Extrathymic T cell Receptor Gene Rearrangement in Human Alimentary Tract

by

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Cover:
The human RAG1 gene organization and the splice forms formed from the newly discovered 5'UTR 1A and 1B.

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Till Mina Föräldrar
Acti labores jucundi
(Uffört arbete är behagligt)
ABSTRACT

**T** lymphocytes regulate the initiation, duration, and magnitude of adaptive immune responses and function as effector cells in cell-mediated immunity. To become immunologically competent they must generate functional antigen receptors. This process takes place in the thymus and requires somatic recombination of T cell receptor (TCR) genes. It is mediated by the endonucleases recombination activating gene-1 (RAG1) and RAG2. Although the thymus regresses at puberty, T cells are present throughout life implying that other tissues must provide the proper milieu for T cell development. This thesis describes extrathymic T cell maturation in man. RAG1, RAG2, and the preTα-chain (pTα), which is exclusively utilized in developing T cells, were used as markers for TCR gene rearrangement. Two new exons (1A and 1B) encoding sequences in the 5' untranslated region (5'UTR) of mRNA were discovered in the human RAG1 gene. The previously described 5'UTR exon (renamed 1C) was located between the new exons and exon 2, the latter containing the entire coding sequence. We found that small intestinal lymphocytes of the T cell lineage expressed the new exons in three different splice forms. RAG1 mRNA containing the 1C exon was not expressed in small intestinal lymphocytes. In contrast, splice forms containing the 1A exon were not expressed in thymocytes. RAG1 and pTα mRNA expressing lymphocytes were seen both within the epithelium and in lamina propria. Thymocyte-like CD2+CD7+CD3−, CD4+CD8+, CD1α+, and IL7-R+ lymphocytes were identified in the small intestinal mucosa. CD2+CD7+CD3− cells had the highest expression levels of mRNA for RAG1 and pTα, suggesting that the small intestinal mucosa is indeed a site for T cell maturation. Small intestinal T lymphocytes were also shown to kill via the Fas/FasL pathway in a TCR/CD3 independent manner and via the perforin/granzyme pathway in a TCR/CD3 dependent manner. The Fas/FasL-mediated cytotoxicity may reflect an ongoing selection process of extrathymically matured T cells. The nasopharyngeal tonsil is the major inductive site for immune reactions against inhaled antigens. Previous demonstration of RAG1 expression in tonsillar B cells was interpreted as antigen driven receptor revision. The present study confirms the expression of RAG1 in B cells. We also found that RAG1, RAG2, and pTα mRNAs were expressed in lymphocytes of the T cell lineage. A small population of cells with the immature phenotype CD2+CD7+CD3− was demonstrated. This population had the highest expression levels of mRNA for RAG1, RAG2, pTα and terminal deoxynucleotidyl transferase. All four splice-forms of RAG1 mRNA were expressed. RAG1 and pTα mRNA expressing cells were mainly located in the proximity of the surface epithelium and in the outer rim of the follicles. These results suggest that the nasopharyngeal tonsil is a site where extrathymic T cell development and antigen driven TCR revision are occurring in parallel. Celiac disease (CD) is a small intestinal enteropathy characterized by permanent intolerance to gluten. Gluten reactive intestinal T cells are central in the pathogenesis and CD can be regarded as a failure to maintain tolerance to this food antigen. Expression of the RAG1 1A/2 splice form was significantly decreased in small intestinal T cell subsets of CD patients suggesting that impaired TCR gene rearrangement could contribute to failure of maintain tolerance in CD. Together, these findings show that both small intestinal and nasopharyngeal tonsillar lymphocytes of T cell lineage have the molecular machinery for antigen receptor rearrangement and that thymocyte-like lymphocytes are present in both tissues. Thus these organs are likely sites of T lymphocyte ontogeny as well as for secondary T cell receptor rearrangement in man.

