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CONTROL OF IMMUNE RESPONSES IN HUMAN ADENOTONSILLAR TISSUE

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Academic Dissertation

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Cover: Transmission electron microscope images of apoptotic and viable adenotonsillar CD4+ CD45R0+ T lymphocytes.

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ABBREVIATIONS

ACAD	Activated T cell autonomous death
AICD	Activation induced cell death
AIF	Apoptosis inducing factor
AIRE	Autoimmune regulator gene
Apaf-1	Apoptotic-protease activating factor-1
APC	Allophycocyanin
ATP	Adenosine triphosphate
Bcl-2	B-cell CLL/lymphoma 2 protein
BH	Bcl-2 homology
Ca	Calcium
CAD	Caspase-activated DNase
CD	Cluster of differentiation
cDNA	Complementary DNA
CHX	Cycloheximide
DAG	Diacylglycerol
DHE	Dihydroethidium
$DiOC_6(3)$	3,3'-dihexyloxacarbocyanine iodide
DNA	Deoxyribonucleic acid
$\Delta\psi_m$	Mitochondrial transmembrane potential
EM	Electron microscopy
FACS	Fluorescence activated cell sorter
FADD	Fas-associated death domain
$FADH_2$	Flavin adenine dinucleotide
Fas / FasL	Fas receptor / Fas ligand
FITC	Fluorescein isothiocyanate
FLIP	FLICE-like inhibitor protein
Foxp3	Forkhead box p3 transcription factor
GSH	Glutathione
GSSG	Oxidized glutathione
H_2O	Water
H_2O_2	Hydrogen peroxide
HEA	Human epithelial antigen
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP_3	Inositol triphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
JAK	Janus kinase
MALT	Mucosa-associated lymphoid tissue
MEF2	Myocyte enhancer factor-2
MHC	Major histocompatibility complex
MnTPCl	Manganese(III) 5,10,15,20-tetra(4-pyridyl)-21*H*,23*H*-porphine chloride tetrakismethochloride
mRNA	Messenger RNA
NAC	N-acetyl-L-cysteine
NADH	Nicotinamide adenine dinucleotide
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor-κB
NK cell	Natural killer cell
O_2	Molecular oxygen
O_2^-	Superoxide anion
^-OH	Hydroxyl radical
PCR	Polymerase chain reaction
PE	R-Phycoerythrin
PECAM	Platelet endothelial cell adhesion molecule
PerCp	Peridinin chlorophyll protein
PUMA	p53 upregulated modulator of apoptosis
rhFas	Recombinant human Fas-Fc chimera protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SOD	Superoxide dismutase
STAT	Signal transducers and activators of transcription
TCR	T cell receptor
Th cell	T helper cell
TNF	Tumor necrosis factor
TRITC	Tetramethylrhodamine isothiocyanate
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VCAM	Vascular cell adhesion molecule
ZAP70	ζ-chain-associated protein kinase of 70 kDa
ZDEVD-fmk	Benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone
ZVAD-fmk	Benzyloxycarbonyl-Tyr-Val-Ala-Asp-fluoromethyl ketone

ABSTRACT

Adenotonsillar tissue in the pharynx is presumably continuously exposed to foreign antigens that can induce immune responses. The purpose of this study was to evaluate mechanisms that control immune responses in adenotonsillar tissue. The entry of foreign antigens into adenotonsillar tissue is thought to occur through the adenoidal epithelial crypt that is constantly infiltrated with leucocytes. The mechanisms that mediate this infiltration were evaluated as these mechanisms have remained unknown. The major players that control immune responses are CD4+ T lymphocytes that help both antibody-mediated and cytotoxic immune responses. Therefore, it was also evaluated, which mechanisms control the survival of adenoidal CD4+ T lymphocytes. Knowledge of the control of lymphocyte survival is important as improper control may lead to autoimmunity, excessive accumulation lymphocytes, as well as neoplasia.

It was found that epithelial cells at the base of the adenoidal crypt expressed platelet endothelial cell adhesion molecule PECAM-1, which has a function in the migration of blood leukocytes through vascular endothelium. Adenotonsillar naïve phenotype CD45RA+ CD4+ T cells, unlike peripheral blood CD4+ T cells, included cells that expressed the activation marker CD69. Memory phenotype CD45R0+ CD4+ T cells almost invariably expressed CD69, and this population also included cells that expressed the activation associated markers CD71, CD38, and HLA-DR. Adenoidal naïve phenotype CD45RA+ CD4+ cells, but not peripheral blood CD4+ cells, included cells that were susceptible to Fas-mediated programmed cell death, apoptosis, upon cross-linking with a high concentration of antibody against the T cell antigen receptor complex (TCR). Such stimulation with a high concentration of CD3 antibody mimics the encounter of the T cell by a high antigen dose. On the contrary, most adenoidal memory phenotype CD45R0+ CD4+ cells were sensitive to rapid and spontaneous apoptosis that was independent on Fas- and TCR- signaling. This apoptosis could be attenuated by various cytokines, such as IL-2, IL-7, IL-15, IL-6, as well as the chemokine CXCL12. Interestingly, unlike reports made in mouse models, it was found that the neutralization of superoxide anions did not rescue memory phenotype T cells from apoptosis, but still inhibited apoptotic DNA degradation.

The finding that the endothelial cell adhesion molecule PECAM-1 is expressed in adenoidal epithelial crypt suggests that it may have a role in leukocyte infiltration into the crypt. This may contribute to the formation of the specialized immune environment in the epithelial crypt. The observations on CD4+ T cell survival suggests that high concentrations of antigens, such as non-pathogenic antigens in inhaled air or swallowed nutrients, may induce peripheral immune tolerance in human adenotonsillar tissue by selectively eliminating naïve phenotype CD45RA+ CD4+ T lymphocytes that may mediate adverse reactions. The finding that the activated memory phenotype CD45R0+ CD4+ T cells require constant survival signals from cytokines implies that the amount of the immune response can be fine-tuned by various cytokines. Superoxide anions do not appear to play a crucial role in inducing apoptosis of adenoidal CD45R0+ CD4+ T cells. However, superoxide anions are not mere toxic by-products of the oxidative phosphorylation, as it was found that they have an active role in the signal transduction

that leads to apoptotic DNA degradation. To conclude, the survival of human adenotonsillar CD4+ T cells is regulated by complex control mechanisms that may involve the dose of the antigen during the initiation of the immune response. Later on, the magnitude of the immune response appears to be regulated by various cytokines of different cytokine superfamilies.

ABSTRACT IN FINNISH
(TIIVISTELMÄ)

Nielussa sijaitseva risakudos altistuu jatkuvasti vieraille antigeeneille, jotka voivat käynnistää immuunivasteita. Tämän väitöskirjatyön tarkoituksena oli selvittää mekanismeja, jotka kontrolloivat immuunivasteita risakudoksessa. Vieraat antigeenit kulkeutuvat risakudokseen oletettavasti epiteelikryptan kautta. Epiteelikryptaan kuljetetaan jatkuvasti myös valkosoluja. Tässä työssä haluttiin tutkia mekanismeja, jotka ohjaavat tätä valkosolujen kuljetusta kryptaan, sillä nämä mekanismit ovat huonosti tunnettuja. Lisäksi selvitettiin tekijöitä, jotka säätelevät immuunivasteissa tärkeiden CD4+ T -solujen ohjelmoitunutta solukuolemaa eli apoptoosia risakudoksessa. Tällaisten tekijöiden tunnistaminen on tärkeää, sillä solujen epätäydellinen tuhoaminen apoptoosin avulla saattaa johtaa solujen liialliseen lisääntymiseen sekä autoimmuunitautien ja jopa syövän syntymiseen.

Kitarisan kryptan pohjalla olevien epiteelisolujen havaittiin ilmentävän leukosyyttien adheesiomolekyyli PECAM-1:ä. Kitarisan naiivit CD45RA+ CD4+ T -solut ilmentävät pinnallaan T solujen aktivaatioon liitettyä proteiinia CD69:ää. Kitarisan muisti-CD45R0+ CD4+ T -solut puolestaan ilmentävät pinnallaan CD69:n lisäksi myös monia muita T solujen aktivaatioon liitettyjä proteiineja, kuten CD71:ä, CD38:aa ja HLA-DR:ää. Kitarisan naiivit CD45RA+ CD4+ T -solut olivat herkkiä Fas-välitteiselle apoptoosille korkealla T-solureseptorivasta-ainekonsentraatiolla stimuloitaessa. Veren vastaavat solut eivät puolestaan olleet herkkiä apoptoosille. Kitarisan muisti-CD45R0+ CD4+ T -solut olivat puolestaan alttiita spontaanille ja nopealle apoptoosille, joka oli riippumaton Fas- ja T-solureseptorisignaloinnista. Tätä spontaania apoptoosia voitiin estää monilla eri sytokiineilla, kuten IL-2:lla, IL-7:llä, IL-15:llä, IL-6:lla sekä kemokiini CXCL12:lla. Lisäksi havaittiin, että reaktiivisten happiradikaalien neutralisointi ei pelastanut T-soluja apoptoosilta, mutta esti apoptoosissa tapahtuvaa DNA:n hajoamista.

PECAM-1-ilmentymä kitarisan epiteelikryptan pohjalla viittaa siihen, että PECAM-1 saattaa ohjata valkosolujen kuljetusta kryptaan ja edesauttaa näin myös antigeenien esittelyä kitarisakudoksessa. Havainnot CD4+ T -solujen eloonjäämisen kontrolloinnista viittaavat siihen, että korkeat antigeenikonsentraatiot, kuten hengitetyn ilman antigeenit tai ravinnon antigeenit, saattavat edistää perifeerisen immuunitoleranssin muodostumista kitarisakudoksessa. Kitarisan aktivoituneet muisti-CD45R0+ CD4+ T -solut tarvitsevat jatkuvasti eloonjäämissignaaleja. Tämä saattaa merkitä sitä, että immuunivasteen voimakkuutta voidaan hienosäätää monilla sytokiineilla, joiden tehtävänä on estää muisti-T- solujen kuolemaa. Reaktiiviset happiradikaalit eivät suoranaisesti kontrolloi muisti-CD45R0+ CD4+ T -solujen eloonjääntiä. Happiradikaalit eivät kuitenkaan ole ainoastaan oksidatiivisen fosforylaation ja apoptoosin haitallisia sivutuotteita, sillä niiden havaittiin välittävän apoptoottisessa DNA:n hajoamisessa tarvittavia signaaleja. Yhteenvetona voidaan sanoa, että monet mekanismit kontrolloivat CD4+ T -solujen apoptoosia kitarisakudoksessa. Päärooli immuunivasteen kontrolloinnissa on mitä ilmeisimmin aluksi antigeenikonsentraatiolla, mutta myöhemmin immuunivasteen voimakkuutta saatetaan säädellä erilaisilla sytokiineilla.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following four original publications, which are referred to in the text by their Roman numerals I to IV.

I **Pajusto M**, Tarkkanen J, Mattila PS (2005) Platelet Endothelial Cell Adhesion Molecule-1 is Expressed in Adenoidal Crypt Epithelial Cells. Scand J Immunol. 61: 82-6.

II **Pajusto M**, Tarkkanen J, Mattila PS (2005) Human primary adenotonsillar naïve phenotype CD45RA+ CD4+ T lymphocytes undergo apoptosis upon stimulation with a high concentration of CD3 antibody. Scand J Immunol, in press.

III **Pajusto M**, Ihalainen N, Pelkonen J, Tarkkanen J, Mattila PS (2004) Human in vivo activated CD45R0+ CD4+ T cells are susceptible to spontaneous apoptosis that can be inhibited by the chemokine CXCL12 and IL-2, -6, -7, and -15. Eur J Immunol. 34: 2771-2780.

IV **Pajusto M**, Toivonen TH, Tarkkanen J, Jokitalo E, Mattila PS (2005) Reactive oxygen species induce signals that lead to apoptotic DNA degradation in primary CD4+ T cells. Apoptosis, in press.

 In addition, some unpublished data are presented.

1. INTRODUCTION

Adenotonsillar tissue is located in the pharynx, which is the point of entry of foreign antigens to the respiratory and digestive tracts. Thereby, it is constantly exposed to antigens that can induce immune responses. Adenotonsillar tissue is prominent in children but rudimentary in adults (Arens, et al., 2002; Vogler, et al., 2000) implying that it may have important biological functions in the maturation of immunity during childhood. However, we still have very little knowledge about the role of adenotonsillar tissue in the human immune system.

Peripheral T cell homeostasis is accomplished by continuous balancing between cell division and programmed cell death, apoptosis (Danial and Korsmeyer, 2004). This is essential in mounting immune responses that are strong enough but at the same time can prevent hypersensitivity reactions, autoimmunity, as well as excessive lymphoproliferation (Arch and Thompson, 1999). CD4+ T lymphocytes are one of the major regulators in immune responses. The T cell receptor repertoire of CD4+ T lymphocytes is originally established in the thymus where self-reactive T lymphocytes are deleted by apoptosis. This leads to immune unresponsiveness against self-antigens, the central immune tolerance is established (Palmer, 2003). Before birth the fetus may encounter foreign antigens through the placenta, but especially after birth the immune system confronts a variety of foreign antigens, including large amounts of antigens that are not harmful, such as nutrients and non-pathogenic inhaled antigens. Unresponsiveness to these non-harmful antigens is established through a mechanism called peripheral immune tolerance. This unresponsiveness is not very well characterized and is, in part, achieved by the function of regulatory T cells (Sakaguchi, 2004). Peripheral immune tolerance is also controlled via pathways that are intrinsic to activated cells and that lead to cell death. One such mechanism is called activation induced cell death (AICD) that is strictly antigen specific and that is triggered via death receptors. Another mechanism, activated T cell autonomous death (ACAD), is distinct from AICD in that it results from loss of survival signals (Lenardo, et al., 1999).

Several unanswered questions remain concerning the peripheral immune tolerance and the control of CD4+ T cell apoptosis in humans. It is not known, which human T cells are susceptible to AICD. Furthermore, it is not known which signals control the survival of activated human CD4+ T cells and whether reactive oxygen radicals play a role. These questions were addressed in this study by evaluating the mechanisms that control apoptotic cell death of human adenotonsillar CD4+ T cells as well as mechanisms that mediate the leukocyte infiltration into the adenoidal epithelial crypt, which is a putative route of entry of pharyngeal luminal antigens into the adenotonsillar tissue.

