression between the two sites.

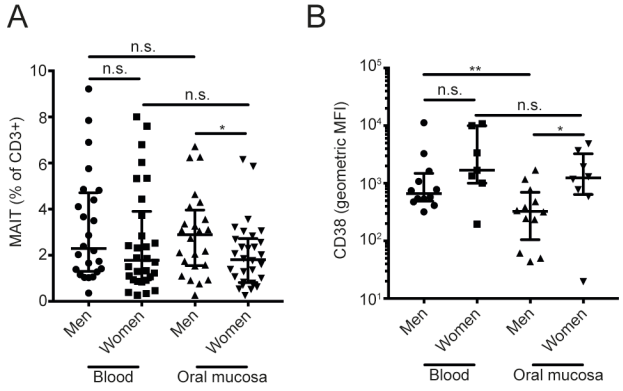

Figure 5. Differences in oral mucosal MAIT cells between men and women. (A) Frequency of MAIT cells in blood and oral mucosa (B) CD38 expression in blood and oral MAIT cells. *$p<0.05$ **$p<0.01$

Sex differences in susceptibility to disease have long been known, and multiple underlying differences in the immune system which may contribute to this have been described (248), most likely driven primarily by sex hormones (249). Adult men have a higher blood $CD8^+$ T cell frequency than women (248). In the context of mucosal immunity, male patients suffering from irritable bowel syndrome had more T lymphocytes in general and $CD8^+$ cells within the T lymphocyte population at the gut mucosal barrier than female patients (250). Men also showed a Th17 bias compared to women among ankylosing spondylitis patients (251). Intriguingly, naïve $CD4^+$ T cells from blood of men are biased towards IL-17 and away from IFNγ expression compared to those of women (252), a similar phenotype as we observed in oral mucosal MAIT cells (**Paper II, figure 5**). It is possible that a similar bias in MAIT cells in men leads to increased IL-17 expressing MAIT cell recruitment to mucosal surfaces in response to the local cytokine milieu, particularly IL-6 at the oral mucosal barrier, which is known to attract Th17 cells (17), thus resulting in higher oral MAIT cell frequencies in men than women.

CD38 is a multifunctional ectoenzyme with cADP-ribose hydrolase activity present on multiple cell types, but in T lymphocytes functionally dependent on the CD3/TCR complex,

and can be used as a marker of activation (253). Systemic expression of CD38 in male mice is negatively correlated with testosterone levels (254). Interestingly, we observed that mean CD38 level in peripheral blood MAIT cells from men tended to be lower than in women, although this trend did not reach statistical significance. On the other hand, Meier *et al.* found that $CD8^+$ T cells from women expressed significantly higher levels of CD38 in response to higher amounts of IFNα released from DCs activated by HIV-1 derived TLR7 ligands (255). Perhaps the difference in CD38 expression by oral mucosal MAIT cells is secondary to sex differences in cytokine production by local APCs in the oral mucosa.

It is also possible that the differences in oral MAIT cells between men and women might be related to differences in the oral microbiota. Oral microbiome is affected by female sex hormones (256), and oral bacteriome differs between men and women (257, 258) as does the oral virome (259). Those differences may bear consequences for MAIT cell migration into the oral mucosa and activation status.

4.2.6.2 Alcohol and Lactobacillus-containing product consumption is associated with the frequency and CD8 expression of oral and blood MAIT cells

Based on frequency of alcohol consumption stated in the questionnaire answers, we divided the donors into two groups: those who consume alcohol on at least a weekly basis and rarer consumers, including non-consumers. We did the same for *Lactobacillus*-containing product (LCP) consumers, including such products as yoghurt and soured milk (260). Frequent alcohol consumers had fewer MAIT cells in the oral mucosa than in the blood, as well as fewer oral mucosal MAIT cells than infrequent/non-consumers (**Figure 6A**). Additionally, while we observed lower representation of $CD8^+$ MAIT cells among oral mucosal MAIT cells compared to blood across the population (**Paper II figure 2**), frequent alcohol consumers showed further reduced $CD8^+$ MAIT subset in both oral mucosa and peripheral blood compared to infrequent/non-consumers (**Figure 6B**).