Syftet med forskningsprojektet var att undersöka om T-celler kan utmognas utanför tymus hos människa och om så var fallet, karaktärisera den mognadsprocess som T-lymfocyter genomgår utanför tymus. Jag fann att tunntarm och den nasopharyngeala tonsillen, ”körteln bakom näsan”, är två organ där extratymsk T-cells utmognad sker hos människa. Att producera T-celler lokalt i tarmen kan vara ett sätt för immunsystemet att åstadkomma ett effektivt skydd samtidigt som immunreaktioner mot nyttiga komponenter, såsom födoämnen, undertrycks (oral tolerans). Som indikation på extratymsk T-cells utmognad användes uttryck av mRNA (budbärar RNA) för RAG1, RAG2 och preTα-kedjan. Två ej tidigare kända gensegment i genen för RAG1 identifierades. De används i tre olika mRNA ”splice”-former, dvs. redigerade kopian av DNA sekvensen i RAG1 genen. De tre nya ”splice”-formerna uttrycktes i både omogna och mogna T-lymfocyter i tunntarmen. De tre varianterna uttrycktes allt i tunntarmen men två av dem uttrycktes inte i tymus. Även mRNA för preTα-kedjan och RAG2 uttrycktes i T-lymfocyter i tunntarmen. Vidare hittades omogna T-lymfocyter vilka hade de högsta uttryckts nivåerna av mRNA för RAG1. Dessutom påvisades tidiga förstadiesceller i tunntarmen. När förekomsten av mekanismer viktiga för eliminering av självreaktiva T-celler i tymus undersöcktes i tarmen, återfanns dessa också hos T-lymfocyterna i detta organ. Sammantagna är
dessa resultat mycket starka indikationer för att det förekommer en lokal T-cells utmognad i tunntarmen i människa.

Det är tänkbart att avsaknad av/bristande extratymisk lokal T-cells utmognad i tarmen skulle kunna leda till oformåga att utveckla tolerans mot födoämnen. Gluten specifika T-lymfocyter i tarmen spelar en viktig roll i sjukdomsbilden vid celiaki (gluten intolerans). Den oönskade immuniteten mot gluten skulle kunna vara en konsekvens av en bristfällig T-cells utmognad eller sekundär omlagring av genen för T cells receptor. Vi har studerat uttrycket och mängden av de olika RAG1 mRNA varianterna i olika undergrupper av T-lymfocyter från tarmen hos celiakipatienter och kontroller. Preliminära resultat visar minskade mängder av en av RAG1 mRNA varianterna (1A/2) hos celiakipatienter i de tre T-cells typer som studerades.

Den nasopharyngeala tonsillen är i ständig kontakt med de partiklar som vi andas in och anses som det organ där immunreaktioner mot mikroorganismer som kommit in genom andningsvägarna startar. Våra resultat visar på uttryck av alla varianter av RAG1 mRNA samt också mRNA för RAG2 och preTα-kedjan i T-celler i tonsillen. Framför allt var uttrycket av dessa gener högst i omogna T-lymfocyter. Även uttrycket av ett enzym som är viktigt för att T-celler ska få en stor variabilitet i sin receptor, ”terminal deoxynucleotidyl transferase” (TdT), påvisades i de omogna T-celler. Dessa resultat tyder på en pågående T-cells utmognad i tonsillen parallellt med att T-celler som är aktiva i immunreaktioner mot främmande ämnen kan förändra sin antigen receptor (TCR).

För att det ska vara möjligt att jämföra hur mycket olika gener uttrycks, d.v.s. olika mRNA nivåer, måste mRNA nivåerna normaliseras. För normalisering använder man en annan gen som finns uttryckt i de flesta celler, s.k. ”housekeeping”-gen, och som inte varierar vid t.ex. olika cellstadier, aktivering eller experimentella förhållanden. I syfte att hitta den mest stabila genen för studier i T-lymfocyter valdes de tre mest använda ”housekeeping”-generna och variationen av dessa studerades vid T-cell aktivering. Våra resultat visade att 18S rRNA varierade minst jämfört med de övriga två och valdes därför som den gen som kunde användas för normalisering av uttryck av andra gener.

Sammanfattningsvis visar våra resultat att extratymisk omlagring av T-cell receptorns gener hos människa kan ske både i tunntarmen och i den nasopharyngeala tonsillen. Resultaten tyder dessutom på att såväl nybildning av T-celler som revidering av befintliga T-cells receptorer i mogna T-celler sker i dessa båda organ.
The thesis is based on the following papers:


III. **Anna Bas**, Göte Forsberg, Olle Hernell, Sten Hammarström, and Marie-Louise Hammarström. Changes in T cell receptor gene rearrangement in the small intestinal mucosa of children with celiac disease. *Manuscript*

IV. **Anna Bas**, Sten Hammarström, Sten Hellström, and Marie-Louise Hammarström. The nasopharyngeal tonsil - a site for T cell receptor gene rearrangement in man. *Submitted*