2. REVIEW OF THE LITERATURE

2.1. The immune system and self-defense

The purpose of the immune system is to protect the host from harmful pathogens. The human immune system consists of innate and adaptive immune responses (Goldsby, et al., 2001). Self-defense against pathogens occurs firstly by the innate immune system that initially recognizes antigens that are non-self. The innate immune system identifies antigens by relatively few types of pattern recognition receptors, such as toll-like receptors, on neutrophils, monocytes, macrophages, dendritic cells, natural killer (NK) cells, and mast cells. NK cells can destroy virally infected cells or malignant cells by cytolysis whereas macrophages can directly phagocyte infectious agents. Dendritic cells are antigen presenting cells that capture and process foreign antigens and then present them to the other cells of the immune system (Janeway, et al., 2005). These innate responses are independent on previous encounters of the antigen and thus do not develop immunologic memory. After the response of the innate immune system, the adaptive immune system, including specialized lymphocytes, T and B cells, can mount an immune response by recognizing antigens that are foreign. In adults, T and B cells are mainly found in lymphoid organs, such as lymph nodes, bone marrow, spleen, and adenotonsillar tissue, as well as in peripheral blood. T and B cells express their antigen receptors on the cell surface, and in addition to this, B cells can produce soluble antigen receptors called antibodies. T and B cells can acquire numerous different specificities by somatic recombination of their receptor genes (Goldsby, et al., 2001; Janeway, et al., 2005). There is also a third lymphocyte population, so called natural killer T (NKT) cells, which have both T cell and NK cell receptors (Kronenberg, 2005). Adaptive immune responses have immunologic memory, which can be defined as an altered, faster and stronger, response that follows after re-exposure to the previously encountered antigen (Goldsby, et al., 2001; Janeway, et al., 2005).

2.1.1. T cells

All lymphocytes differentiate from the common pluripotent stem cells in the bone marrow. Lymphoid progenitor cells give rise to T cells, B cells, and natural killer (NK) cells. Precursor T lymphocytes migrate into the thymus to undergo their maturation. T cells can be divided into T helper (Th) cells, which express CD4 and cytotoxic killer T cells, which express CD8 membrane glycoproteins on their surfaces. CD4+ helper T cells provide help in cytotoxic immune responses against intracellular pathogens (Th1, Th type 1 responses) as well as help for antibody synthesis (Th2 responses) (See 2.1.2. for classification). Cytotoxic CD8+ T cells function by killing other cells like those infected with pathogenic microbes. T cells recognize antigens only when they are processed to antigenic peptides and presented on the surface of the antigen presenting cells by so-called MHC (major histocompatibility complex) molecules. CD4+ T cells recognize antigenic peptides presented by cell surface MHC class II molecules, whereas CD8+ T

cells recognize antigenic peptides presented by MHC class I molecules (Goldsby, et al., 2001; Janeway, et al., 2005).

2.1.2. CD4+ T cells

CD4+ helper T cells have a central role in the regulation of several different T cell responses (Kaufmann, 1993). T cells secrete cytokines, which are a group of intercellular signalling proteins that regulate immune responses as well as growth and differentiation of the cells (Belardelli, 1995; Curfs, et al., 1997). Cytokines, such as interleukin-2 (IL-2), may have different functions depending on the target cell or other cytokines present. Thereby, IL-2 usually promotes survival and proliferation of the T cells, yet it also plays a role in inducing CD4+ T cells to become susceptible to controlled cell death (Jenkins, et al., 2001). As mentioned above, there are different functional types of CD4+ T cells. Th1 type CD4+ T cells produce inflammatory cytokines, such as interferon-γ (IFN-γ), tumor necrosis factor-β (TNF-β), and IL-2 and promote cytotoxic immune responses by helping CD8+ T cells and macrophages, which are important in immune defense against intracellular pathogens. Th2 type CD4+ T cells produce cytokines such as IL-4, IL-5, IL-6, and IL-13, which are involved in the regulation of antibody responses by B cells (Coffman, et al., 1988; Mosmann, et al., 1986). In addition to the cytokine production pattern, there are several different cell surface markers that distinguish Th1 and Th2 CD4+ T cells from each other. Th1 CD4+ T cells express the chemokine receptors CXCR-3 and CCR-5, whereas the receptors CXCR-4, CCR-3, CCR-4, and CCR-8 are mainly expressed on Th2 CD4+ T cells (Syrbe, et al., 1999). Chemokines are produced by a variety of cells and they can attract cells to inflammatory sites (Moser and Loetscher, 2001; Moser, et al., 2004). Thereby, specific chemokines can preferentially mobilize, for example, either Th1 or Th2 CD4+ T cells to the sites of inflammation.

In addition to Th1 and Th2 type CD4+ T cells, a third type of CD4+ T cells, regulatory T cells, has been identified. Regulatory T cells, previously also known as suppressor T cells (Chatenoud, et al., 2001), are increasingly recognized as central players in the regulation of immune responses as well as in preventing pathological self-reactivity (Sakaguchi, 2004). There are many types of regulatory T cells and the characterization of these different cell types is not yet fully established. For example Th3 and T regulatory type 1 (Tr1) cells may regulate immune responses by the release of suppressive cytokines such as transforming growth factor (TGF) -β and IL-10 (Weiner, 2001). The functional alteration or reduction of regulatory T cells can lead to spontaneous development of various organ-specific autoimmune diseases, such as autoimmune thyroiditis or type 1 diabetes (Itoh, et al., 1999; Sakaguchi, et al., 1995). Regulatory T cells are defined by the expression of the transcription factor Foxp3 (forkhead box p3) that can control the expression of many other genes and is a key regulator of the development of the regulatory cells (Hori, et al., 2003). The large majority of Foxp3-expressing regulatory T cells are found within the MHC class II restricted CD4+ T cell population and express CD25 that is the high affinity receptor for IL-2. CD25 may also be expressed on non-regulatory T cells in settings of immune activation such as during an immune response to certain pathogens (Sakaguchi, 2004; Shevach, 2002).

2.1.3. Early maturation of T cells and thymic selection – development of central tolerance

T lymphocytes originate from the bone marrow and migrate to the thymus to mature. The thymus is located in the throracic cavity between the heart and the sternum. It is prominent during the fetal development but usually withered in adults. During their early maturation, T cells begin to reassemble their T cell receptor variable (V), diversity (D), and joining (J) gene segments by somatic recombination resulting in a T cell population in which each T cell has a unique T cell receptor (Goldrath and Bevan, 1999). The T cell receptors are able to recognize processed antigenic peptides only when they are bound to MHC molecules on antigen presenting cells, such as dendritic cells, during a process called antigen presentation. As the assembly of T cell receptor gene segments occurs in more or less stochastic fashion during somatic recombination, most developing T cells do not bind to self-peptide MHC-complex at all and are eliminated by programmed cell death, apoptosis. Those cells that are able to bind self-peptide MHC-complex proliferate in a process called positive selection (Goldsby, et al., 2001; Janeway, et al., 2005).

T cells that are positively selected undergo another step of the selection called negative selection. In this process, the cells that have very high affinity T cell receptors to MHC-peptide complex undergo programmed cell death (Palmer, 2003). This process deletes T cells that are reactive to self and thus would attack self-components (Ardavin, 1997). The purpose of the positive and negative selection is to generate a T cell population that is able to bind to MHC molecules only when the MHC molecule is associated with a processed foreign antigenic peptides, but not when the antigen binding groove of the MHC molecule is occupied by self-peptides (Farr and Rudensky, 1998). The negative selection by clonal deletion is called the development of central tolerance.

It has been shown that many peripheral self-antigens are actively transcribed in the thymus and then presented to developing T cells, thereby driving the negative selection. One of the transcriptional regulators that is responsible for such promiscuous transcription in the thymus is encoded by the autoimmune regulator gene, AIRE (Derbinski, et al., 2001; Pitkanen and Peterson, 2003). The thymic selection finally results in T cells that are both self-MHC restricted and self-tolerant and thus capable to respond against foreign antigens. Altogether over 95% of early immature T cells are eliminated during these rigorous selection processes in the thymus (Goldsby, et al., 2001).

2.1.4. Secondary lymphoid organs and adenotonsillar tissue

Lymphoid organs and tissues fall into two categories, primary (central) and secondary (peripheral). Primary lymphoid organs are the sites where lymphocytes differentiate from progenitor cells, namely the thymus and bone marrow. Secondary lymphoid organs comprise the tissues where the actual immune responses occur. The secondary lymphoid

organs, such as the spleen, lymph nodes, and mucosa-associated lymphoid tissues (MALT), can be further classified according to the body regions, which they are defending. The spleen takes care of the antigens in blood whereas the lymph nodes respond to antigens that are transported by the lymph that drain various tissues in lymphatic vessels. MALT, which includes the adenoids and the tonsils, bronchus-associated lymphoid tissue, as well as the Peyer's patches of the intestine, respond to antigens that have entered the body through mucosal barriers (Roitt, et al., 1998).

Antigens are presented to the naïve antigen inexperienced CD4+ T cells by antigen presenting cells, such as dendritic cells, within the T cell areas of the secondary lymphoid organs (Jenkins, et al., 2001). When a naïve T cell encounters a processed antigenic peptide bound to an MHC molecule on the cell surface it proliferates and differentiates. The cell first acts as an effector cell and finally becomes a memory T cell (Iezzi, et al., 1998). T cell activation through the antigen receptor causes a change in the isoform usage of the common leukocyte antigen CD45 from the naïve type CD45RA to the memory type of CD45R0 (Akbar, et al., 1988). The expression of CD45RA and CD45R0 isoforms discriminate two discrete cell populations in adenotonsillar tissue that present naïve and memory CD4+ T cell populations (Mattila and Tarkkanen, 1998). Memory CD45R0+ CD4+ T cells are functionally different from naïve CD45RA+ CD4+ T cells as they express a different pattern of cell surface markers and also respond differently to antigens (Budd, et al., 1987; Inaba, et al., 1999). Memory cells are able to respond to antigens faster and stronger and are less dependent on accessory cell co-stimulation than naïve cells (Croft, et al., 1994; Dutton, et al., 1998).

Adenotonsillar tissue is a part of the MALT in the pharynx. It is composed of the adenoids (pharyngeal tonsil), which is located in the nasopharynx and can be visualized with a mirror, the tonsils (palatine tonsil), which is a paired organ in the oropharynx, and the lingual tonsil, which is a small collection of lymphoid tissue in the base of the tongue. Collectively, the adenoids, the tonsils, and the lingual tonsil are called the Waldaeyer's ring and they can together be considered to form a somewhat ring-like structure (Bluestone, et al., 2003). The location of the adenoids, the tonsils, and the lingual tonsil in the pharynx is illustrated in Figure 1. Adenotonsillar tissue is prominent during childhood. In adults, the adenoids are normally rudimentary and the tonsils are quite small (Arens, et al., 2002; Vogler, et al., 2000) suggesting that it presumably has its main biological functions early during childhood. The location of adenotonsillar tissue is optimal to confront foreign antigens as the major route of entry of foreign antigens to respiratory and digestive tracts are through the pharynx. In the adenotonsillar tissue, the invaginated epithelial crypts are the ideal candidates for the point of entry of pharyngeal luminal antigens into the adenoidal tissue (Koshi, et al., 2001). The crypts are consistently infiltrated with leucocytes and thus appear to have active immune functions, forming a special lymphoepithelial structure (Ruco, et al., 1995).

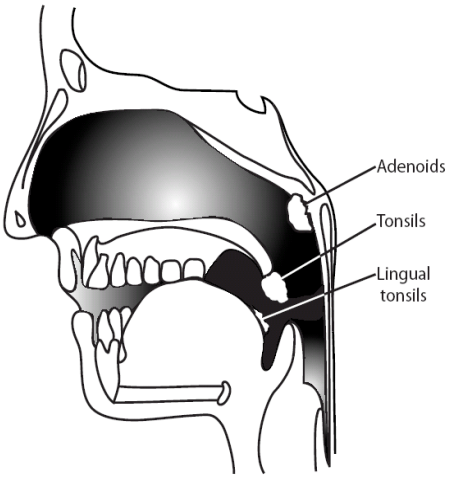

Figure 1: The location of the adenotonsillar tissue in the pharynx. The adenoids are located in the posterior wall of the nasopharynx, whereas the tonsils are located on the lateral walls of the oropharynx, and the lingual tonsils at the base of the tongue. The adenoids, the tonsils, and the lingual tonsils compose the Waldaeyer's ring of lymphoid tissue in the pharynx.

The adenotonsillar tissue can be chronically infected by pathogens. To treat these conditions, adenotonssillar tissue can be removed by adenoidectomy (removal of the adenoids) and by tonsillectomy (removal of the tonsils) (Gates, 1999; Lanphear, et al., 1997). Usually adenoidectomy is performed because of recurrent or persistent childhood otitis media, when the adenoids are considered to be chronically infected and serve as a source of pathogens causing otitis media (Hammaren-Malmi, et al., 2005; Mattila, et al., 2003). Infections may also cause enlargement of the adenoids, which can further cause obstructive symptoms that can be relieved by adenoidectomy (Bluestone, et al., 2003; Sade and Luntz, 1991). Tonsillectomy is usually performed because of tonsillar hyperplasia causing obstructive symptoms, peritonsillar abscesses, and chronic tonsillitis (Wetmore, et al., 2000).

2.2. Control of peripheral T cell responses

Upon exposure to pathogens the immune system needs to mount a rapid immune response but yet it has to avoid excessive and unwanted responses as well as responses to self. This is achieved by immune tolerance (Van Parijs and Abbas, 1998). Despite the selection of developing T cells in the thymus that results in the development of central immune tolerance, there are various mechanisms that maintain and strengthen immune tolerance in peripheral tissues. These mechanisms are collectively called peripheral immune tolerance. This can be accomplished in part by the action of regulatory T cells that suppress specific peripheral immune responses (See 2.1.2.). Peripheral tolerance to various antigens can also be induced by a mechanism called T cell anergy. In a state of anergy, a T cell is intrinsically functionally inactivated following an antigen encounter,

but it remains alive for an extended period of time in a hyporesponsive stage (Schwartz, 2003; Janeway, et al., 2005). In addition, stimulated T cells can be downregulated by soluble factors through a mechanism called immune deviation, in which one Th cell subset is the preferentially activated over another (Rocken and Shevach, 1996; Gao, et al., 1998). Peripheral immune responses are controlled also by the elimination of T cells through mechanisms that are intrinsic to the activated T cell. Two types of such mechanisms have been identified (Akbar and Salmon, 1997). Firstly, there is activation induced cell death (AICD), which is induced by death receptors on the T cell surface upon signals initially triggered through the T cell receptor (Nagata, 1997; Thornberry and Lazebnik, 1998). This results in the death of T cells of unwanted specificities and serves to maintain peripheral immune tolerance (Budd, 2001). The other mechanism, called activated T cell autonomous death (ACAD), results from loss of survival signals for activated T cells and it attenuates already established T cell responses. Both AICD and ACAD occur through a precisely controlled cell death mechanism, apoptosis (Hildeman, et al., 2003b; Lenardo, et al., 1999; Van Parijs and Abbas, 1998).