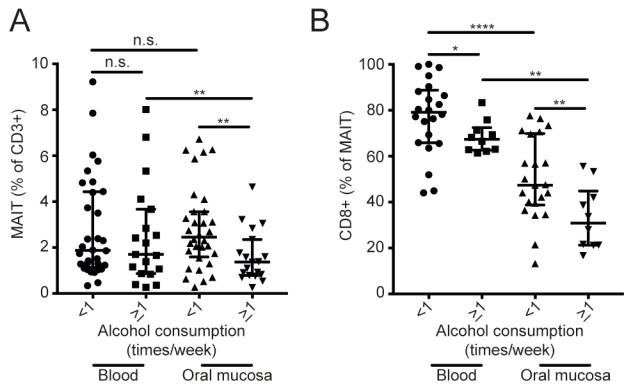

Figure 6. Effects of alcohol consumption on blood and oral MAIT cells. (A) Frequency of MAIT cells in blood and oral mucosa (B) Frequency of $CD8^+$ cells within the MAIT population. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001

We initially considered the possibility that the difference in the oral mucosal MAIT cell population in alcohol consumers may be due to the local effect of alcohol. Alcohol has long been known as a factor in local oral tissue damage (261). However, we did not observe any

effect on oral MAIT population in donors reporting use of alcohol-based mouthwash (Listerine) versus non-alcohol based (Flux). As alcohol-based mouthwash has previously been shown to contribute to development of oral cancer through local tissue effect (262), this suggests that the effect of alcohol may be systemic. Additional evidence against local effect of alcohol is that alcohol consumption alone does not affect the oral microbiome (263), and hence likely secondary immune parameters. Liver damage due to chronic hepatitis C virus (HCV) infection causes an irreversible loss and dysfunction of MAIT cells in the blood (239) and it is possible that alcohol-induced liver damage may similarly affect the MAIT cell compartment at non-liver sites. This possible interpretation is strengthened by our observation of a reduced $CD8^+$ MAIT cell population in the blood of frequent alcohol consumers, despite that the overall MAIT cell population is maintained.

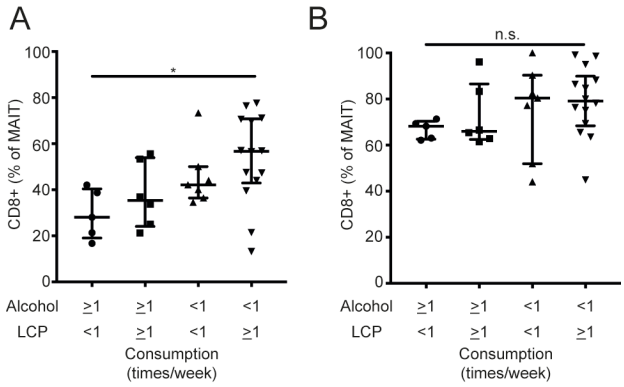

Figure 7. Effects of combined alcohol and *Lactobacillus*-containing product (LCP) consumption on the size of the $CD8^+$ MAIT compartment in the (A) oral mucosa and (B) peripheral blood. *p<0.05

Interestingly, we found that the relative loss of oral mucosal $CD8^+$ MAIT cells in frequent alcohol consumers was counteracted by the frequent consumption of LCPs (**Figure 7A**). At the same time, contrary to alcohol, LCP consumption had no effect on blood $CD8^+$ MAIT cell frequency (**Figure 7B**). This suggests that the effect of soured milk products is local.