V. **Anna Bas**, Göte Forsberg1, Sten Hammarström, and Marie-Louise Hammarström. Utility of the housekeeping genes 18S rRNA, β-actin, and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) for normalisation in real-time quantitative RT-PCR analysis of gene expression in human T lymphocytes. *Submitted*

1Both authors contributed equally.
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AICD</td>
<td>activation induced cell death</td>
</tr>
<tr>
<td>AMC</td>
<td>adenoid mononuclear cell</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BMC</td>
<td>bone marrow mononuclear cell</td>
</tr>
<tr>
<td>CD</td>
<td>celiac disease</td>
</tr>
<tr>
<td>CIA</td>
<td>chronically infected adenoid</td>
</tr>
<tr>
<td>CP</td>
<td>cryptopatch</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DN</td>
<td>double negative</td>
</tr>
<tr>
<td>DP</td>
<td>double positive</td>
</tr>
<tr>
<td>ETCM</td>
<td>extrathymic T cell maturation</td>
</tr>
<tr>
<td>FAE</td>
<td>follicle-associated epithelium</td>
</tr>
<tr>
<td>FasL</td>
<td>Fast apoptosis stimulating protein-ligand</td>
</tr>
<tr>
<td>GALT</td>
<td>gut associated lymphoid tissue</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate-dehydrogenase</td>
</tr>
<tr>
<td>GC</td>
<td>germinal center</td>
</tr>
<tr>
<td>HOA</td>
<td>hypertrophic obstructive adenoid</td>
</tr>
<tr>
<td>IEL</td>
<td>intraepithelial lymphocyte</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>LFV</td>
<td>lymphocyte filled villi</td>
</tr>
<tr>
<td>LP</td>
<td>lamina propria</td>
</tr>
<tr>
<td>LPL</td>
<td>lamina propria lymphocyte</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer cell</td>
</tr>
<tr>
<td>NKT</td>
<td>natural killer T cell</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PP</td>
<td>Peyer’s patch</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>5' RACE</td>
<td>rapid amplification of the 5' cDNA end</td>
</tr>
<tr>
<td>RAG</td>
<td>recombination activating gene</td>
</tr>
<tr>
<td>RSS</td>
<td>recombination signal sequence</td>
</tr>
<tr>
<td>TdT</td>
<td>terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>helper T cell</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>T&lt;sub&gt;REG&lt;/sub&gt;</td>
<td>regulatory T cell</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
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PART I – GENERAL INTRODUCTION

GENERAL OVERVIEW

HISTORICAL PERSPECTIVE OF IMMUNOLOGY

The English word immunity originates from the Latin term immunis, meaning protected. Immunology grew out of the observation that individuals who recovered from certain infectious diseases were protected from those diseases. The first documented case of immunity can be traced back to a plague outbreak in Athens 430 B.C. Thucydides documented in his dialogue "History of the Peloponnesian war” that only those who had survived an attack of plague (including himself) could nurse the sick because they did not experience the disease a second time.

For a long time, disease was regarded as a punishment from God. It was considered that those who survived had repented their sins and made their peace with God. This fatalistic approach to death and disease made it difficult to develop an understanding of the immune system. It was not until the fifteenth century, when Chinese and Turks, who had religions more conducive to analytical observation, made the first attempt to induce immunity. This was done to protect against smallpox, caused by the variola virus. Chinese medical practitioners transferred pus from the sores of an affected individual to people who were unaffected by inserting dried pus to small cuts in their skin (a technique called variolation), thus inducing immunity.

The person who brought variolation to Europe was Lady Mary Worthy Montagu, the wife of the British ambassador to Constantinople. She had observed the positive effects of variolation and had the technique applied to her own children. The technique of variolation was improved by the English physician Edward Jenner in 1798. Jenner noticed that milkmaids, who were infected with and had recovered from the mild disease cowpox, were subsequently immune to smallpox, an often fatal disease. He reasoned that protection against smallpox could be induced by inoculation with the fluid from a cowpox pustule. His theory proved right when a boy, previously inoculated with pus from cowpox sores, did not develop the deadly disease smallpox after exposure to pus from active smallpox. In this manner Jenner began the science of immunology.

It was nearly 100 years before the technique of vaccination was applied to other diseases. Louis Pasteur demonstrated that it was possible to attenuate, or weaken, a pathogen and administer the attenuated strain as a vaccine. In
1885, Pasteur administrated the first vaccine to a human, a boy who had been bitten by a rabid dog. The boy survived and later became a custodian at the Pasteur Institute.