2.2.1. Apoptotic cell death

Apoptosis, programmed cell death, is a universal mechanism that plays a critical role in the development and in the normal tissue homeostasis in multicellular organisms (Cohen, et al., 1992; Danial and Korsmeyer, 2004). Apoptotic cell death was first characterized by Kerr et al. (1972) who identified two different forms of cell deaths. Necrotic cell death usually results from death by accident, such as infarction of an organ or drug injury. It is characterized by swelling of the cell, plasma membrane disruption and destruction of the cellular organelles as well as release of the intracellular contents leading to inflammation. The second type of cell death is apoptosis, which has distinct morphological features including blebbing of the plasma membrane, shrinkage of the cell, and condensation and fragmentation of the nuclear chromatin. Apoptotic cells disintegrate into small membrane-enclosed vesicles, apoptotic bodies, containing intact organelles (Wyllie, et al., 1980). Cells undergoing apoptosis begin to express phosphatidyl-serine on their surface, which allows the phagocytocing macrophages to recognize and remove the apoptotic cells (Fadok, et al., 1998; Fadok, et al., 1992). The apoptotic bodies and cells are then degraded by phagocytocing macrophages without a noticeable inflammatory response (Kerr, et al., 1972). In T lymphocytes, apoptosis plays an essential role in maintaining T cell repertoire and in deletion of autoreactive T cells, thus limiting immune responses (Osborne, 1996; Rathmell and Thompson, 1999; Rathmell and Thompson, 2002). There are two major pathways that can induce apoptotic cell death in activated T cells. Apoptosis can be triggered either by external, death receptor-mediated, pathway (Ashkenazi and Dixit, 1998; Budihardjo, et al., 1999; Nagata, 1997) or through intrinsic, mitochondrial-mediated, signalling pathway (Green and Kroemer, 2004; Green and Reed, 1998). Finally, after triggering of apoptosis, the external and intrinsic pathways converge and result in the activation of caspases, a family of cysteine proteases, and the final execution of apoptosis (Daniel, et al., 2001; Gupta, 2001; Thornberry, 1998). These two major apoptotic pathways are illustrated in Figure 2 and explained more detailed in the following paragraphs.

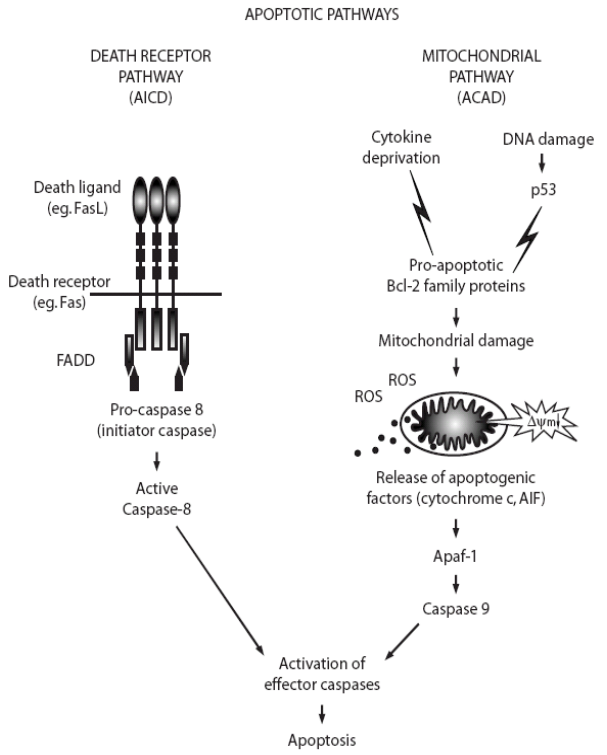

Fig 2. A simplified diagram of two major apoptotic pathways in human cells. Cells may undergo apoptosis via death receptor pathway (left pathway in the figure) or through intrinsic, mitochondrial pathway (right pathway in the figure). The death receptor pathway is triggered when death receptor ligands (eg. FasL) bind to their receptors (eg. Fas). This induces receptor clustering and the formation of a death inducing signalling complex. FADD (Fas-associated death domain) then further recruits pro-caspase-8 molecules resulting in the activation of the initiator caspase 8. Mitochondrial pathway can be initiated by various external stimuli, such as growth factor deprivation or DNA damage. This results in the activation of pro-apoptotic members of the Bcl-2 (B-cell CLL/lymphoma 2) family of proteins and further to the release of apoptogenic factors (eg. cytochrome c and apoptosis inducing factor, AIF) from mitochondria. Cytochrome c binding to Apaf-1 (Apoptotic protease-activating factor-1) results in the binding and activation of initiator caspase 9. Both pathways activate effector caspases, such as caspase-3, that function in the final execution of apoptosis. There is a significant interplay between the death receptor and the mitochondrial apoptotic pathways. The initial activation of caspase 8 and other caspases by the death receptor pathway can lead to the cleavage of the Bcl-2 family protein Bid that result in the subsequent permeabilization of the mitochondrial outer membrane and the activation of the mitochondrial apoptotic pathway. Such feedback loops may amplify apoptotic cell death cascades leading to complete execution of apoptosis after the initial decisive events have taken place.

2.2.2. Activation induced cell death (AICD)

AICD was first demonstrated by the experiments in mice where several days of IL-2 treatment of CD4+ T cells in vitro resulted in sensitivity to apoptosis upon T cell receptor

stimulation through Fas death receptor (Brunner, et al., 1995; Dhein, et al., 1995; Ju, et al., 1995). As AICD has been demonstrated when T cells are stimulated through the T cell receptor (Hornung, et al., 1997; Wong, et al., 1997), it is thought to have important physiological functions in the prevention of autoimmune reactions when T cells encounter high concentrations of antigens (Siegel, et al., 2000). The interaction between Fas ligand (FasL) and the Fas death receptor (Fas) results in triggering of apoptosis and thus has a major role in the regulation of AICD (Alderson, et al., 1995; Lenardo, et al., 1999).

The contact between the T cell antigen receptor (TCR) on the T cell and the processed antigenic peptide bound to MHC on the antigen presenting cell forms a specialized junction, so-called immunological synapse (Bromley, et al., 2001; Huppa and Davis, 2003). Immunological synapse consists of TCR and the surrounding, well-organized ring of several different adhesion molecules (Grakoui, et al., 1999). This interaction initiates a wide range of intracellular signaling events that finally lead to the activation of transcription factors regulating the expression of various genes, including cytokine genes (Friedl, et al., 2005).

The earliest signaling events after TCR ligation include tyrosine phosphorylation, which involves the activation of Src family tyrosine kinases, namely Lck and Fyn, and the phosphorylation of phospholipase C (Roitt, et al., 1998). The active forms of Lck and Fyn phosphorylate proteins in the T cell receptor complex, resulting in the phosphorylation and activation of a complex called the immunoreceptor tyrosine-based activation motif (ITAM), that is found in TCR-associated chains, as well as ζ-chain-associated protein kinase of 70 kDa (ZAP70) tyrosine kinase (Germain and Stefanova, 1999; Huppa and Davis, 2003). The activation of ZAP70 phosphorylates downstream targets that activate mitogen-activated protein (MAP) kinase pathways. Phospholipase C, on the other hand, hydrolyzes the membrane lipid phosphatidylinositol 4,5-biphosphate producing diacylglycerol (DAG) and inositol triphosphate (IP_3). DAG activates proteine kinase C, a family of serine-threonine protein kinases that in turn phosphorylate Ras (Huang and Wange, 2004). IP_3 is water-soluble and diffuses through the cytoplasm to the endoplasmic reticulum, where it opens calcium channels releasing calcium from its intracellular stores inside the endoplasmic reticulum into the cytoplasm. Calcium alters many cellular processes, in part by binding to regulatory proteins, such as calmodulin and calcineurin (Hunter, 2000). Calcineurin is a calcium-calmodulin dependent serine/threonine phosphatase, which targets NFAT (nuclear factor of activated T cells) and is a transcriptional regulator of IL-2 and other cytokine gene expression (Bierer, et al., 1990; Crabtree, 2001; Mattila, et al., 1990). TCR ligation thus finally results in the transcriptional activation of the IL-2 gene. Besides NFAT, the transcription of IL-2 and other genes that are important for T cell activation is also dependent on the formation and activation of other transcription factors, including activator protein-1 (AP-1) and the nuclear factor kappa-β (NF-κB) (Rao, et al., 1997; Rothenberg and Ward, 1996). The NF-κB induction in T cells upon TCR stimulation is dependent on protein kinase C activation (Jamieson, et al., 1991). Interestingly, it has been shown that in addition to IL-2, NFAT gene family regulates also the transcription of Fas-L (Latinis, et al., 1997). Stimulation with a high concentration of CD3 antibody may result in the upregulation of

FasL but stimulation with a low concentration of CD3 antibody fails to upregulate FasL. It is not known which intracellular pathways result in FasL transcription in AICD. These mechanisms may include the transcription factor Nur77 that has been suggested to play a decisive role in TCR mediated apoptosis, mainly characterized in thymocytes (Toth, et al., 2001; Woronicz, et al., 1994; Woronicz, et al., 1995). It has been reported that the myocyte enhancer factor-2 (MEF2) is the prime controller of Nurr77 transcription within the cell and that the transcriptional activity of MEF2 is regulated via calcium-dependent repressor Cabin1 (Esau, et al., 2001; Youn and Liu, 2000).

FasL is a type II membrane protein that is expressed on T lymphocytes upon activation (Suda, et al., 1995; Suda, et al., 1993; Tanaka, et al., 1995). The receptor for FasL, Fas death receptor (Itoh, et al., 1991; Nagata, 1997), belongs to the tumor necrosis factor (TNF) receptor superfamily consisting of over 20 members (Itoh, et al., 1991; Oehm, et al., 1992; Trauth, et al., 1989; Watanabe-Fukunaga, et al., 1992; Zheng, et al., 1995). Fas death receptor mediates apoptosis in a wide variety of cell types (Nagata, 1994a; Nagata, 1994b). FasL is a trimer and its binding to Fas death receptor on cell surface induces trimerization of Fas (Nagata, 1999). This further induces the cytoplasmic recruitment of adapter protein FADD (Fas-associated death domain) to the cytoplasmic tail of Fas through the interaction of the respective death domains (Krammer, 1999; Pinkoski and Green, 1999). The opposite end of FADD contains two death effector domains that are able to activate caspase-8 or its enzymatically inactive homologue FLIP (FLICE-like inhibitor protein) (Thome and Tschopp, 2001). For example, the stimulation of cells through IL-2 receptor normally promotes cell survival, but in some cases it may also increase the transcription and cell surface expression of FasL and also decrease the levels of FLIP (Holtzman, et al., 2000; Refaeli, et al., 1998). The signaling through the Fas death receptor is illustrated in Figure 2.

Caspases are synthesized as inactive pro-caspases, which undergo proteolytic cleavage upon activation (Budihardjo, et al., 1999). Caspases are highly conserved through evolution and all known caspases possess an active-site cysteine and cleave substrates after aspartic acid residues (Hengartner, 2000). Active caspase-8, belonging to so-called initiator caspases, further activates the execution phase of the apoptosis by activating caspase-3 and other downstream caspases, so-called effector caspases (Thornberry, 1998; Thornberry and Lazebnik, 1998). Signaling through Fas has also an opposite role in the regulation of T cells: besides providing apoptotic signals in previously activated cells, it can also act as a co-stimulatory molecule (Alderson, et al., 1993; Budd, 2002). This is mediated by caspase-8, which, in addition to providing apoptotic signals, can also mediate cellular stimulation by activating NF-κB (Su, et al., 2005).

It has been shown in mice that AICD affects primarily unprimed T cells and in lesser extent antigen-primed T cells, suggesting that it is important in the beginning of the immune response (Desbarats, et al., 1999; Inaba, et al., 1999). The essential role of Fas-FasL interactions in the induction of AICD is also evident when comparing the ability of Th1 and Th2 type cells to undergo AICD. Cloned Th1 cells that express high levels of FasL are susceptible to AICD whereas Th2 cells that express only low levels FasL are capable of undergoing AICD only in the presence of Th1 cells (Ramsdell, et al., 1994).

Furthermore, certain site-specific mechanisms are developed for the maintenance of tolerance through lymphocyte apoptosis in immune privileged sites, like in the eyes or testis, where inflammatory responses could lead to serious injuries (Abbas, 1996). Thereby, if activated T cells succeed to enter the eye, they are sentenced to die through the Fas-FasL pathway and the hazardous immune response is avoided as the cells in these sites express FasL (Griffith, et al., 1995). Activated human peripheral blood T cells from asthmatic individuals are more resistant to Fas-mediated apoptosis than T cells from nonasthmatic individuals suggesting that the ineffective activation of Fas signaling may also promote the development of T cell dependent inflammation (Jayaraman, et al., 1999). Moreover, mutations in Fas and Fas ligand genes in mice (Chu, et al., 1993; Lynch, et al., 1994; Watanabe-Fukunaga, et al., 1992) as well as in man (Fisher, et al., 1995; Rieux-Laucat, et al., 1995; Siegel, et al., 2000) causes abnormal T cell apoptosis resulting in autoimmune responses and excessive lymphoproliferation, implying the important role of apoptosis in peripheral tolerance to self-antigens and in lymphocyte homeostasis (Nagata, 1999).