Lactobacillus species are capable of robust riboflavin synthesis (264) and one could speculate that frequent consumption of products containing *Lactobacilli* may affect oral MAIT cell population by local riboflavin metabolite antigen concentration. While *Lactobacilli* are present at various sites in the oral cavity, including the epithelial surface, they are less robust colonizers than many keystone oral microbes (265). However, oral application of *Lactobacillus*-containing preparations enables their efficient colonization and growth at sites throughout the body (266), suggesting that frequent consumption could maintain a higher *Lactobacillus* representation within the mucosa. Another explanation for the increased $CD8^+$ oral mucosal MAIT cell population might be that *Lactobacillus* species, which are known to efficiently inhibit growth of multiple bacterial pathogens (155, 267, 268), act by maintaining a healthy oral microbial composition, thus preventing oral MAIT cell exhaustion and activation-induced loss of CD8 expression. The same effect can be mediated by *Lactobacillus* strains dampening local MAIT cell activation (269). The possibility of reduced MAIT cell

activation is supported by the observation that LCP consumption did not affect total oral MAIT cell frequency.

4.3 BACTERIAL PROFILE OF APICAL PERIODONTAL LESIONS

As part of the research presented in **paper III**, we identified bacterial taxa associated with periodontal lesions to elucidate their correlation to patient data and possible involvement in MAIT cell activation.

4.3.1 Total bacterial load in AP tissues

Measuring 16S rRNA gene copy number by qPCR indicated similar levels of bacteria present in control and AP tissue (**Paper III figure 3b**). However, in AP tissue with fistulas, 16S gene copies were about 10 times more numerous than in control tissues, and a weak positive correlation was found between 16S gene copy number and patient age in AP tissues (**Paper III table 1A**).

4.3.2 Similar microbial composition between AP and control tissues

We next analyzed the microbial composition of AP and control tissues through 16S rRNA gene-based microbiome sequencing, grouping the data at phylum and genus levels (**Paper III figures 6-9**). In terms of phyla, dominant taxa in both AP and control tissues were *Proteobacteria*, *Bacterioidetes*, *Firmicutes*, *Fusobacteria*, *Spirochaetes* and *Synergistetes*; no difference was found in the relative abundance of taxa between AP and control tissues (**Paper III figures 6 and 8**). At the genus level, dominant taxa in both AP and control tissues were *Burkholderia-Paraburkholderia*, *Pseudomonas*, *Achromobacter* and *Fusobacterium* with, similarly to phyla, no significant differences in relative abundance between AP and control tissues (**Paper III figures 7 and 9**). We also observed a large variation between patients in microbial composition of AP lesions.

4.3.3 IL-17$^+$ MAIT cell involvement in immune reaction in AP lesions

Next, we sought to elucidate the potential availability of riboflavin-producing bacterial taxa in the AP lesions that could be potential sources of MAIT cell-activating antigens at the site (**Paper III figure 10**). We found that the cumulative relative abundance of riboflavin-producing bacterial taxa (identified in **paper III table 3**) was significantly higher in AP tissues than in control tissues (**Paper III figure 10a**). Furthermore, the riboflavin biosynthesis pathway gene RibD was expressed at a higher level in AP lesions than in control tissues (**Paper III figure 10d**), even though other genes from the same pathway, RibA and RibC, were not (**Paper III figure 10b-c**).

We then used sparse partial least squares discriminant analysis (sPLS-DA) to identify correlations between TCR and cytokine expression levels and bacterial taxa abundance (**Paper III figure 11**). In total, we found 23 different bacterial taxa which were negatively correlated to Cα, Vα7.2-Jα33 and IL-17A expression within AP lesions. Of those, the majority were identified as riboflavin producers (**Paper III table 3**). Together, the data

suggest that MAIT cell antigens are present within AP lesions, and that MAIT cells function as local IL-17 producers that may be involved in the pathogenesis of AP.

4.4 HSV1 AND INKT CELLS INFLUENCE IL-15 EXPRESSION PROFILE IN INFECTED KERATINOCYTES

4.4.1 Upregulation of the IL-15/IL-15Rα complex in HSV1-infected keratinocytes

4.4.1.1 The IL-15/IL-15Rα complex is upregulated in human keratinocytes shortly after HSV1 infection at both mRNA and protein levels