Even though Pasteur proved that vaccination worked, he never understood the mechanisms involved. In 1880 the experimental work of Emil von Behring and Shibasaburo Kitasato provided the first insight into the mechanisms of immunity. They took serum from animals infected with diphtheria and injected it into healthy animals. When these animals were later exposed to diphtheria they were found to be resistant to infection. This method of conferring infection resistance is known as "passive immunization". A serum antitoxin was suggested as the protective agent. In the 1930s it was shown that this antitoxin was a substance called antibody, produced specifically against the diphtheria microbe. Since immunity was mediated by antibodies in the body fluids (also known as humors) it was called humoral immunity.

Independent to the discovery of serum antibody was the discovery by Elie Metchnikoff in 1883 that cells also contribute to the immune state of an animal. He observed that certain white blood cells could engulf microorganisms. He named these cells phagocytes. Metchnikoff observed that phagocytic cells were more active in immunized animals and hypothesized that those cells, rather than antibodies, were the major effectors of immunity (a concept named cell mediated immunity). Studies on the role of lymphocytes and phagocytic cells in immunity in the 1950s formed the basis of our current view of the immune system, that of an integrated action of both cellular and humoral factors.

THE IMMUNE SYSTEM

The immune system protects the host against infectious agents. In vertebrates it is divided into two branches: the innate- and the adaptive immune system.

Innate immunity consists of mechanical barriers such as skin and mucosal membranes, various physiological barriers like the fever response, low pH, and complement activated via the alternative pathways. In addition, phagocytic cells like neutrophils and macrophages, and also antimicrobial factors such as defensins, lysozyme, lactoferrin, and NO are important effectors. NK cells and sometimes γδT cells are considered to be other cellular components of innate immunity. While some of the host defense molecules
are constitutively expressed, others are induced or enhanced upon pathogen recognition by so called pattern recognition receptors. These receptors recognize structures present in microorganisms but not in endogenous cells, e.g. lipopolysaccharide [1]. In contrast to adaptive immunity, the receptors used in innate responses are germ-line encoded, which means that the specificity of each receptor is genetically predetermined and cannot be improved by gene rearrangement. Innate immunity is considered to be the first line of defense and serves to limit infection in the early hours after exposure to an infectious agent.

The characteristic features of the adaptive immune system are specificity, diversity, and memory. The central elements of the adaptive response are T and B lymphocytes. Each lymphocyte expresses a single kind of structurally unique receptor, which is generated somatically during their development. Since the receptors of T and B cells are not germ-line encoded, they are not predestined. An extremely diverse repertoire in the lymphocyte population is gained through a process of random gene-segment recombination. Upon antigen encounter, the cell is triggered to proliferate and differentiate into effector cells. The end result of a primary immune response is the generation of long-term memory that leads to an elevated response upon re-encounter with the same pathogen.

T lymphocytes exhibit a wide range of functions such as control of B lymphocyte activation, interaction with phagocytic cells helping them to destroy intracellular pathogens, and destruction of cells that have become infected by a virus or other intracellular pathogens. T cells generate these effects either by releasing various communication molecules carrying messages between immune cells (cytokines), or by direct cell-cell contact signaling.

Activated B cells combat extracellular pathogens by producing pathogen-specific antibodies in the form of different classes of immunoglobulin (Ig) molecules (IgM, IgG, IgD, IgE, and IgA). After B cell receptor (BCR) recognition of an intact antigen, a humoral immune response is initiated resulting in differentiation into plasma cells and the production of antibodies with the same specificity as the BCR [2].

The combined action of innate and adaptive immunity constitutes an efficient system for protection from infectious diseases and is the key factor for survival of long-lived complex vertebrates. Immunocompetent lymphocytes are generated in the primary lymphoid organs; the B cells differentiate into functional cells in the liver of the fetus and the bone marrow of the adult, while the T cells mature in the thymus. Secondary lymphoid organs such as the spleen, peripheral lymphoid nodes, tonsils, and Peyer’s...
patches (PPs) represent organized immune-inductive tissues in which mature B and T cells are primed against encountered antigens to become effector or memory cells. Primed cells are later distributed to tertiary immune compartments, more commonly referred to as effector sites (e.g. the mucosal lamina propria and epithelia) where they respond quickly and vigorously to antigens encountered at these sites.