2.2.3. Activated T cell autonomous death (ACAD)

The other type of activated T cell death, activated T cell autonomous death, ACAD, which has also been called passive cell death or death by neglect, occurs as a result of loss of survival signals at the end of the immune response when the T cell – antigen presenting cell engagement ends (Lenardo, et al., 1999; Van Parijs and Abbas, 1998). In contrast to AICD, ACAD is independent of TCR- and Fas-signaling and more likely the members of the B-cell CLL/lymphoma 2 (Bcl-2) family of proteins regulate ACAD through the mitochondrial apoptotic pathway (Hildeman, et al., 2002; Strasser, et al., 1995; Van Parijs, et al., 1998). The Bcl-2 family of both pro- and anti-apoptotic proteins can be divided into three classes, which show sequence and structural similarity in the Bcl-2 homology (BH) regions (Adams and Cory, 1998). The anti-apoptotic proteins Bcl-2, Bcl-xL, A1/Bfl-1, Bcl-w, Boo/Diva/Bcl-B, and Mcl-1 share three of the four BH regions. A subgroup of the pro-apoptotic Bcl-2 family members, including Bax, Bak, Bok/Mtd, Bcl-x_s, and Bcl-G_L, have two or three common BH regions. The other pro-apoptotic subgroup, so called BH3-only proteins includes Bid, Bad, Bcl-G_s, Bik/Nbk, Bim/Bod, Blk, Bmf, Hrk/DP5, Noxa, and PUMA/Bbc3, which share only one short BH3 region (Marsden and Strasser, 2003). The pro-apoptotic Bcl-2 family proteins induce apoptosis by disrupting the integrity of the outer mitochondrial membrane, which results in the release of apoptogenic factors from the intermembrane space (Luo, et al., 1998; Opferman and Korsmeyer, 2003; Strasser, 2005). All BH3-only proteins can also bind with high affinity to pro-survival Bcl-2 family proteins and thereby trigger apoptosis when overexpressed by neutralizing the functions of the pro-survival proteins (Strasser, 2005). The expression of anti-apoptotic members of the Bcl-2 family proteins on the inner mitochondrial membrane inhibit apoptosis by forming heterodimers with the other pro-apoptotic Bcl-2 family of proteins, thus preventing the permeabilization of the membrane and maintaining the mitochondrial integrity (Van Parijs, et al., 1998).

BH3-only protein of the Bcl-2 family, PUMA (p53 upregulated modulator of apoptosis), is needed for p53-dependent apoptosis (Nakano and Vousden, 2001). p53 tumor-suppressor protein is essential for restricting inappropriate cell proliferation and cancer development under conditions of cellular stress, such as DNA-damage, oncogene activation, hypoxia and oxidative stress (Levine, 1997; Lohrum and Vousden, 1999). Loss or mutations in p53 is a decisive step in the development of most cancers. The p53 acts as a transcription factor that directly binds to DNA in a sequence-specific manner and activates the expression of numerous genes, including the BH3-only protein PUMA. PUMA associates with mitochondria and it induces apoptosis when overexpressed in various cell lines. Furthermore, PUMA knockout mice have been reported to have increased resistance to apoptosis in various cells including lymphocytes (Jeffers, et al., 2003). It has been found that PUMA acts by modulating the activity of Bax, another pro-apoptotic member of the Bcl-2 family, thus facilitating the release of cytochrome c from the mitochondria (Yu, et al., 2001). In addition to p53, the expression of PUMA is also regulated by p53-independent stimuli, including glucocorticoids and serum deprivation (Han, et al., 2001; Villunger, et al., 2003). Another BH3-only protein, Bim, is required for IL-2 and IL-7 withdrawal-induced apoptosis, implying that it is crucial in shut downing immune responses against acute infections (Strasser, 2005). Furthermore, gene targeting experiments in mice have revealed that Bim has important roles also in hematopoietic cell homeostasis as well as in the prevention of autoimmunity (Bouillet, et al., 1999).

Mitochondrial apoptotic pathways have a complex and expanding role in the induction of apoptosis especially in the cases where apoptosis takes place in response to loss of survival factors and cellular stress, as in oxidative stress (Wang, 2001). These signals can lead to the permeabilization of the mitochondrial outer membrane and allow the release of cytochrome c and other apoptogenic factors, such as apoptosis inducing factor (AIF) (Joza, et al., 2001; Susin, et al., 1999), from the mitochondrial intermembrane space into to cytosol, which further can activate caspases or directly damage DNA (Green and Reed, 1998; Liu, et al., 1996; Martinou and Green, 2001). Cytochrome c release into the cytoplasm catalyzes the oligomerization of mitochondrial Apaf-1 (Apoptotic protease-activating factor-1), which promotes the activation of pro-caspase 9 and the formation of a complex called apoptosome in the cytosol. This further leads to the activation of caspase 9 and subsequent activation of caspase 3 and finally apoptotic destruction of the cell. Besides caspases, there are also other mitochondrial factors that mediate apoptosis (Green and Reed, 1998). AIF, for example, can reach the nucleus upon release from the mitochondrial intermembrane space and stimulate apoptotic chromatin condensation and DNA fragmentation. It can also further augment apoptosis by disrupting the mitochondrial transmembrane potential by a caspase-independent pathway and thus promote mitochondrial release of cytochrome c (Susin, et al., 1999). The signaling through the mitochondrial pathway is illustrated in Figure 2.

Mitochondrion is the center for oxidative phosphorylation and the energy production inside the cell. Mitochondria generate most of energy required by the aerobic cells in the form of adenosine triphosphate (ATP). ATP is formed as a product of oxidative phosphorylation through the mitochondrial respiratory chain, a process taking place in the

inner mitochondrial membrane. The mitochondrial respiratory chain in the inner mitochondrial membrane consists of complexes I, II, III, and IV and two mobile electron carriers cytochrome c and ubiquinone. These respiratory chain protein complexes act in sequence in order to accept reducing equivalents from NADH (nicotinamide adenine dinucleotide) or $FADH_2$ (flavin adenine dinucleotide) and transfer them through the series of oxidation-reduction reactions finally to O_2 (Newmeyer and Ferguson-Miller, 2003). Electrons from reducing substrates, such as NADH and succinate, are transferred from complex I (NADH ubiquinone oxidoreductase) or complex II (succinate ubiquinone oxidoreductase), respectively, to ubiquinone, and further to complex III. Complex III is cytochrome c oxidoreductase, which reduces cytocrome c. Cytochrome c then further transfers electrons to complex IV, cytochrome c oxidase, and finally to O_2 (Poyton and McEwen, 1996; Stryer, et al., 2002). Cytochrome c has thus important functions in both the vital oxidative phoshorylation and in cell death, apoptosis (Chandra, et al., 2002). Electron flow through complexes I, III, and IV results in pumping of protons out of the mitochondrial matrix to the intermembrane space. This generates mitochondrial transmembrane potential ($\Delta\psi_m$) across the membrane. The reverse flow of the protons from the intermembrane space into the matrix drives ATP-synthesizing complex, F_0F_1-ATPase, to produce ATP (Stryer, et al., 2002).

In addition to the ATP production, the mitochondrial transmembrane potential, $\Delta\psi_m$, is also needed for the regulation of metabolite transport and for the mitochondrial protein import (Ricci, et al., 2004). One of the most important and earliest mitochondrial apoptotic events in the cell is the loss of $\Delta\psi_m$. In consequence, $\Delta\psi_m$ can be used to measure the cellular viability (Zamzami, et al., 1995). Loss of $\Delta\psi_m$ results in uncoupling of oxidative phosphorylation, generation of superoxide radicals, and Ca^{2+} flux into the cytosol (Hirsch, et al., 1997) leading to apoptosis.

Molecular O_2 is effectively converted to water during oxidative phosphorylation. At the same time, small amounts of intermediates of O_2 reduction can escape the process and are thus constantly produced as by-products in the ATP synthesis in mitochondria. During this process superoxide anions, O_2^-, are formed from single electrons and molecular oxygen that escape the mitochondrial respiratory chain. In addition other intermediates of oxygen reduction are formed. These intermediates of oxygen reduction are collectively called reactive oxygen species (ROS), which have either unpaired electrons or the ability to take electrons from other molecules (Cai and Jones, 1998). ROS are toxic to cells and as cells are repeatedly under attack from ROS, effective detoxification mechanisms have been developed to inactivate them.

Superoxide dismutase (SOD) (Buttke and Sandstrom, 1994; Voehringer, 1999) is an important mitochondrial enzyme that catalyses the conversion of toxic superoxide anions O_2^- to hydrogen peroxide (H_2O_2) and molecular oxygen (Stryer, et al., 2002). Hydrogen peroxide can in turn form a highly reactive and toxic hydroxyl radical ($^{\cdot}OH$) in the presence of reduced metal atoms unless it is detoxified to water and molecular oxygen by glutatione peroxidase or catalase (Schriner, et al., 2005) Glutathione peroxidase oxidizes glutathione (GSH), a major thiol within the cell, to oxidized glutathione (GSSG) in a reaction where hydrogen peroxide is detoxified to water and molecular oxygen. GSSG in

turn is toxic and is fast converted back to GSH in the reduction reaction that is catalyzed by the enzyme glutathione reductase (Stryer, et al., 2002). The essential detoxification reactions that are mediated by SOD, catalase, and glutathione peroxidase are presented in Figure 3.

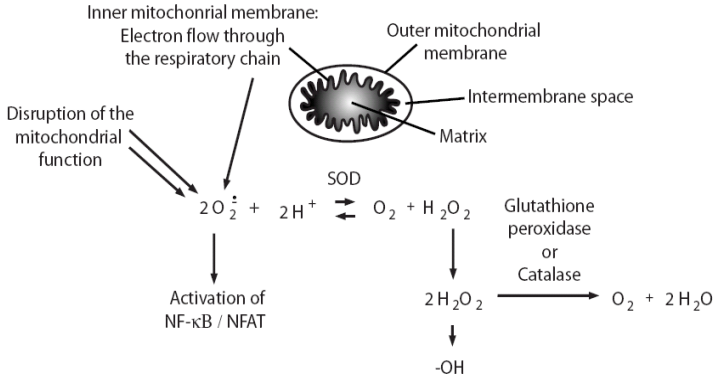

Figure 3. The essential detoxification mechanisms of reactive oxygen species. Small amounts of superoxide anions O_2^- are unavoidably formed during oxidative phosphorylation. These are toxic but they are eliminated by superoxide dismutase (SOD) that catalyses the conversion of superoxide anions O_2^- to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). H_2O_2 forms a toxic hydroxyl radical ($^{\cdot}OH$) unless it is further detoxified to water (H_2O) and O_2 by glutatione peroxidase or catalase. During apoptosis the formation of reactive oxygen species is greatly enhanced and the detoxification mechanisms fail to eliminate them.

The major intracellular thiol, GSH, is a tripeptide that contains one sulfhydryl group. It is thus able to buffer and remove free radicals and plays a key role in several detoxification reactions (Voehringer, 1999). Besides being essential in detoxification reactions, GSH is important in mediating signal transduction and gene expression (Arrigo, 1999; Sies, 1999). Furthermore, it has been shown that glutathione depletion in human peripheral blood mononuclear cells inhibits the cell cycle transition from G1 to S phase, implying that GSH is crucial also for cell cycle progression (Messina and Lawrence, 1989). N-acetyl-L-cysteine (NAC) is a thiol-containing antioxidant that is able to effectively raise the intracellular GSH levels and detoxify free radicals thus preventing DNA damage (Malins, et al., 2002). For example in the HIV infection, GSH levels are low in plasma, erythrocytes as well as in individual T cell subsets and NAC is routinely used in order to replenish GSH and further improve the immunological functions of the T cells (De Rosa, et al., 2000). NAC has also been reported to inhibit the death of oligodendrocytes induced by both cytotoxic stimuli and trophic factor deprivation (Mayer and Noble, 1994).

Disintegration of the mitochondrial function during apoptosis disrupts the proper function of the mitochondrial respiratory chain resulting in the permeabilization of the mitochondrial membrane and the formation of excessive amounts of reactive oxygen species, ROS (Dussmann, et al., 2003). ROS-mediated reactions play a role in discrete pathogenic processes, including carcinogenesis, as they can for example directly damage DNA and act as tumor promoters (Adler, et al., 1999). Recently, it has also been

suggested that ROS play a pivotal role in mediating apoptosis of in vivo activated mouse peripheral T cells (Hildeman, et al., 1999) by down-regulating the expression of the anti-apoptotic Bcl-2 protein (Hildeman, et al., 2003a; Tripathi and Hildeman, 2004). Even though ROS are toxic by-products, they also function in modulating various cellular processes including signal transduction and gene expression (Adler, et al., 1999; Los, et al., 1995).

2.3. Unresolved issues in the control of peripheral immune responses with particular emphasis on human adenotonsillar tissue

Despite the advances in understanding the immune system, several questions remain concerning the control of human CD4+ T cell apoptosis as well as the regulation of human peripheral immune tolerance.

All children are born with adenotonsillar tissue. However, the exact role of adenotonsillar tissue in the maturation of the immune system of the growing child is unknown. An elusive structure is the adenotonsillar epithelial crypt that is thought to mediate the antigen transport from the pharyngeal lumen to the adenoidal tissue. This epithelial crypt is constantly infiltrated with leukocytes, but the mechanisms that mediate this characteristic leukocyte infiltration are unknown.

Even though it is well known that the mutations in Fas and FasL genes cause autoimmune diseases and lymphoproliferative disorders in humans (Fisher, et al., 1995; Rieux-Laucat, et al., 1995; Siegel, et al., 2000), it is not known, which human T cells are susceptible to Fas-mediated AICD that is presumably induced by a high antigen concentration. As adenotonsillar tissue is critically located at the point of entry of foreign antigens, it is a candidate organ where deletion of CD4+ T cells occurs upon contact with high concentrations of antigens such as nutrients and various harmless inhaled antigens.

It is also unknown, which human CD4+ T cells are susceptible to ACAD and which signals control their survival. Reactive oxygen radicals have been suggested to play important roles in controlling T cell apoptosis in mice (Hildeman, et al., 2003b; Hildeman, et al., 1999) but it is questionable, whether anti-oxidants or related compounds could modulate human immune responses. It is also unknown whether ACAD is dependent on TCR stimulation in humans.

Knowledge of the control of peripheral immune responses in adenotonsillar tissue may reveal important therapeutic windows, which might be used in the treatment of various hyperinflammatory disorders of the upper respiratory tract, including chronic otitis media with effusion, chronic sinusitis and polyposis, and not to mention respiratory allergy, including asthma.

3. AIMS OF THE PRESENT STUDY

The purpose of this study was to search for mechanisms that control immune responses in adenotonsillar tissue by evaluating the characteristics of the adenoidal epithelial crypt, a potential route of antigen entry into the adenoids, as well as the signals that influence the survival of adenoidal CD4+ T cells.

The specific aims were:

1. To evaluate the mechanisms that may mediate leukocyte infiltration in the adenoidal epithelial crypt (I).

2. To understand the mechanisms involved in controlling the apoptosis of adenoidal naïve T cells (II).

3. To evaluate the mechanisms which control the survival of adenotonsillar memory phenotype T lymphocytes (III).

4. To analyze the role of reactive oxygen species and mitochondria in the apoptosis of human adenoidal memory phenotype T lymphocytes (IV).