It was previously shown that expression of IL-15 is upregulated in HSV1-infected human monocytes (209). Since skin keratinocytes are usually the first cell type infected in the course of primary HSV1 infection, we investigated the dynamics of IL-15 and IL-15Rα expression in the human immortal keratinocyte cell line HaCaT. We infected HaCaT cells with HSV1 strain F virus for 6, 12 or 18 hours and found that the surface expression of both IL-15 and IL-15Rα was progressively upregulated. At the early 6 hours post infection (hpi) time point IL-15 was upregulated by about 50% and IL-15Rα not at all at, while at 18 hpi both IL-15 and IL-15Rα surface expression was upregulated to between 4 and 5 times the level seen in uninfected cells (**Paper IV figure 1B**). We also found that *IL15* and *IL15RA* mRNA was upregulated at 3 hpi, albeit *IL15* only by about 30% and *IL15RA* at over 2 times compared to uninfected cells (**Paper IV figure 1C**). Together, these results show that HSV1 infection induce IL-15/IL-15Rα complex expression both at mRNA and at surface protein levels. This induction *in vivo* may play a role in the local immune response to primary HSV1 infection (reviewed in **section 1.4.2.3**), and could also play a secondary role as a proliferative signal to self- or neighboring keratinocytes in order to restore barrier integrity, as IL-15 induces epidermal proliferation in HaCaT cells (270).

4.4.1.2 TLR3 is involved in the upregulation of IL-15Rα

In HSV1-infected monocytes, IL-15 upregulation is induced by TLR2 (209). However, IL-15 is produced in response to various stimuli depending on cell type (60, 271-273), and we thus investigated the mechanism involved in IL-15/IL-15Rα upregulation in HSV1-infected HaCaT cells (**Paper IV figure 2**). HSV1-induced IL-15Rα expression was partially blocked by the addition of bafilomycin A1, which inhibits endosome acidification and is an indirect inhibitor of endosome-associated PRRs (**Paper IV figure 2A**). This suggested that such PRRs might be involved. HSV1 is known to be detected by TLRs 2, 3 and 9, aside from cytosolic DNA sensors (179-183). We first screened HaCaT cells for TLR expression by qPCR (**Paper IV figure 2B and suppl. figure 1**) and found high level of TLR3, low levels of TLR2 and no TLR9 expression. Further, we treated HaCaT cells with zymosan (TLR2 ligand), poly(I:C) (TLR3 ligand) and *E. coli* DNA (TLR9 ligand) (**Paper IV figure 2C**). Of the three, only poly(I:C) induced upregulation of IL-15Rα, with no effect on IL-15Rα expression induced by either zymosan or *E. coli* DNA.

To further investigate if IL-15Rα upregulation by HSV1 is indeed dependent on TLR3, we treated HSV1-infected HaCaT cells with the specific TLR3 inhibitor Cu CPT 4a or the NF-κB inhibitor TPCA-1 (**Paper IV figure 2D-E**). We found that HSV1-induced upregulation of IL-15Rα surface expression at 15 hpi was completely blocked by both inhibitors, acting either alone or together. Similarly, at the transcriptional level, induction of *IL15RA* mRNA at 3 hpi was inhibited by these inhibitors both in HSV-1 infected and poly(I:C)-treated cells. Those results confirm that IL-15Rα upregulation in HSV-1 infected keratinocytes is driven by TLR3 recognition and signaling.

While the results presented here contrast with previous research in human monocytes by Ahmad *et al.*, which indicated TLR2 as the driver of IL-15 upregulation in HSV1 infection (209) it should be noted that IL-15 is upregulated by DCs in response to poly(I:C), which points to TLR3 involvement (273). As infected keratinocytes in the skin can be expected to exhibit similar APC responses as local DCs, engagement of this signaling pathway may be involved in primary skin HSV1 infection.

4.4.2 HSV1 downregulates IL-15/IL-15Rα expression in a viral replication-dependent manner

4.4.2.1 The IL-15/IL-15Rα complex is downregulated in human keratinocytes at later timepoints in HSV1 infection

To investigate the dynamics of IL-15/IL-15Rα expression in HSV1 infection further, we measured surface expression of IL-15 and IL-15Rα, as well as *IL15* and *IL15RA* mRNA levels, over a 36 hour infection time course (**Figure 8, paper IV figure 3A-B**). We found that after peaking at 18 hpi (for surface protein expression), or 3 hpi (for mRNA expression), both surface protein and mRNA levels declined to levels below those in uninfected cells. Moreover, measurement of levels of intracellular IL-15/IL-15Rα during a time course assay through a late 48 hpi time point revealed that intracellular protein was also severely downregulated to about 5% of uninfected control levels for IL-15 and about 45% of uninfected control levels for IL-15Rα (**Paper IV figure 3C**).