LYMPHOCYTES

Lymphocytes are small white blood cells, usually no larger than 6-10 µm in diameter. A characteristic feature of non-activated lymphocytes is the large round nucleus that occupies ~ 90% of the cell volume. The lymphocytes fall into three sets, the T-, B- and natural killer (NK) cells.

T Lymphocytes

T cell progenitors are conceived in the bone marrow and probably most are developed and educated in the thymus. Pre T lymphocytes enter the bloodstream, which delivers them to the thymus where they develop into mature T lymphocytes (so called because they are "thymus dependent" or thymus derived"). T cells can be divided into subsets according to the constitution and specificity of their antigen receptors, the markers they express, and their functions. According to the composition of the T cell receptor (TCR), T lymphocytes fall into two categories: αβ T cells and γδ T cells. αβ T cells express receptors composed of one α and one β polypeptide chain and γδ T lymphocytes express one γ and one δ chain. The majority of T cells developed in the thymus express an αβ TCR. The α and β chains together form the antigen-specific binding unit while the closely associated CD3 complex transduces signals into the cell upon antigen contact. T cells recognize protein antigens. In order to be recognized, proteins have to be presented to the T cell in the form of short peptides bound to specialized antigen presenting molecules, the major histocompatibility complex (MHC) molecules, on the surface of a self-cell (antigen presenting cell). The MHC molecules are highly polymorphic and fall into either of the two classes MHC class I or MHC class II. The specificity of the αβ TCR differentiates T lymphocytes into those that recognize peptides presented by MHC class I or MHC class II molecules. The γδ T cells can recognize antigens in their native form, without presentation on MHC molecules. Mature αβ T lymphocytes
belong to two main subsets defined by the expression of CD4 and CD8 molecules, CD4⁺CD8⁻ and CD4⁻CD8⁺. Normally, CD4⁺CD8⁻ cells constitute 60% and CD4⁺CD8⁺ cells 40% of the T cells [3]. Based on their function, αβ T lymphocytes can be divided into two main subsets, helper T cells (Th), which are CD4⁺CD8⁻, and cytotoxic T lymphocytes (CTL), which are CD4⁺CD8⁺.

**T helper cells (Th):** Naïve CD4⁺CD8⁻ T cells can differentiate into at least two classes of cells during an immune response: Th1 cells, which secrete the cytokines interferon-γ (IFN-γ) and interleukin-2 (IL-2), and Th2 cells which secrete IL-4 and IL-5 [4]. Th1 lymphocytes are responsible for cell-mediated immunity whereas Th2 cells are responsible for humoral immunity [4, 5]. The Th1-Th2 hypothesis has evolved to also include cytokines that are not necessarily secreted by CD4⁺ T cells but that promote the development of either Th1 or Th2 cells. Thus cytokines such as IL-12, although not secreted by T cells, have been assigned to the Th1-associated group of cytokines, whereas cytokines such as IL-10 and IL-13 have been assigned to the Th2-associated group of cytokines [4]. T cells that simultaneously produce both Th1 and Th2 cytokines are usually referred to as Th0, while T cells producing high amounts of transforming growth factor β (TGF-β) have been termed Th3 and are generally involved in down-regulation of immune reactions [6].

**Cytotoxic T lymphocytes (CTLs):** Upon activation, naïve CD8⁺ T cells become effector cells and acquire the capacity to lyse infected and transformed cells. The differentiation and activation of CD8⁺ effector T cells is promoted by IL-2 and IFN-γ, i.e. by Th1 lymphocytes [5]. The fully competent cytotoxic T lymphocytes (CTLs) lyse their targets either by cytolytic granule exocytosis or by Fas/Fas-ligand (FasL) cross linking [7]. Following TCR ligation, the cytoplasmic vesicles containing the pore-forming protein perforin and proteases known as granzymes are secreted. Perforin causes pore formation in the plasma membrane of target cells, allowing granzymes to enter and trigger nuclear damage and cell death [8]. The second apoptosis-inducing strategy requires ligation of FasL on the activated CTLs with its cell-death transducing receptor, Fas, on the target cell [9]. CTLs also produce several cytokines, such as IFN-γ and TNF-α, that interfere with pathogen gene expression and replication [7]. CD8⁺ T cells not only kill, but also secrete a number of cytokines and thus contribute to regulation of the immune response [10, 11]. Similar to Th cells, CTLs can persist in the body as memory cells [12].