4. MATERIALS AND METHODS

Detailed descriptions of the materials and methods are presented in the original articles I to IV.

4.1. Tissue specimens (I, II, III, IV)

Adenoids and tonsils were obtained from children, aged 1 to 4 years, who underwent adenoidectomy or tonsillectomy at Helsinki University Central Hospital because of infections or hyperplasia. Peripheral blood was obtained from healthy adults aged 20 to 40 years or from some children who underwent adenoidectomy. This study was evaluated and approved by the ethical review committee of the Helsinki University Central Hospital.

4.2. Immunohistochemistry (I)

The frozen adenoidal tissue sections (thickness 5 μm) were stained with mouse monoclonal antibodies. The bound antibody was detected using the Vectastain ABC peroxidase mouse IgG Kit (Vector Laboratories, Burlingame, CA, USA). Formalin-fixed, paraffin-embedded tissue sections (thickness 5 μm) were deparaffinized and stained with mouse monoclonal antibodies. The bound antibody was visualized with the Envision staining kit (DAKO, Glostrup, Denmark), using a peroxidase-conjugated secondary antibody and diaminobenzidine as the chromogenic substrate. The following mouse monoclonal antibodies were used for the stainings: anti-CD3 (clone PC3/188A; DAKO), anti-CD20 (clone L26; DAKO), anti-PECAM-1 (clone JC/70A; DAKO), anti-VCAM-1 (clone 1.4C3; DAKO), anti-ICAM-1 (clone 6.5B5; DAKO), anti-cytokeratin 5/6 (clone D5/16 B4; DAKO), anti-cytokeratin 8 (clone CAM 5.2; BD Biosciences, San Jose, CA, USA), and anti-pan-cytokeratin (clone AE1/AE3; DAKO).

4.3. Immunofluorescence (I)

The frozen tissue sections (thickness 5 μm) were first stained with mouse monoclonal antibodies against PECAM-1 or VCAM-1 and with rabbit polyclonal anti-keratin antibody (cat. no. A0575, DAKO). For the double immunofluorescence detection, the tissue sections were stained thereafter with FITC-conjugated (FITC, fluorescein isothiocyanate), affinity purified donkey anti-rabbit IgG (711-095-132, 1.5 mg/ml, dilution 1/300, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) as well as with TRITC-conjugated (TRITC, tetramethylrhodamine isothiocyanate) affinity purified goat anti-mouse IgG (115-025-100, 1.3 mg/ml, dilution 1/200, Jackson ImmunoResearch Laboratories, Inc.).

4.4. Enrichment of the adenoidal epithelial cells (I)

Adenoidal tissue samples were mechanically homogenized using a Medimachine tissue disaggregation machine (DAKO), and filtered through a 50 μm pore-size filter (DAKO) to obtain a single cell suspension. After washing with PBS, the tissue homogenates were incubated 30 minutes in 10% human AB blood group serum (Finnish Red Cross, Helsinki, Finland) in RPMI-1640 medium (containing 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin; Sigma, St. Louis, MO, USA) in order to block the unspecific Fc-receptor binding. Homogenates were thereafter washed again with PBS, and suspended in separation buffer (0.5% human serum albumin, 2 mM EDTA in PBS, pH 7.4) at a density of 10 x 10^6 cells in 100 μl, and stained by adding 15 μl of magnetically labeled antibody against Ep-CAM (HEA Microbeads, Miltenyi Biotec, Bergisch Gladbach, Germany) per 10 x 10^6 cells. Subsequently, the cell suspension was incubated for 30 minutes at +6°C to +8°C, washed once with ice-cold separation buffer, and suspended in 1 ml of separation buffer. The cell suspension was then eluted through magnetized LS columns (Miltenyi Biotec) and the cells that were bound into the column were collected as enriched epithelial cells.

4.5. Purification of CD45RA+ CD4+ and CD45R0+ CD4+ T lymphocyte populations (II, III, IV)

Adenotonsillar tissue specimens were homogenized and filtered through a 50 μm pore-size filter as described above. Mononuclear cells were purified from tissue homogenates as well as from peripheral blood by Ficoll density gradient centrifugation (Ficoll-Paque, Amersham Biosciences Ab, Uppsala, Sweden), washed twice with PBS and suspended in culture medium (RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin; Sigma).

Isolation of naïve phenotype CD45RA+ CD4+ T cells and memory phenotype CD45R0+ CD4+ T cells was carried out in two steps using antibodies conjugated with magnetic beads followed by purification with magnetic column (LS separation columns and Midi MACS magnet, Miltenyi Biotec, Bergisch Gladbach, Germany). CD4+ cells were first purified through positive selection after which other cells than T cells and either CD45R0+ cells or CD45RA+ cells were depleted in order to purify CD45RA+ CD4+ T cells or CD45R0+ CD4+ T cells, respectively (See 4.6. for purity). In brief, cells were suspended in separation buffer (0.5% human serum albumin, 2 mM EDTA in PBS, pH 7.4) at 10 million cells in 80 μl and stained by adding 15 μl of magnetically labeled anti-CD4 antibody (CD4 MultiSort MicroBeads, Miltenyi Biotec) per 10 million cells. The suspension was incubated at +6 to 8°C for 15 min, washed once with ice-cold separation buffer, suspended in 1 ml of separation buffer, and eluted through magnetized LS separation column. The column was washed three times with 3 ml of the separation buffer, removed from the magnet, and the cells attached into column were eluted with 5 ml of ice-cold separation buffer. Magnetic particles were removed enzymatically by incubating the cell suspension with 150 μl Release Reagent (Miltenyi Biotec) for 15 min at +6 to 8°C. After washing, the cells were suspended in separation buffer (50 μl per 10

million cells) and 30 µl of Stop Reagent (Miltenyi Biotech) was added per 10 million cells in order to stop the reaction for releasing magnetic particles. The suspension was then incubated for 15 min at +6 to 8°C, and washed once with separation buffer.

Depletion of non-T cells from the purified CD4+ cell population was performed using hapten-conjugated anti-CD11b, anti-CD16, anti-CD19, anti-CD36, and anti-CD56 antibodies (Pan T cell isolation kit, Miltenyi Biotec). Briefly, the purified CD4+ cells were suspended in separation buffer (80 µl per 10 million cells) and 15 µl of hapten-conjugated antibodies were added per 10 million cells. The cells were incubated for 15 minutes at +6 to 8°C, washed twice, suspended in separation buffer (80 µl per 10 million cells), and 15 µl of Anti-Hapten MicroBeads (Miltenyi Biotech) were added per 10 million cells. To further deplete possible remaining non-T cells, 15 µl of magnetically labeled anti-CD19, anti-CD33, anti-CD14, anti-CD1a and anti-CD8 antibodies (Miltenyi MicroBeads, Miltenyi Biotec) per 10 million cells were added. To purify either CD45RA+ CD4+ T cells or CD45R0+ CD4+ T cells, 15 µl of magnetically labeled anti-CD45R0 or anti-CD45RA antibodies (Miltenyi MicroBeads, Miltenyi Biotec) were added per 10 million cells, respectively. The suspension was then incubated for 20 minutes at +6 to 8°C, washed once, suspended in 1 ml of separation buffer, and eluted through magnetized LS column. The column was washed 3 times and the unbound cells were collected. In addition to this two-step purification, CD45RA+ CD4+ and CD45R0+ CD4+ T cells were also purified without the anti-CD4 antibody by depleting non-CD4 cells using Pan T cell kit (Miltenyi Biotec) together with CD19, CD33, CD14, CD1a, and CD8 MicroBeads (Miltenyi Biotec), and either CD45R0 or CD45RA MicroBeads (Miltenyi Biotec) as described above. All tissue specimens were prepared on ice immediately after the surgery.

4.6. Flow cytometric analysis of cell surface antigens (I, II, III, IV)

Surface marker staining for flow cytometry was performed by incubating the cells with the antibodies for 20 minutes at +4°C. Subsequently, the cells were washed with PBS and fixed with 4% paraformaldehyde in PBS. When needed, the cells were treated with 10% human AB blood group serum in RPMI 1640 medium in order to inhibit unspecific Fc-receptor binding before the antibody staining. Flow cytometric analysis of the cell surface antigens was performed by BD FACSCalibur flow cytometer (BD Biosciences).

The enriched epithelial cells were stained with FITC-conjugated anti-EpCAM antibody, a surface marker specific for epithelial cells (HEA-FITC, Miltenyi Biotec), CyChrome-conjugated anti-CD45 antibody (clone H130, BD Biosciences), and either with PE-conjugated anti-PECAM-1 antibody (anti-CD31, clone WM-59; BD Biosciences) or PE-conjugated IgG1κ immunoglobulin isotype control (BD Biosciences).

The following antibodies were used in order to evaluate the purity of the magnetically purified CD45RA+ CD4+ and CD45R0+ CD4+ T cells fractions: PerCP-conjugated (PerCp, peridinin chlorophyll protein) anti-CD4 (clone SK3, BD Biosciences, San Jose, CA, USA), FITC-conjugated anti-CD3 (clone SK7, BD Biosciences), and PE-conjugated

(PE, R-Phycoerythrin) anti-CD45RA (clone HI100, BD Biosciences), or PE-anti-CD45R0 (clone UCHL1, BD Biosciences), respectively. The cells in the purified CD45RA+ CD4+ or CD45R0+ CD4+ T cell population were generally 99% positive for both CD3 and CD4 and more than 90% positive for CD45RA or CD45R0. An example of the purity of one purified CD45R0+ CD4+ T cell population is given in publication III (III, Fig. 8 A).

The expression of different activation antigens on the cell surface was investigated by using the following antibodies: PE-anti-CD69 (clone FN50, BD Biosciences), PE-anti-HLA-DR (clone G46-6, BD Biosciences), PE-anti-CD71 (clone YDJ.1.2.2., Immunotech, Marseille, France), PE-anti-CD38 (clone HIT2, BD Biosciences), FITC-anti-CD45R0 (clone UCHL1, BD Biosciences), PerCP-anti-CD4 (clone SK3, BD Biosciences) or CyChrome-anti-CD4 (clone RPA-T4, BD Biosciences). The expression of different cytokine receptors on the surface of the adenoidal CD45R0+ CD4+ T cells was studied by using the following antibodies: APC-conjugated (APC, Allophycocyanin) anti-CD25 (clone M-A251, BD Biosciences), biotin-conjugated anti-CD122 (clone MIK-β3, BD Biosciences), PE-anti-CD126 (clone M5, BD Biosciences), and PE-anti-CXCL12 receptor antibody (clone 12G5, BD Biosciences). Streptavidin-PerCP (BD Biosciences) was used as a second step reagent with biotin.

4.7. In vitro treatment of cells (II, III, IV)

Cells were incubated at a density of 5 x 10^5 cells/ml in culture medium (RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin 100 U/ml, and streptomycin 100 μg/ml, Sigma) at +37°C in humidified 5% CO_2 atmosphere in 96-well plates (Costar, Cambridge, MA, USA) in a volume of 0.2 ml or in 24-well plates (Costar) in a volume of 0.5 ml. To stimulate cells through TCR, the plates were coated overnight with 0.1 μg/ml of anti-CD3 (clone UCHT1, R&D Systems, Minneapolis, MA, USA) accompanied with 5 μg/ml of anti-CD28 (clone 37407.111, R&D Systems) in PBS or with 0.1 μg/ml to 10 μg/ml of anti-CD3 alone in PBS. The coating antibody solution was then removed by suction, after which the cells suspended in culture medium were added into the coated wells. To interfere with the Fas-receptor signaling, recombinant human Fas - Fc chimera (TNFRSF6, R&D Systems, Minneapolis, MA, USA) or Fas ligand antibody (clone NOK1, R&D Systems) was added in culture media at 1 μg/ml and 5 μg/ml, respectively. The following human recombinant cytokines were used in order to study the effect of the different cytokines: interleukin 2 (R&D Systems) at 7.5 ng/ml, interleukin 15 (R&D Systems) at 25 ng/ml, interleukin 7 (R&D Systems) at 7.5 ng/ml, interleukin 6 (R&D Systems) at 10 ng/ml, and CXCL12/SDF-1a (R&D Systems) at 90 ng/ml. The pan-caspase inhibitor ZVAD-fmk was used at 100 μM or 50 μM and the caspase 3 selective inhibitor ZDEVD-fmk was used at 100 μM (Enzyme Systems Products, Livermore, CA, USA). Wortmannin (Alexis Biochemicals, Carlsbad, CA, USA) and SH-6 (Alexis Biochemicals) were used at 1 μM and 10 μM, respectively.

To study the role of superoxide anions in apoptosis, the cells were treated with the following drugs: superoxide dismutase mimetic, manganese(III) 5,10,15,20-tetra(4-pyridyl)-21*H*,23*H*-porphine chloride tetrakis(methochloride) (MnTPCl; Sigma) was used at 200 μM, N-acetyl-L-cysteine (NAC; Sigma) was used at 10 mM, and N^G-Methyl-L-arginine acetate salt (Arginine; Sigma) was used at 100 μM. The following mitochondrial uncouplers influencing the mitochondrial oxidative phosphorylation were used to affect the coupling between substrate oxidation and ATP synthesis: oligomycin (Sigma) was used at 1.25 μM, antimycin (Sigma) was used at 3 μM, and sodium azide (Merck, Rahway, NJ, USA) was used at 2 mM. The pan-caspase inhibitor ZVAD-fmk (BD Biosciences) was used at 50 μM. The protein synthesis inhibitor cycloheximide (CHX; Sigma) was used at 350 nM. The pH value of the culture medium was adjusted to 7.4 when needed.

4.8. Assays for apoptosis

4.8.1. Measurement of caspase-3 activity (III, IV)

Caspase 3 activity was measured with time-resolved fluorometry by using LANCE Caspase-3 kit (Wallac, Turku, Finland). Briefly, 1 x 10^5 cells per sample were lysed on ice for 15 min in DELFIA Lysis Buffer (Wallac) diluted 1/10 in Caspase Reaction Buffer (Wallac) with 10 mM dithiothreitol in a total volume of 50 μl. To measure caspase-3 activity, 5 μl of the cell lysate from the each sample was incubated in a total volume of 20 μl of Caspase Reaction Buffer containing 200 nM of caspase-3 substrate and 10 mM dithiothreitol for 90 minutes at +37°C in black 384 plates (Wallac). Fluorescence emitted by the released Europium was measured with VICTOR^2 fluorometer (Wallac).