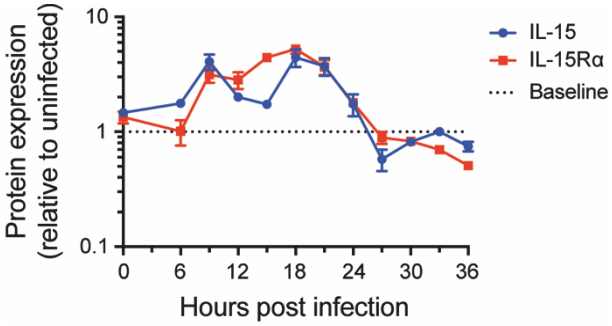

Figure 8. Dynamics of IL-15/IL-15Rα complex surface expression over 36 hours of primary HSV1 infection.

4.4.2.2 The downregulation of the IL-15/IL-15Rα complex is dependent upon HSV1 replication

To determine whether the downregulation of the surface IL-15/IL-15Rα complex is dependent on viral factors, we measured levels of surface IL-15Rα in HaCaT cells treated with poly(I:C) for 18 and 36 hours (**Paper IV figure 3D**). After 36 hours of poly(I:C) treatment, surface IL-15Rα returned to levels similar to untreated control, but did not go lower. This suggested that a viral mechanism might be driving IL-15/IL-15Rα surface downregulation at later time points. In order to investigate this possibility, we measured surface levels of IL-15 and IL-15Rα in HSV1-infected HaCaT cells treated with phosphonoacetic acid (PAA) or acyclovir, inhibitors of viral replication and late gene expression (274, 275) (**Paper IV figure 3E-F**). We observed at least partial inhibition of HSV1-driven IL-15Rα surface downregulation in cells treated with either agent, as well as inhibition of IL-15 downregulation in cells treated with acyclovir. This suggests that IL-15/IL-15Rα complex downregulation in HSV1-infected keratinocytes at later time points is caused by an active viral mechanism dependent on viral DNA replication.

The loss of intracellular protein may be due to several factors. For example, surface-bound protein may be released from the surface. However we have not detected any IL-15 in the supernatant of HaCaT cells in the HSV1-infection assays. Another possibility is that the IL-15/IL-15Rα complex may be degraded. Under normal conditions, the complex recycles through the endosome back to the surface, and IL-15Rα, which is not ubiquitinylated, protects the complex from degradation (276). However, in HSV1 infection, two possibilities for protein degradation are present. First, HSV1 infection induces an autophagic reaction, which leads to degradation of both viral and cellular proteins (277). Second, the viral protein ICP0 has a robust ubiquitin E3 ligase activity, which is known to target various host cell proteins for proteasomal degradation (278). Either of those processes may lead to degradation of existing IL-15/IL-15Rα and, in the absence of new protein being produced, depletion of the complex altogether.

4.4.3 iNKT cells maintain the IL-15/IL-15Rα upregulation in HSV1-infected keratinocytes through IFNγ release

iNKT cells are known to play a part in the immune response to HSV1 (reviewed in **section 1.4.2.2**). Previous research has shown that HSV1-infected primary keratinocytes do not downregulate CD1d and retain the ability to activate iNKT cells (194). In the HaCaT keratinocyte cell line, we found that CD1d is not significantly downregulated by HSV1 infection at either early or late time points (**Paper IV figure 4A**), and thus the cells may retain the ability to activate iNKT cells through TCR engagement. To determine whether iNKT cells are capable of influencing the dynamics of IL-15/IL-15Rα, we co-cultured them with infected HaCaT cells for 18 and 42 hours in the presence or absence of anti-IFNγ blocking antibody (**Figure 9, paper IV figure 4B**). We found that in the presence of iNKT cells, infected HaCaT cells at 42 hpi maintained the level of IL-15Rα expression seen at 18

hpi. Furthermore, the addition of anti-IFNγ blocking antibody significantly reduced that effect.