**Regulatory T cells (T\(_{\text{REG}}\)):** Two subsets of CD4⁺ T\(_{\text{REG}}\) cells have been proposed, natural and adaptive [13]. They differ in terms of their specificity,
development, mechanism of action, and co-stimulatory signaling.

The natural T\(_{\text{REG}}\) cells are so designated because they are always present in normal individuals and carry out their regulatory functions during normal surveillance of self-antigens. They develop in the thymus, and are thereafter exported to peripheral tissues where it is proposed that they function to prevent the activation of self-reactive T cells. These thymus-induced T\(_{\text{REG}}\) cells typically express high levels of CD25 as well as the co-stimulatory molecule cytotoxic T-lymphocyte antigen 4 (CTLA4), and their peripheral homeostasis is controlled by signaling through CD28. An in vitro model has shown that natural T\(_{\text{REG}}\) cells function by a cytokine-independent mechanism, which presumably involves direct interactions with responding T cells or APC [14]. It has been argued that natural T\(_{\text{REG}}\) cells should be most effective at suppressing autoreactive T cell responses locally, in non-inflammatory settings.

Adaptive T\(_{\text{REG}}\) cells are generated from mature T cell populations, either from classical T cell subsets or natural T\(_{\text{REG}}\) cells, under certain conditions of antigen stimulation. Their CD25 expression is variable and unlike natural T\(_{\text{REG}}\) cells, they might not require co-stimulation through CD28 for their development and function. Furthermore, in contrast to natural T\(_{\text{REG}}\) cells, which are fully functional at the time of thymic export, the development of adaptive T\(_{\text{REG}}\) cells in the periphery might be triggered by low affinity antigen or altered TRC signal transduction [13]. Adaptive T\(_{\text{REG}}\) cells mediate their inhibitory activities by producing immunosuppressive cytokines, such as TGF-\(\beta\) and IL-10, which inhibit production of inflammatory cytokines like IFN-\(\gamma\) and IL-2 [15, 16]. However, direct cell-cell contact might be required to initiate the suppressive cascade. It has been proposed that adaptive T\(_{\text{REG}}\) cells might be induced during self-damaging inflammatory reactions to microbes or transplanted tissue or in settings of inflammatory autoimmune disease that are more similar to the infectious settings. Although CD4\(^{+}\) T cells seem to play the central role in the suppression of immune responses, T\(_{\text{REG}}\) cells can also be found among CD8\(^{+}\), \(\gamma\delta\) TCR\(^{+}\), and Natural Killer T cells [17-19].

**Natural Killer T Cells:** Natural Killer T cells (NKT cells) comprise a unique subset of T cells that co-express TCR and NK cell related surface markers. In mice, they are usually defined as NK1.1\(^{+}\)\(\alpha\beta\) TCR\(^{+}\) cells [20]. NKT cells are primarily comprised of CD4\(^{+}\)CD8\(^{-}\) or CD4\(^{+}\) cells and are frequent in the liver and bone marrow but rare in spleen, peripheral lymph nodes, and blood [20]. Human NKT cells also express NK cell markers, such as NKR-P1A, CD56, CD57 and CD122 and their frequency is low in peripheral blood (0.1-0.5%) and relatively high in liver (4-5% of immune cells) [20]. T lymphocytes expressing NK cell markers have been found in human small
General Overview

NKT cells have been shown to recognize glycolipid antigens in the context of the non-classical MHC class I molecule CD1d [24]. They produce both Th1 and Th2 cytokines and have cytotoxic capacity [25].