4.8.2. Detection of plasma membrane phosphatidyl-serine translocation (II, III, IV)

Phosphatidyl-serine translocation from the inner side of the plasma membrane to the cell surface during apoptosis (Fadok, et al., 1992) can be detected using Annexin-V, which is a calcium dependent phospholipid-binding protein with high affinity for phosphatidyl-serine (Vermes, et al., 1995). Plasma membrane phosphatidyl-serine translocation was assessed using Annexin-V-Fluos Staining kit (Roche Molecular Biochemicals, Mannheim, Germany). The cells were first washed with PBS, after which a mixture containing annexin V-FITC and propidium iodide in HEPES-buffer was added. The suspension was incubated 15 minutes at RT after which 250 μl of HEPES-buffer was added, and the samples were analyzed with BD FACS Calibur flow cytometer.

4.8.3. Measurement of DNA degradation (III, IV)

Apoptotic DNA degradation was determined with propidium iodide staining of permeabilized cells (Krishan, 1975; Nicoletti, et al., 1991). The cells were first washed, suspended in PBS, and fixed with an equal volume of methanol at -20°C overnight.

Subsequently, the cells were washed and incubated in 200 IU/ml RNAse A (Sigma) in PBS for 30 minutes at +37°C. Finally, the cells were incubated overnight in 50 μg/ml propidium iodide solution (Sigma) in PBS at +4°C after which the cellular DNA content was determined by FACSCalibur Flow Cytometer (BD Biosciences). Cells in the Sub-G0 area of the cell cycle were counted as cells with apoptotic DNA degradation.

4.8.4. Measurement of the formation of DNA strand breaks (III, IV) and the generation of oligosomal DNA fragments (IV)

The formation of DNA strand breaks during apoptosis was assessed by terminal deoxynucleotidyl transferase FITC-dUTP nick end labeling (TUNEL, In Situ Cell Death Detection Kit, Roche) according to manufacturer's instructions. The cells having fragmentated DNA were detected with flow cytometry using FACSCalibur Flow Cytometer (BD Biosciences) as well as with fluorescence microscopy by using Zeiss Axioplan imaging system (Carl Zeiss Vision GmbH, München-Hallbergmoos, Germany).

The generation of oligosomal DNA framents was detected by agarose gel electrophoresis using the Apoptotic DNA ladder kit (Roche Molecular Biochemicals). DNA samples (1.6 μg / lane), mixed with gel loading buffer (Blue Juice Gel Loading buffer, Invitrogen, Carlsbad, CA, USA), were resolved in a 1.2% Tris Borate EDTA (TBE) agarose gel. Oligosomal DNA fragments were then visualized with UV light using ChemiDoc XRS gel documentation unit (BioRad, Hercules, CA, USA) after ethidium bromide staining.

4.8.5. Analysis of intracellular superoxide, and mitochondrial membrane potential ($\Delta\psi_m$) (IV)

In order to determine the levels of intracellular superoxide anions, the cells were stained with 3 μM dihydroethidium (Molecular Probes, Leiden, The Netherlands) for 40 min at +37°C in culture medium. Dihydroethidium is oxidized to fluorescent ethidium in the presence of superoxide anions (Castilho, et al., 1999). Fluorescence was measured with FACS Calibur flow cytometer with a 585/42 nm FL2 bandpass filter.

For the evaluation of the mitochondrial membrane potential ($\Delta\psi_m$), cells were treated with 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) (Molecular Probes). $DiOC_6(3)$ is a fluorochrome which incorporates into cells depending upon their $\Delta\psi_m$ (Petit, et al., 1990; Zamzami, et al., 1995). Unwashed cells were incubated in culture medium with 25 nM $DiOC_6(3)$ for 40 min at +37°C, after which fluorescence was measured with FACS Calibur flow cytometer with a 530/30 nm FL1 bandpass filter.

4.8.6. Electron microscopy (IV)

In order to study the cellular morphology with electron microscopy, the cells were fixed with 2% glutaraldehyde in 100 mM sodium cacodylate, pH 7.4, for 1 hour at RT. Fixative

with two-fold strength was carefully added into the culture medium in order to avoid any damage to the cells due to fixation. After fixation the cells were gently washed with 100 mM sodium cacodylate and processed for transmission electron microscopy as previously described (Yu, et al., 2003). After sectioning and staining with uranyl acetate and lead citrate the cells were analyzed with transmission electron microscope (JEOL 1200 EX II, Jeol Ltd., Tokyo, Japan).

4.9. Quantitative real time PCR (III)

Cells were lysed in Nucleic Acid Purification Lysis Solution (Applied Biosystems, Branchburg, NJ, USA; 1 x 10^6 cells per 250 µl Lysis solution). RNA was extracted from the cell lysates with ABI Prism 6100 Nucleic Acid PrepStation (Applied Biosystems) according to the manufacturer's protocol. Reverse transcription of the RNA to cDNA was performed using 1.25 U/µl of MultiScribe Reverse Transcriptase (Applied Biosystems) in a reaction containing 5.5 mM Magnesium Chloride, 500 µM of each deoxyNTP, 2.5 µM Random Hexamers and 0.4 U/µl Rnase Inhibitor in 1 x TaqMan RT Buffer. Reverse transcription reaction was performed with ABI Prism 7000 Sequence Detection System (Applied Biosystems) by first incubating the plate at 25°C for 10 min and then at 48°C for 30 min followed by inactivation at 95°C for 5 min.

Quantification of IL-2 mRNA expression was performed using Pre-developed TaqMan Assay reagents for IL-2 (Applied Biosystems, Assay ID 4309882P). The quantity of specific mRNA was adjusted by using ribosomal 18S mRNA as an endogenous control. The primers and probe for 18S mRNA were from Applied Biosystems (Assay ID 4319413 E). After synthesis of cDNA the amplification conditions were: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 sec at 95°C and 1 min at 60 °C using ABI Prism 7000 Sequence Detection System (Applied Biosystems) in a final volume of 25 µl in 1 x TaqMan Universal PCR Master Mix (Applied Biosystems). TaqMan probe contains a reporter dye FAM or VIC at the 5'-end of the probe and a quencher dye TAMRA or MGB (MGB, minor groove binder) at the 3'-end of the probe. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence by Förster-type energy transfer (Förster, 1948). During the reaction, if the target of interest is present, the 5' → 3' exonuclease activity of the AmpliTaq Gold DNA polymerase leads to the release of the reporter from the quencher dye and results in increased reporter fluorescence. Fluorescence intensity is thus proportional to the amount of specific PCR product generated (Heid, et al., 1996).

4.10. Statistical analyses (II, III, IV)

The Student's *t*-test was performed using StatView software (SAS Institute Inc., Cary, NC, USA).

5. RESULTS AND DISCUSSION

5.1. PECAM-1 is expressed in adenoidal crypt epithelial cells (I)

Structure of the adenoidal tissue resembled the structure of the lymph node (I, Fig. 1). The B cell rich area in the adenoids was immediately below the adenoidal epithelial surface whereas in the lymph node the B-cell rich area is located immediately below the capsule of the lymph node. The T-cell rich area in both of these tissues is located deeper under the B cell rich area (Lewis and Harriman, 2001). Furthermore, the adenoidal epithelial interfollicular crypts, as stained with a pan-cytokeratin antibody, were located between the lymphoid follicles (I, Fig. 1 A & Fig. 2 A). This location resembles the location of afferent lymphoid vessels that transport antigens and leukocytes to lymph nodes (Lewis and Harriman, 2001). The structure of the adenoidal epithelial crypt was characteristic of simple epithelia (Reibel and Sorensen, 1991) as the basal layer of the crypt was positive for cytokeratin 5 and 6 (I, Fig. 2 B) whereas the apical layer was found to be positive for cytokeratin 8 (I, Fig. 2 C). Especially the base of the adenoidal epithelial crypt was infiltrated with frequent leukocytes (I, Fig. 2), including T and B cells (unpublished observations), which is consistent with previous findings that show the leukocyte infiltration in the epithelial crypt (Koshi, et al., 2001; Ruco, et al., 1995).

The expression of different endothelial cell adhesion markers was studied in order to reveal potential mechanisms that mediate infiltration of leukocytes into the adenoidal epithelial crypt. Cellular adhesion molecules PECAM-1 (CD31; platelet endothelial cell adhesion molecule-1), VCAM-1 (CD106; vascular cell adhesion molecule-1), and ICAM-1 (CD54; intercellular adhesion molecule-1) play a major role in the migration of blood leukocytes through vascular endothelium of blood vessels (Bevilacqua, 1993; Luscinskas, et al., 2002; Wiedle, et al., 2001). Epithelial cells in the base of the epithelial crypt were found to express PECAM-1 (I, Fig. 3 A & Fig. 4). PECAM-1 expression was further confirmed in epithelial cells by enriching epithelial cells using magnetically labelled antibody against Ep-CAM and then analyzing the enriched cell population with flow cytometry. Simultaneous staining with the epithelial marker Ep-CAM (Balzar, et al., 1999), leukocyte marker CD45, and PECAM-1 clearly showed that it was indeed the epithelial cell fraction that contained the cells expressing PECAM-1 (I, Fig. 5). PECAM-1 has a function in transendothelial migration of lymphocytes from the vascular lumen into tissue in the vascular endothelium (Newman, 1997) suggesting that its expression in adenoidal crypt epithelial cells may have a role in the migration of leukocytes into the epithelial crypt, which is extensively infiltrated with leukocytes (Ruco, et al., 1995). Of interest is also that PECAM-1 expression has previously been found in epithelial cells of the thymus (Tenca, et al., 2003).

Another cell adhesion molecule, VCAM-1, was mainly found at the orifice but not at the base of the adenoidal epithelial crypt (I, Fig. 3 B & Fig. 4). VCAM-1 expression has previously been found in epithelial cells of the tonsil (Ruco, et al., 1995) as well as

bronchi (Atsuta, et al., 1997) and its expression may be regulated by the surrounding microenvironment, such as viruses (Papi and Johnston, 1999). The expression of ICAM-1 was found in isolated cells throughout the epithelial crypt (I, Fig 3 C). ICAM-1 has a function as a co-receptor for rhinoviruses and it has been also previously found in adenotonsillar epithelium (Winther, et al., 1997). Notably, PECAM-1 and VCAM-1 have different ligands that mediate leukocyte binding. PECAM-1 binds with homotypic interactions to itself, to CD38, and to αvβ3 integrin, whereas VCAM-1 binds to α4β1 and α4β7 integrins (Bevilacqua, 1993; DeLisser, et al., 1994; Fawcett, et al., 1995; Piali, et al., 1995; Deaglio, et al., 1998). Thus, the expression of PECAM-1 in the base of the epithelial crypt and the expression of VCAM-1 in the opening of the crypt suggest that these different compartments of the crypt may be distinct in their ability to recruit leukocytes.

5.2. Adenoidal CD4+ T cells express activation markers (II, III)

Adenotonsillar tissue is located at the point of entry of inhaled and swallowed antigens in the pharynx. Thereby, adenotonsillar lymphocytes are presumably constantly exposed to foreign antigens. As these antigens may induce immune responses, we wanted to study the activation status of adenotonsillar T lymphocytes. It was found that adenoidal naïve phenotype T cells express higher levels of the activation marker CD69 as compared to peripheral blood naïve phenotype T cells (II, Fig. 1). Adenoidal memory phenotype T cells were found to express high levels of activation markers CD69, CD71, HLA-DR, and CD38 whereas most of the peripheral blood memory phenotype T cells showed only very low expression for each of these markers (III, Fig. 1). Differencies in the expression of the activation marker CD69 on the surface of adenoidal and peripheral blood memory phenotype CD45R0+ CD4+ T cells and naïve phenotype CD45R0- CD4+ T cells is shown in Figure 4. The expression of the activation markers on the surface of the adenoidal cells suggests that the cells in adenoidal tissue may be exposed to foreign antigens *in vivo* and that the microenvironment inside the tissue may be able to maintain the activation phenotype. As the cells are thus presumably activated, they may also be sensitive to activation-induced apoptosis.

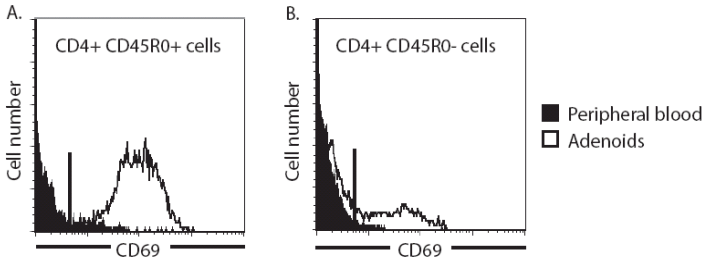

Figure 4. The expression of CD69 activation marker on the surface of adenoidal and peripheral blood memory phenotype CD45R0+ CD4+ and naïve phenotype CD45R0- CD4+ T lymphocytes. Adenoidal and peripheral blood T cells were stained with PerCp-conjugated anti-CD4 antibody, FITC-conjugated anti-CD45R0 antibody, and PE-conjugated anti-CD69 antibody. The histograms show the expression of the activation marker CD69 on the cells that are CD4+ and either CD45R0+ (A: CD45R0+ CD4+ T cells; T cell population with a memory phenotype) or CD45R0- (B: CD45R0- CD4+ T cells; T cell population with a naïve phenotype). Approximately 85% of the adenoidal CD45R0+ CD4+ T cells (A: white) and 17% of the adenoidal CD45R0- CD4+ T cells (B: white) were positive for CD69, whereas only 1% of the peripheral blood CD45R0+ CD4+ T cells (A: black) and CD45R0- CD4+ T cells (B: black) were positive for this marker.