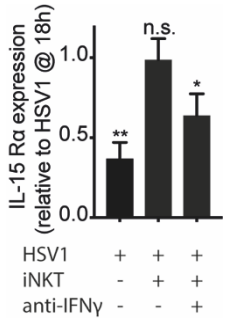

Figure 9. IL-15Rα expression on HaCaT cell surface at 42h post infection (relative to 18h). *p<0.05 **p<0.01

IFNγ is robustly produced by iNKT cells in response to CD1d-presented antigen (279) and thus IFNγ in the co-culture might have been effected by TCR engagement by HaCaT cells presenting endogenous danger signal antigen on CD1d. However, IL-15 can itself induce IFNγ production in DCs, NK cells, T lymphocytes and ILCs (5, 273, 280, 281), and an interesting possibility is that keratinocyte-produced IL-15 and iNKT-produced IFNγ could take part in a positive feedback loop in response to HSV1 infection.

IL-15 induction is normally associated with type I interferons IFNα and β (272), rather than IFNγ. However, in human keratinocytes, IFNγ induces *IL15* mRNA expression and IL-15 release into the supernatant (60) and in monocytes, IFNγ stimulation results in upregulation of surface-bound IL-15 specifically (282). This is similar to what we observe in keratinocytes, suggesting that iNKT-produced IFNγ may counteract HSV1-driven downregulation of IL-15 by inducing its expression. Such mechanism however might be in turn inhibited by viral immune evasion mechanisms blocking the protein synthesis machinery of the infected cell (reviewed in **section 1.4.3**). Another way of IFNγ-induced maintenance of IL-15 upregulation in infected keratinocytes might be through interference with viral infection progress. IFNγ slows down HSV1 infection progress by inhibiting viral replication through induction of nitric oxide synthase (283, 284). As our results show, IL-15/IL-15Rα downregulation is dependent on HSV1 replication (**Paper IV figure 3 E-F**). The presence of iNKT cells also slows down the progress of infection *in vitro* (**Paper IV figure 4C**). These findings suggest that IL-15/IL-15Rα upregulation in infected keratinocytes might be maintained by iNKT cell-produced IFNγ by blocking viral replication, while the upregulation itself might still be driven by TLR3 signaling, or through synergy with IL-15 production induced by IFNγ. The paper's findings are summarized in **paper IV figure 5**.

4.4.4 Possible clinical application of iNKT cell activation in treatment of drug-resistant HSV1 infection

HSV1 infection usually carries low risk of complications, but it can involve serious acute symptoms, and cause potentially lethal neurological disease, in the elderly and immunocompromised, particularly in patients suffering from TLR3 deficiency (168, 184).

Thus, effective treatment strategies against primary and recrudescent HSV1 infection are necessary. The most common treatment in HSV1 infection is acyclovir, and several other antiviral drugs can be used, but single- and multidrug-resistant HSV1 strains cause problems in clinical care (285). Alternative treatment strategies are being actively sought, and proposed strategies include novel synthetic pharmaceuticals, naturally occurring antivirals, and antibody-based immunotherapy (286-289). A viable alternative might be cell-based immunotherapy. In the research presented here, we have shown that iNKT cells can slow down progress of HSV1 infection in keratinocytes (**Paper IV figure 4C**), and potentially induce upregulation of surface IL-15 through IFNγ release (**Paper IV figure 4B**), thus contributing to the broader immune response against HSV1, known to be dependent on IL-15 signaling (196, 208). Activation of iNKT cells through local administration of αGalCer is thus one possible anti-HSV1 treatment strategy. Using αGalCer as a vaccine adjuvant has been shown to enhance antiviral protection (197, 290), and in fact Iversen *et al.* demonstrated that local administration of αGalCer conveys protection against intravaginal HSV2 infection in mice by iNKT expansion and activation (103). This research, together with the iNKT-HSV1 interaction explored herein, raises the possibility of using αGalCer or other CD1d-presented antigens to treat drug-resistant HSV1 infection in susceptible patients.