γδ T-cells: γδ T cells develop early in ontogeny and precede the development of αβ T cells. After birth, intrathymic differentiation shifts towards αβ T cell production and virtually all progenitors later produced differentiate into αβ T cells. γδ T cells constitute only a small proportion of the lymphocytes in human blood but are widespread and frequent within epithelia [26, 27]. In the peripheral blood of humans, most γδ T cells utilize the Vδ2 and Vγ9 gene segments in their TCR, whereas γδ T cells of the gut and cord blood predominantly utilize Vδ1 in combination with Vγ8 [21, 28, 29]. In contrast to αβ T cells, γδ T cells in man usually do not express CD4 or CD8 and are thus double negative (DN). There exists, however, a small population of γδ T cells in human small intestine that express the CD8 marker [21]. It is possible that these γδ T lymphocytes are induced to express CD8 upon activation as seems to be the case in gingiva during chronic inflammation [30, 31]. The biological functions of γδ T cells are not yet well defined, but they are thought to participate in the innate responses since they seem to have the capacity to respond quickly and without the requirement for expansion of a specific clone [18]. γδ T cells are able to secrete cytokines such as IFN-γ and RANTES (Regulated on Activation, Normal T cell Expressed and Secreted) [18, 32] and to execute cytolytic activities in response to activation [32, 33]. Furthermore, γδ T cells have been reported to play a role in maintaining epithelial integrity by producing epithelial-cell specific fibroblast growth-factor, which can induce growth or differentiation of epithelial cells and in this way reduce mucosal damage following tissue injury [34]. Non-peptide antigens, such as alkylamines that are derived from bacteria, are recognized by γδ T cells utilizing the Vδ2 gene segment [35]. The recognition of these non-peptide molecules does not require MHC presentation [36]. The γδ T cell subset utilizing Vδ1 recognize heat-shock protein 60 [27], MHC class I polypeptide-related sequences (MICA and MICB) which are expressed by stressed intestinal epithelial cells [37-39] and lipids presented by CD1c molecules expressed on professional antigen presenting cells [39, 40].
B Lymphocytes

The antigen receptor of B cells consists of a membrane bound Ig molecule that functions as the antigen-binding unit. The Ig is composed of two identical heavy and two identical light chains, each containing both variable and constant regions. The variable regions determine the antigen specificity while the constant region of the heavy chain determines the biological function. During an immune response, the activated B cells may exchange the constant part of their Ig while retaining the antigen specificity, a process termed isotype switching [41]. In contrast to the TCR on αβ T cells, the BCRs recognize unprocessed antigen and bind to complementary structures (epitopes) on the antigen itself rather than to peptide-MHC complexes displayed on antigen presenting cells. Upon antigen encounter, and with the help of Th cells, the B cells become activated, diversify their Ig genes by somatic hypermutation (increase of the antibody affinity due to mutations in the antigen binding region), and differentiate into plasma cells and long-lived memory B cells. Plasma cells secrete immunoglobulins at high rates. Without help from Th lymphocytes, stimulated B cells differentiate into IgM secreting plasma cells without further diversification of their Ig genes by somatic hypermutation and without establishment of B cell memory.

Natural Killer Cells

Without the need for prior activation, NK cells recognize and kill target cells that have been modified by infection or transformation. They are a key component of the innate immune system and participate in the first line of defense before the development of T and B cell mediated adaptive immunity. The absence of the CD3 complex and antigen-specific cell surface receptors distinguishes NK cells from T lymphocytes. The effector functions of NK cells, i.e. cytokine production and cytolytic activity, are regulated by activating- and inhibitory receptors expressed on their surface. In humans, the inhibitory receptors include killer cell Ig-like receptors and CD94/NKG2 heterodimers [42]. Inhibitory receptors prevent NK cell activation against cells that express self-MHC class I molecules. In contrast, when self-MHC class I molecules are absent or reduced, NK cells no longer receive inhibitory signals and the target cell is killed [43]. NK cell activation/inhibition is controlled by a balance of positive and negative signals mediated by the inhibitory and activating receptors [43]. Because the negative signal is dominant, it can override those involved in cell activation [44].
ANTIGEN PRESENTING CELLS

Initiation of the adaptive immune response depends mainly on T cell recognition of foreign peptides bound to MHC molecules on the surface of an antigen presenting cell (APC). The MHC molecules fall into either of the two classes MHC class I or MHC class II. MHC class I molecules are expressed on nearly all nucleated cells in the body and present peptides from intracellular proteins. MHC class II molecules present processed extracellular antigens and are expressed by APCs, which include monocytes/macrophages, dendritic cells (DCs) and B cells. In addition to MHC molecules, APCs also express so-called co-stimulatory molecules on their surface. These are CD80 and CD40, which interact with their respective ligands CD28 and CD40L on the T cells, resulting in a second, unspecific signal that is usually required for T cell activation and a subsequent adaptive immune response. DCs are believed to be the most potent APCs. They are efficient at antigen uptake and processing and express high levels of CD80. APCs present antigen to $\alpha\beta$ T cells. CD4$^+$ $\alpha\beta$ T cells are restricted to antigen presentation on MHC class II molecules while CD8$^+$ $\alpha\beta$ T cells are restricted to antigen presentation on MHC class I molecules. APCs can be situated in the bodily tissues and migrate to secondary lymphoid organs upon antigen uptake or be stationary in secondary lymphoid organs where they capture antigens from the lymph that passes. Some epithelial cells express MHC class II molecules and therefore can present antigens. Their capacity to function as APCs is debated since they usually lack co-stimulatory molecules. A special type of antigen presenting cell is the follicular dendritic cell present in germinal centers. These cells do not process antigens. Instead they present intact, native antigen to B cells in the germinal center reaction thereby rescuing B cells with desired specificity from apoptosis.