5.3. Adenoidal naïve phenotype CD45RA+ CD4+ T cells are sensitive to Fas-mediated apoptosis upon contact with high concentration of anti-CD3 antibody (AICD) (II)

When stimulated with a high concentration of immobilized anti-CD3 antibody, which mimics a contact with a high antigen dose, adenoidal CD45RA+ CD4+ T cells, but not peripheral blood CD45RA+ CD4+ T cells, were susceptible to apoptosis (II, Fig. 2). The apoptosis of CD45RA+ CD4+ T cells was triggered only by a high concentration of immobilized anti-CD3 antibody (10 μg/ml) whereas lower concentrations did not have significant effects on apoptosis (II, Fig. 4). The apoptosis, induced by a high concentration of anti-CD3 antibody, could be inhibited by interfering with the Fas-receptor death pathway with rhFas or anti-FasL antibody (II, Fig. 5). However, the effect was only partial. This suggests that the interaction between Fas and FasL could be interfered only partially. There may also be other previously unknown mechanisms besides the Fas-pathway that contribute to the apoptosis in adenoidal CD45RA+ CD4+ T cells upon stimulation with high concentration of CD3 antibody. The apoptosis of adenoidal CD45RA+ CD4+ T cells thus resembled the TCR triggered and Fas-mediated AICD-type apoptosis, which has previously been observed in mice (Brunner, et al., 1995; Dhein, et al., 1995; Ju, et al., 1995). As the adenotonsillar tissue is located in the pharynx, in the close proximity to pharyngeal lumen, it is constantly exposed to high concentrations of antigens. It can be hypothesized that this exposure to high concentrations of antigens may induce AICD in adenotonsillar CD45RA+ CD4+ T cells, a feature of adenotonsillar tissue that has not been recognized previously. The size of the adenotonsillar tissue declines after childhood (Vogler, et al., 2000), implying that it may be important especially early in life in the development of the immune unresponsiveness

against antigens that have not succeeded to induce effective central immune tolerance during the thymic selection. Thus, high concentrations of antigens, such as various different inhaled airborne antigens or swallowed nutrients may induce peripheral immune tolerance by selectively eliminating naïve CD45RA+ CD4+ T cells in human adenotonsillar tissue.

CD45RA+ CD4+ T cells were purified using anti-CD4 antibodies conjugated with magnetic beads and by subsequently depleting non-T cells as well as CD45R0+ T cells. This purification strategy may result in stimulation of cells by the anti-CD4 antibody. We thus evaluated whether stimulation through CD4 could influence the effect of CD3 stimulation. The cells were purified with anti-CD4 antibody (positive enrichment) and also without anti-CD4 antibody (depletion) by depleting all other cells except CD4+ CD45RA+ T cells. It was found that the anti-CD4 antibody did not have any significant effect on the induction of apoptosis (II, Fig. 3).

5.4. Adenoidal memory phenotype CD45R0+ CD4+ T cells are sensitive to Fas- and caspase-independent apoptosis that can be inhibited by different cytokines (ACAD) (III, IV)

Unlike adenoidal naïve phenotype CD45RA+ CD4+ T cells or peripheral blood naïve or memory phenotype T cells, adenoidal memory phenotype CD45R0+ CD4+ T cells were sensitive to spontaneous apoptosis when incubated *in vitro* as measured with phosphatidyl-serine translocation (III, Fig. 2 A & C and IV, Fig. 1 C & D) as well as with caspase-3 activation (III, Fig 3). This spontaneous apoptosis of adenoidal CD45R0+ CD4+ T cells was also associated with DNA degradation (III, Fig 4 and IV Fig. 1 A & B) and the formation of DNA strand breaks (III, Fig. 5) as well as the loss of mitochondrial membrane potential (IV, Fig. 2 A & B), and the generation of reactive oxygen radicals (IV, Fig. 2 C & D). Characteristic morphological features of apoptosis, such as condensation of chromatin and sickle shaped nuclear morphology could be observed with electron microscopy after 4 hours of culture (IV, Fig. 8). Different apoptotic features observed after 4 hours of incubation *in vitro* in adenoidal CD45R0+ CD4+ T cells are illustrated in Figure 5.

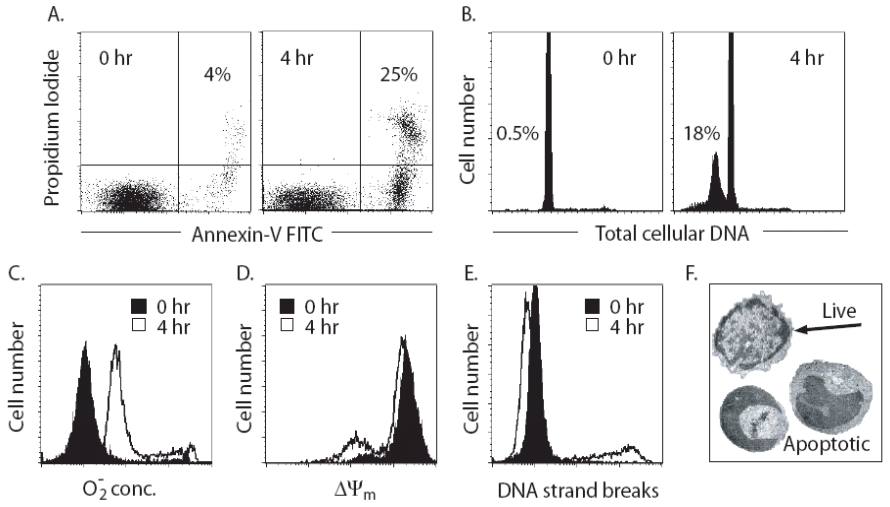

Figure 5. Characteristic features of the spontaneous growth factor deprivation-induced apoptosis of adenoidal CD45R0+ CD4+ T cells: A) phosphatidyl-serine translocation (III, IV), B) DNA degradation (III, IV), C) formation of superoxide anions (IV), D) loss mitochondrial membrane potential ($\Delta\psi_m$) (IV), E) DNA strand break formation (TUNEL) (III, IV), and F) chromatin condensation and changes in nuclear morphology (IV). Plasma membrane phosphatidyl serine translocation could be detected with Annexin-V staining in 25% of the cells after 4 hours of incubation (A: right panel, 4 hr) whereas only 4% of the cells were Annexin-V positive before incubation (A: left panel, 0 hr). Before incubation 0.5% (B: left panel) and after 4 hours of incubation 18% (B: right panel) of the cells had degraded DNA as measured with propidium iodide staining of the permeabilized cells. After 4 hours of incubation 15% of the cells had high superoxide anion (O_2^-) concentration (C: white histogram) and 20% had low mitochondrial membrane potential ($\Delta\psi_m$) (D: white histogram) as detected by dihydroethidium and $DiOC_6(3)$ stainings, respectively. The formation of DNA strand breaks could be detected in 17% of the cells after 4 hours of incubation (E: white histogram) by terminal dUTP nick end labeling method (TUNEL). Changes in nuclear morphology, such as sickle-shaped nuclear morphology and condensation of chromatin (F) could be observed by transmission electron microscopy after 4 hours of incubation.

To evaluate whether the CD4 antibody used in the purification process influenced the apoptosis of the CD45R0+ CD4+ T cells, the cell populations were also purified without the anti-CD4 antibody by depleting all other cells except CD45R0+ CD4+ T cells. It was found that the anti-CD4 antibody used in the purification did not have any significant effect on the survival of adenoidal CD45R0+ CD4+ T cells (III, Fig. 2 B).

The spontaneous apoptosis of adenoidal CD45R0+ CD4+ T cells could not be inhibited by disrupting the Fas-FasL engagement (III, Fig. 6 A & B) implying that the apoptosis was independent on Fas-signaling. Even though the pan-caspase inhibitor ZVAD-fmk and the caspase-3 selective inhibitor ZDEVD-fmk completely inhibited caspase-3 activity, they failed to significantly inhibit apoptosis as measured with phosphatidyl-serine translocation (III, Fig. 6 A & C). The same phenomenon has been previously observed in human peripheral blood T cells after in vitro activation and IL-2 withdrawal (Ferraro-Peyret, et al., 2002). Thus, it can be proposed that caspases appear not to have a primary initiating role in apoptosis of human *in vivo* activated adenoidal memory

phenotype CD45R0+ CD4+ T cells. Stimulation through the T cell antigen receptor by anti-CD3 and anti-CD28 antibodies did not rescue cells from apoptosis (III, Fig. 6 A & D) nor did a high concentration of anti-CD3 antibody augment apoptosis (data not shown). However, the stimulation with anti-CD3 and anti-CD28 antibodies induced the transcription of IL-2 (III, Fig. 6 D), implying that the stimulation still resulted in the productive signal transduction through TCR. Our results are consistent with previous reports which show that primed, antigen-experienced memory T cells, are relatively resistant to Fas-mediated and TCR-induced AICD-type cell death as compared to naïve cells (Desbarats, et al., 1999; Inaba, et al., 1999).

Several external stimuli were found to provide survival signals for adenoidal memory phenotype CD45R0+ CD4+ T cells. The apoptosis of adenoidal memory phenotype CD45R0+ CD4+ T cells resembled ACAD type cell death as it could partly be inhibited by various cytokines, such as CXCL12/SDF-1, IL-2, IL-7, IL-15, and IL-6 (III, Fig. 7). IL-2, IL-7, and IL-15 bind the same cell surface receptors that share the common cytokine receptor γ chain (Fehniger and Caligiuri, 2001; Kondo, et al., 1993). The signaling through the common cytokine receptor γ chain has been found to promote cell survival and proliferation in different cell types (Akbar, et al., 1996; Nakajima, et al., 1997; Salmon, et al., 1994; Schluns and Lefrancois, 2003). IL-6 receptor, on the other hand, belongs to the gp130 receptor cytokine superfamily (Bravo and Heath, 2000; Taga and Kishimoto, 1997). The signaling through the IL-6 receptor has previously been reported to inhibit apoptosis in mouse splenic and lymph node CD4+ T cells (Teague, et al., 1997). Interestingly, it has also been reported that the blockade of the IL-6 signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation in human Crohn disease (Atreya, et al., 2000). It was found that almost all CD45R0+ CD4+ T cells expressed the receptor for IL-6 (CD126; III, Fig. 8 B). The majority of the CD45R0+ CD4+ T cells expressed CXCR4, the receptor for the chemokine CXCL12 (III, Fig 8 B), which is a member of the G protein-coupled receptor superfamily (Berger, et al., 1999). Interestingly, CXCL12, which has previously been shown to promote cell survival in different cell types upon serum deprivation (Suzuki, et al., 2001; Zhou, et al., 2002), was able to inhibit apoptosis in our study even in the presence of 10% serum (III, Fig. 7). IL-10, which has previously been reported to inhibit peripheral blood T cell apoptosis upon TCR and IL-2 stimulation (Cohen, et al., 1997), did not rescue T cells from apoptosis in our model (data not shown).

We found that the adenoidal memory phenotype CD45R0+ CD4+ T cell population cover a mixture of different sub-populations as defined by the expression of IL-2 receptor α chain (CD25) and CXCL12 receptor CXCR4 (III, Fig. 8 C). Moreover, our group has previously reported that distinct sub-population can be resolved in adenoidal CD45R0+ CD4+ T cell population based on the expression of CCR5 and CD62L (Mattila, et al., 2000). Thereby, it can be supposed that the anti-apoptotic cytokines that were found in our study may promote the survival of certain sub-populations in adenoidal CD45R0+ CD4+ T cell population based on their homing receptor or cytokine receptor expression pattern. This may further regulate immune responses by selectively supporting the survival of distinct T cell populations in certain cytokine environments. The combination of all of these cytokines had even more effective inhibitory effect on apoptosis (III, Fig.

7), suggesting that a single cytokine may promote survival only in a subpopulation of cells. Taken together, inflammation may maintain cell proliferation and survival through the presence of various cytokines in microenvironment, whereas at the end of immune responses when the inflammation is fading the cells may undergo ACAD-type cell death.

The extracellular cytokine signals are transmitted through their specific receptors on the cell surface into the nucleus via receptor-coupled signal transduction systems. Cytokine binding to the specific receptor usually trigger Janus kinases (JAKs) to phosphorylate and activate cytosolic STAT (signal transducers and activators of transcription) proteins, so called JAK-STAT pathway (Leonard and O'Shea, 1998). Other cytokine signaling pathways include the mitogen-activated protein kinase pathway (Yang, et al., 2003), interferon regulatory factors (Taniguchi, et al., 2001), as well as the NF-κB pathway (Denk, et al., 2000). The signal transduction events may also include the protein kinase Akt. The serine-threonine protein kinase encoded by Akt proto-oncogene can be activated by a variety of growth factors via signals transduced by the phosphatidylinositol 3-kinase (Datta, et al., 1996). We found in our work that phosphatidylinositol 3-kinase inhibitors Wortmannin and SH-6 (Kozikowski, et al., 2003) promoted apoptosis in adenoidal CD45R0+ CD4+ T cells (III). An activated form of Akt has been previously reported to promote cell survival by various mechanisms (Kandel and Hay, 1999; Vivanco and Sawyers, 2002). It can for example interfere with the activity of the Bcl-2 family members (Ahmed, et al., 1997; del Peso, et al., 1997). Akt can be activated by IL-2 (Kelly, et al., 2002; Van Parijs, et al., 1999), IL-6 (Laszlo and Nathanson, 2003), and CXCL12 (Suzuki, et al., 2001; Zhou, et al., 2002). Our data suggests that Akt can provide survival signals in adenoidal CD45R0+ CD4+ T cells.

5.5. Effects of the mitochondrial respiratory chain inhibitors, new protein synthesis, caspases, intracellular thiols, and nitric oxide on apoptosis of adenoidal CD45R0+ CD4+ T cells (IV)

The role of mitochondria in executing apoptosis of adenoidal CD45R0+ CD4+ T cells was studied by treating cells with drugs that inhibit the respiratory chain of mitochondria, namely, antimycin that inhibits electron transport at complex III (Tzung, et al., 2001; Zhuang, et al., 1998), sodium azide that arrests electron transport at complex IV (Wilhelm, et al., 1997), or oligomycin that inhibits ATP synthase (Tzung, et al., 2001; Zhuang, et al., 1998). It was found that all these drugs inhibited the cellular manifestations of apoptosis, such as DNA degradation, plasma membrane phosphatidyl-serine translocation, caspase-3 activation, as well as the generation of superoxide anions (IV, Fig. 3) implying the important role of mitochondria during apoptosis. The notion that these drugs also inhibited the formation of superoxide anions implies that the superoxides presumably play a role in the execution of apoptosis and that those likely originated from mitochondria.

It was found that the inhibition of protein synthesis with cycloheximide and the inhibition of caspase-3 activity with the pan-caspase inhibitor ZVAD-fmk partially inhibited apoptotic DNA degradation as well as the formation of single-strand DNA breaks during

apoptosis (IV, Fig. 6). New protein synthesis and caspase activity thus augmented DNA degradation and DNA strand break formation. As the caspase inhibitors inhibited DNA degradation it can be supposed that caspase-dependent endonucleases, such as caspase activated DNase (CAD) (Enari, et al., 1998; Nagata, et al., 2003) may have significant roles in the apoptotic DNA degradation of adenoidal memory CD45R0+ CD4+ T cells.