5 CONCLUSIONS AND FUTURE OUTLOOK

In this thesis project, we aimed to characterize the involvement of MAIT cells and iNKT cells in the immune defense of two body barrier tissues, oral mucosa and skin. This involved developing methods to study MAIT cell responses, characterization of oral MAIT cells in health and periodontal disease, and analysis of iNKT cell involvement in the immune response against primary HSV1 infection.

In **paper I**, we established a standardized methodology for *in vitro* analysis of MAIT cell activation, cytokine production, proliferation, and cytotoxicity in response to bacterial antigens. The assays, optimized for measuring responses to *E. coli*, can be adapted to involve other pathogens and APCs. In the context of oral mucosal MAIT cell responses, this can involve specific local pathogens, like *C. albicans* or *P. gingivalis*.

In **paper II**, we performed the first comprehensive characterization of the MAIT cell population in the oral mucosa. We discovered that buccal mucosal MAIT cells exhibit an activated, perforin^{low} phenotype, and that the major CD103^{+} resident MAIT cell subpopulation is biased towards IL-17 production. Further characterization of the tissue resident MAIT cell population in the oral mucosa and other barrier tissues may identify multiple cell subsets exhibiting different surface markers and distinct functionality. Of particular interest is the epithelial tissue residency marker CD49a, which defines an IFNγ^{+}, as opposed to IL-17^{+} population in the conventional CD8^{+} T_{RM} cells in the human skin (6). Identification and investigation of distinct polyfunctional subsets of tissue resident MAIT cells may shed more light on MAIT cell involvement within the body barrier immune network.

Additional data was collected to investigate associations between the characteristics of oral mucosal MAIT cells and a variety of health and lifestyle variables provided through self-reporting by tissue donors. We identified differences in the abundance and activation status of steady-state oral MAIT cells between men and women, as well as the correlation between alcohol and *Lactobacillus*-containing product consumption and the local oral mucosal MAIT cell population. These data provide some leads on possible variables influencing the development of diseases affecting the oral mucosa, in particular ones where MAIT cell involvement can be identified.

In **paper III**, we sought to identify the involvement of MAIT cells in a common and clinically important oral disease, apical periodontitis. MAIT cells were present within the AP tissues analyzed, as shown by either qPCR analysis or flow cytometry, and the local cytokine signature, as defined by qPCR, pointed to cytokines characteristic of MAIT cell responses. Additionally, the abundance of bacterial taxa expressing MAIT cell antigens was negatively correlated to MAIT cell and cytokine signatures, pointing to an active involvement of MAIT cells in the maintenance of AP lesions studied here. Further in-depth studies will lead to further understanding of MAIT cell involvement in recognition and elimination of AP-

associated microbiota, as well as their origin (migration from blood or from neighboring mucosal tissue) and their effector function profile.

In **paper IV**, we investigated human keratinocyte expression of the IL-15/IL-15Rα complex in response to HSV1 infection, and the iNKT cell involvement in its control. We found that the IL-15/IL-15Rα complex is upregulated by HSV1-infected keratinocytes in a TLR3-dependent manner, and that this upregulation is counteracted by a viral mechanism dependent on viral replication. In turn, iNKT cells are able to inhibit the virus-driven IL-15/IL-15Rα downregulation through IFNγ release. This study lays the groundwork for the identification of a possible novel viral immune evasion mechanism specifically targeting IL-15 expression in infected cells, a cytokine which is critical in the immune response against the virus (196). In addition, the study sheds light on the specific role of iNKT cells in the control of HSV1 infection, a role which until now had been incompletely characterized.

Invariant innate-like T lymphocyte populations are present in tissue sites across the human body, and have been implicated in the immune response to a number of bacterial and viral infections, as well as being associated with some autoimmune and inflammatory conditions. The field of invariant T cell immunology has been growing steadily since their original discovery, and the work presented in this thesis represents a significant contribution to the understanding of role these T cell subsets play in the immune defense at body surfaces.

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7 APPENDIX

7.1 HEALTH AND LIFESTYLE QUESTIONNAIRE

Questionnaire

Your name:_____

Sex		Age	
Height		Weight	

Please provide your answer below by indicating Yes (Y) or No (N)

General health
Are you taking any medication? (Y/N)
If Yes, please indicate which ……..
Have you taken any antibiotics in the past 3 months? (Y/N)
If Yes, please indicate which and duration……
Do you use vitamin supplements? (Y/N)
If Yes, please indicate which and frequency……
Do you suffer from any allergies/chronic inflammatory conditions? (Y/N)
If Yes, please indicate which ……
Have you had any infections in the past 3 months? (Y/N)
If Yes, please indicate which ……

Smoking and food habits
Do you smoke? (Y/N)
If yes, how many cigarettes a day? (NUMBER) Which brand(s)?.....
Do you use snus? (Y/N)
If yes, how many times a day? (NUMBER)…….
Do you drink alcohol? (Y/N) Alcohol type/content?
If yes, how often? (daily/weekly/monthly)……
Do you eat yoghurt, filmjölk, or other products containing "good bacteria"? (Y/N)
If yes, please indicate which products ……
And how often…….
Do you eat any other foods based on fermentation processes (e.g. mozzarella, chorizo, surkål)? (Y/N/Don't know)
If yes, please indicate which products ……
And how often…….
Are you vegetarian? (Y/N)

Oral hygiene
How many times a day do you brush your teeth? (NUMBER) …….
Do you use mouthwash? (Y/N)
If yes, what brand and how often?……
Have you had any dental treatment the past 3 months? (Y/N)
- If yes, which treatment did you receive…….
Have you had mouth ulcers the past 3 months? (Y/N)

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7.2 CLINICAL PROTOCOL

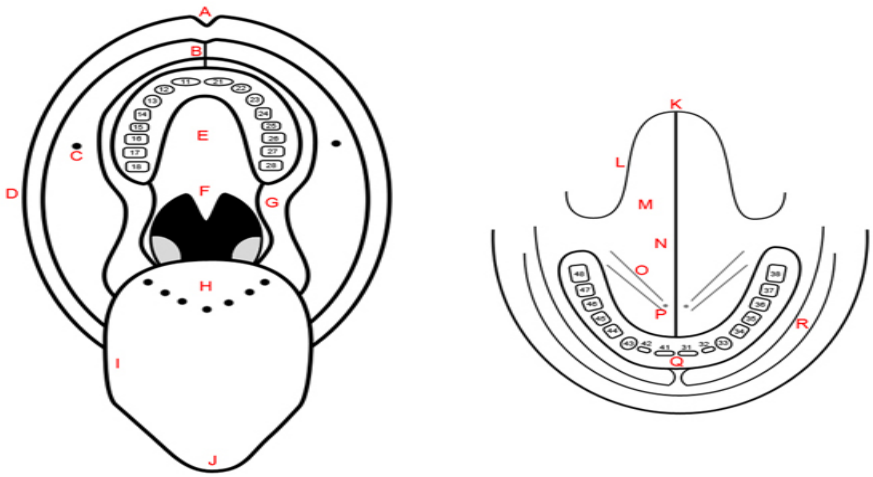
MAIT Study: Clinical Protocol Date for biopsy:…….

Patient

1. Patient code: ……
2. Ethnic origin: Europe ( ) Africa ( ) Asia ( )

Sampling procedure

3. Anesthesia: Nerve block ( ) Infiltration ( ) Xylocain-Adrenalin( )
4. Punsch ( ) Scalpel ( )
5. Total number of biopsies ( ) Mark excision site(s) below:



Biopsy data

6. Inflammation grade: 0 ( )healthy, 1 ( )erythema, 2 ( )erythema/ulcer, 3 ( ) erythema/ulcer/psuedomembrane
7. Diagnosis Mucosa:……
8. Diagnosis Dental:……..
9. Diagnosis Medical, if applicable…….....
10. Clinician´s name: ……..

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