N-acetyl-L-cysteine (NAC), a thiol supplement that can help to replenish intracellular thiols (Malins, et al., 2002; Zamzami, et al., 1995), was used in order to assess the role of intracellular thiols in apoptosis. Intracellular thiols, of which glutathione is the most abundant, can promote the detoxification of reactive radicals by serving as an electron donor (Adler, et al., 1999) and thus protect cells against apoptosis. NAC has previously been shown to act as an inhibitor against trophic factor withdrawal mediated cell death (Mayer and Noble, 1994). In addition, NAC has been reported to increase the survival of HIV infected individuals and to increase glutathione levels in T cells suggesting that NAC administration may increase the survival of T cells in vivo (De Rosa, et al., 2000; Herzenberg, et al., 1997). Interestingly, it has also been reported that NAC can prevent the induction of Fas on human peripheral blood T cells and thus protect cells from Fas-mediated apoptosis (Delneste, et al., 1996). In our work, NAC had a small but noticeably inhibitory effect on apoptotic plasma membrane phosphatidyl-serine translocation and caspase-3 activation, but it did not appear to influence other apoptotic events and thus did not seem to inhibit apoptotic death per se (IV, Fig. 4).

Nitric oxide is an important messenger in diverse signal transduction processes (Lamattina, et al., 2003). Nitric oxide has been reported to have a role in apoptosis in endothelial cells and neurons (Davis, et al., 2001). In these cells superoxide anions can react with nitric oxide and produce peroxynitrite, which can induce apoptosis (Chung, et al., 2001; Lin, et al., 2004; Lipton, et al., 1993; Salgo, et al., 1995). In order to study the role of nitric oxide in the apoptosis of adenoidal CD45R0+ CD4+ T cells, the cells were treated with L-arginine that inhibits nitric oxide synthase (Estevez, et al., 1999). This treatment did not significantly influence apoptosis in our settings, suggesting that nitric oxide does not have an essential role in mediating apoptosis in adenoidal memory phenotype CD45R0+ CD4+ T cells (IV, Fig. 4). However, it has been reported that the *in vitro* culture of mouse lymphocytes in the absence of survival signals leads to the up-regulation of inducible nitric oxide synthase and that nitric oxide may play an important role in lymphocyte apoptosis (Sade and Sarin, 2004).

5.6. Reactive oxygen species mediate DNA degradation during apoptosis of adenoidal CD45R0+ CD4+ T cells (IV)

In order to study the role of reactive oxygen species during the apoptotic process, we used a synthetic superoxide dismutase mimetic MnTPCl, manganese(III) 5,10,15,20-tetra(4-pyridyl)-21*H*,23*H*-porphine chloride tetrakis(methochloride) to catalyze the inactivation of superoxide anions (Gauuan, et al., 2002). Superoxide dismutase mimetics are oxidoreductases that contain Mn, Cu, or Fe at the reactive site and are able to catalyze the dismutation of O_2^- to O_2 and hydrogen peroxide (H_2O_2). H_2O_2 is toxic to cells as it

can form toxic hydroxyl radicals and it can further be detoxified by glutathione peroxidase or catalase (see Figure 3) (Schriner, et al., 2005). SOD mimetics can therefore protect against a variety of oxidant-induced injuries (Day, et al., 1995) as they can also possess catalase activity (Day, et al., 1997).

It was found that the treatment of adenoidal memory phenotype CD45R0+ CD4+ T cells with the superoxide dismutase mimetic MnTPCl almost completely inhibited the generation of superoxide anions (IV, Fig. 4 A). Moreover, it also efficiently inhibited apoptotic DNA degradation as measured by the amount of total cellular DNA (IV, Fig. 4 B, Fig 5 A & Fig 7 A) and had a notable inhibitory effect on caspase-3 activation (IV, Fig. 4 D). It also decreased the formation of oligosomal DNA fragments during apoptosis as analyzed by agarose gel electrophoresis (IV, Fig. 5 B & C).

Interestingly, MnTPCl did not rescue the memory phenotype CD45R0+ CD4+ T cells from apoptosis as measured with plasma membrane phosphatidyl-serine translocation (IV, Fig. 4 C), and the inhibitory effect on the loss of mitochondrial membrane potential was only marginal (IV, Fig. 4 E). Our data indicate that superoxide anions can, however, directly mediate signals that lead to apoptotic DNA degradation in human CD4+ T cells although it did not influence the initiating events of apoptosis.

Although MnTPCl inhibited apoptotic DNA degradation almost completely, it did not have a strong effect on the apoptotic DNA fragmentation as measured by the generation of free 3' DNA strand breaks with TUNEL method even during a culture period of up to 24 hours (IV, Fig. 6 & Fig. 7 B). One possible explanation is that superoxide anions did not presumably activate DNA endonucleases, which drive the formation of simple DNA strand breaks. While detoxification of superoxide antions by MnTPCl almost completely inhibited the loss of nuclear DNA, superoxide anions may activate DNA exonucleases that degrade DNA after the initial formation of simple DNA strand breaks by endonucleases. It is possible, that reactive oxygen species mediate signals through for example NF-κB (Nakamura, et al., 1997), which may further promote the synthesis of new exonucleases.

As MnTPCl did not inhibit the apoptotic loss of mitochondrial membrane potential, it probably failed to inhibit the leakage of the mitochondrial apoptotic factors (Higuchi, 2003), such as AIF (Susin, et al., 1999) and endonucleases, like endonuclease G (van Loo, et al., 2001), which are involved in caspase-independent formation of simple DNA strand breaks. Furthermore, it was also found that the detoxification of superoxide anions with MnTPCl did not influence the characteristic morphological changes, including condensation of chromatin and sickle shaped nuclear morphology (Ziegler and Groscurth, 2004), during apoposis (IV, Fig. 8).

Our results contradict with the results that have been previously reported with in vivo activated mouse lymph node T cells as these cells could be rescued from apoptosis by eliminating the formation of ROS using MnTBAP (Hildeman, et al., 2003b; Hildeman, et al., 1999; Hildeman, et al., 2002; Hildeman, et al., 2002; Tripathi and Hildeman, 2004), an another synthetic superoxide dismutase mimetic. In these studies, it has been shown

that the detoxification of superoxide anions up-regulate the expression of anti-apoptotic Bcl-2 protein (Hildeman, et al., 2003a). Thus, it can be suggested that the different characteristics of the different synthetic superoxide dismutase mimetics may lead to apparently contrasting results (Gauuan et al., 2002). Also, it is presumable, that in the above studies the mouse lymph node cells, that were activated in vivo with superantigen injections, resulted in more vigorous activation status of these cells as compared to human in vivo activated adenoidal CD4+ T cells in our model, and therefore it can be suggested that in the mouse cells the reactive oxygen species more likely mediated the critical events leading to apoptosis. Yet another explanation is that the superoxide mimetic used by us, MnTPCl, failed to sufficiently inactivate the formation of hydrogen peroxide even though it inactivated the generation of superoxide anions. Thereby, hydrogen peroxide, which might have been eliminated sufficiently in the previous reports, may have caused the apoptotic cell death in our setting. Nevertheless, it seems plausible that the detoxification of reactive oxygen species is unable to prevent apoptotic death of activated CD4 T cells in all settings, such as in adenoidal memory phenotype CD45R0+ CD4+ T cells.

Interestingly, it has been reported that the overexpression of catalase in mitochondria in transgenic mice results in reduced generation of hydrogen peroxide accompanied with reduced oxidative DNA damage (Schriner, et al., 2005). More notably, these mice had an increased lifespan (Schriner, et al., 2005). Thus, although in our setting detoxification of superoxide anions could not rescue cells from growth factor withdrawal induced acute apoptosis, reactive oxygen species can have roles in promoting death in more chronic situations as in the prolonged process of aging.

6. SUMMARY AND CONCLUSIONS

Peripheral T cell homeostasis is accomplished by continuous balancing between proliferation and apoptosis. This is essential in establishing prompt immune responses but yet, at the same time, in avoiding hypersensitivity reactions, autoimmunity, as well as lymphoproliferative disorders. The anatomical location of adenotonsillar tissue in the pharynx suggests that it is constantly exposed to high doses of foreign antigens, such as various nutrients and inhaled airborne antigens, which can induce immune responses. As the size of adenotonsillar tissue declines after childhood and it is normally rudimentary in adults, it probably possesses its main biological functions early in life. The aim of this study was to search for mechanisms that control immune responses in adenotonsillar tissue by evaluating the possible mechanisms that mediate leukocyte infiltration into adenoidal epithelial crypt, which is a potential route of antigen entry into the adenoidal tissue from pharyngeal lumen, as well as the signals that influence the survival of adenoidal CD4+ T cells.

It was found that epithelial cells in the outer opening of the adenoidal crypt expressed vascular cell adhesion molecule, VCAM-1, whereas epithelial cells at the base of the crypt, in a region that is extensively infiltrated with leucocytes, expressed platelet endothelial cell adhesion molecule, PECAM-1. This suggests that the base and the orifice of the crypt may be distinct in their ability to recruit leukocytes as PECAM-1 and VCAM-1 bind to different ligands. As PECAM-1 has a function in transendothelial migration of lymphocytes in vascular endothelium, it is possible that PECAM-1 expression in adenoidal epithelial cells has a function in the migration of leukocytes into the epithelial crypt.

Adenotonsillar CD4+ T cells were found to be highly distinct from resting peripheral blood CD4+ T cells in that adenotonsillar CD4+ T cells expressed activation antigens and were susceptible to apoptosis. Adenotonsillar naïve phenotype CD45RA+ CD4+ T cells expressed activation marker CD69 whereas memory phenotype CD45R0+ CD4+ T cells, in addition to CD69, expressed also CD71, CD38, and HLA-DR. Apoptosis of adenoidal CD45RA+ CD4+ T cells could be induced with a high concentration of anti-TCR antibody, which mimics the high dose of antigen. This apoptosis was Fas-dependent and thus resembled AICD-type cell death. On the contrary, apoptosis of memory phenotype CD45R0+ CD4+T cells resembled the ACAD-type cell death as it could be inhibited by various cytokines and it was independent on Fas or TCR signaling. The proper function of the mitochondrial respiratory chain is disrupted during apoptosis. This results in the permeabilization of the mitochondrial membrane and in the formation of superoxide anions. The ACAD-type cell death of memory phenotype CD45R0+ CD4+ T cells could not be inhibited by the elimination of superoxide anions, which were formed during apoptosis. Even though the elimination of the superoxide anions by a synthetic superoxide dismutase mimetic MnTPCl did not inhibit apoptosis, it still inhibited DNA degradation during apoptosis. This suggests that superoxide anions are not mere toxic by-products that are formed during apoptosis, but can also have an active role in executing apoptotic events in adenoidal memory phenotype CD45R0+ CD4+ T cells.

To conclude, the control of CD4+ T cell responses in human adenotonsillar tissue may first be achieved by the deletion of those naïve CD45RA+ CD4+ T cells that are reactive to high concentrations of antigens, via TCR triggered and Fas mediated apoptosis. This may induce peripheral immune tolerance by selectively deleting naïve CD45RA+ CD4+ T cells that are reactive against antigens that are present in high concentrations. Such antigens may be for example swallowed nutrients as well as pollen or animal-derived dust in the inhaled air. After clonal expansion, the magnitude of the immune response can be fine-tuned by various cytokines that can inhibit the death of activated memory phenotype CD45R0+ T cells. Furthermore, it was found that the superoxide anions formed during apoptosis are not critical in inducing apoptosis of memory phenotype CD45R0+ T cells but have a function in apoptotic DNA degradation. A model suggesting how the fate of the CD4+ T cells is controlled in human adenotonsillar tissue is presented in Figure 6.

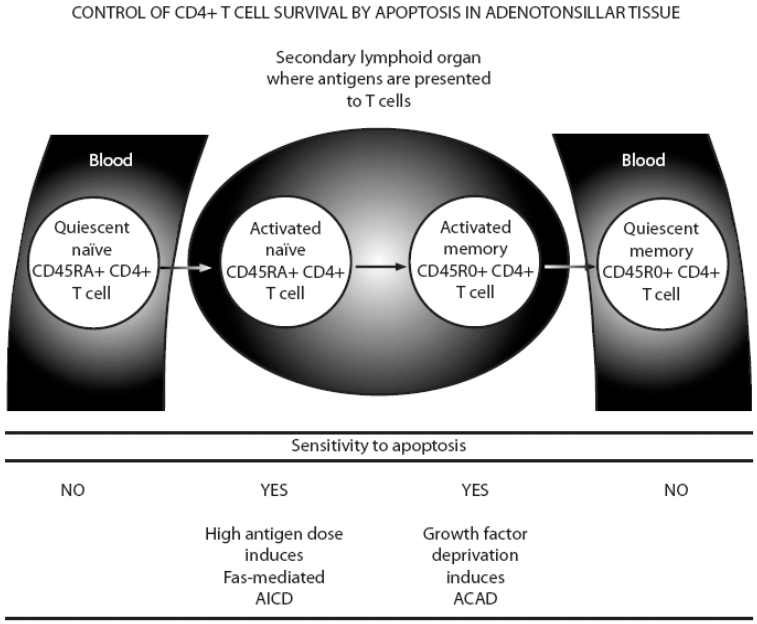

Figure 6: A model of the fate of CD4+ T cells in secondary lymphoid organs, such as in adenotonsillar tissue. Quiescent naïve CD45RA+ CD4+ T cells migrate to secondary lymphoid organs, such as adenotonsillar tissue, via blood. Foreign antigens are then presented to naïve T cells by antigen presenting cells within the secondary lymphoid organ. When the naïve T cell encounters high doses of antigenic peptide, it becomes activated and sensitive to Fas-mediated AICD. Later on, a successful contact with the antigenic peptide in association with a MHC molecule induces a change in the isoform usage of the cell surface CD45 from the naïve type CD45RA+ to the memory type of CD45R0+. After the antigenic challenge is attenuated, most of the activated memory phenotype CD45R0+ CD4+ T cells may undergo spontaneous, growth factor deprivation induced ACAD, which is not dependent on Fas-signaling. However, some of the activated memory T cells survive and return to the blood as quiescent memory CD45R0+ CD4+ T cells, which can respond to foreign antigens faster and establish the immunologic memory.

Our results elucidate some of the key aspects of immune responses in the upper respiratory tract. Detailed characterization of these responses may help to identify mechanisms involved in chronic inflammatory responses in a variety of upper respiratory tract diseases such as otitis media with effusion, chronic sinusitis, nasal polyposis, hypersensitivity reactions, allergy, as well as asthma. This kind of novel basic information may be essential for the future development of effective treatments for these and other inflammatory diseases.

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