IMMUNOLOGICAL MEMORY

Immunological memory can be defined as the ability of the immune system to respond with enhanced vigor and greater rapidity upon secondary exposure to previously encountered antigens. Most of the T and B cells that arise via the activation process during an immune response die by apoptosis (a mechanism called activation induced cell death, AICD) within a few days after the infection has been warded off. However, some of the activated, antigen-specific T and B cells are able to escape death and persist in the body as
memory cells that continually survey the body in order to provide protection against secondary attacks by the same agent. In the case of T cells, memory is believed to be maintained by continuing low-level proliferation [45] and by the action of IL-15 and IL-15Rα, and by IL-7 [46, 47]. The long-term survival of memory cells does, however, not seem to depend on low-level stimulation by residual antigen, as was previously believed, and in case of T cells, also seem to be independent on MHC molecules [48, 49], although MHC-TCR interactions might be required to maintain memory-cell function [50]. This suggests that there is either an intrinsic ability of memory lymphocytes to survive for extended periods or dependence on as yet undefined extrinsic factors.

Two distinct types of memory T cells have been identified: central memory cells present in secondary lymphoid tissues, and effector memory cells residing in non-lymphoid tissues [51]. Sallusto et al. showed that human peripheral blood T cells expressing chemokine receptor 7 (CCR7) were unable to rapidly produce effector cytokines upon stimulation, whereas CCR7- T cells could do so. These subsets were termed central and effector memory subsets, respectively, due to the non-lymphoid tissue migration pattern suggested by the lack of CCR7. In addition, it has recently been suggested that a similar hierarchy may also exist in the memory B cell compartment [52, 53]. McHeyzer-Williams and colleagues have demonstrated that a distinct, novel population of germinal center derived memory B cells arises following immunization with protein in alum. Unlike the classical B220+ population, these cells are negative for B220 expression and appear to more readily differentiate to antibody-secreting cells upon activation.
In the human embryo, blood formation takes place in the liver and, to a lesser extent, in the spleen. In the fifth month of development, stem cells settle down in the bone marrow, where they remain for the rest of the individual's life. Blood formation in human liver and spleen ceases just before birth and the blood cells are thenceforth produced in the bone marrow. Of the blood cells produced in an adult, only the T lymphocytes differentiate outside the bone marrow. The remaining cells go through the hemopoietic progression in the bone marrow and then enter the circulating blood, which contains erythrocytes (red blood cells), platelets, and leukocytes. Leukocytes are divided into two families: mononuclear and polymorphonuclear. Mononuclear leukocytes include lymphocytes and the monocytes (which are the largest of all blood cells, they constitute 1-6% of all blood leukocytes and transform into macrophages upon leaving the blood circulation). Polymorphonuclear leukocytes have granulated cytoplasm; they are therefore often referred to as granulocytes. They are the neutrophils (which preferentially infiltrate infected and inflammatory sites and eliminate foreign objects by phagocytosis), eosinophils (which enter similar sites as neutrophils and kill foreign antigens mainly by degranulation but also by phagocytosis), and basophils (the rarest of all blood cells, have receptors for IgE and degranulate when activated by antigen interaction to surface bound IgE).

Lymphocytes are, after neutrophils, the dominant leukocytes of blood, representing 20-45% of all white blood cells [3]. Among these, T cells are most abundant. The majority of T lymphocytes express CD4⁺CD8⁻ (~ 57%) [54], about 40% are CD8⁺CD4⁻, ~ 4% are of the γδ T cell lineage, and ~ 2% are CD4⁺CD8⁺ [54]. B and NK cells comprise about 15-30% and 10-15% respectively, of all circulating lymphocytes [3, 44].

The human small intestine is a thin-walled tube extending from the pylorus of the stomach to the colon. The small intestine consists of three segments: duodenum, jejunum, and ileum. The intestinal wall, which is studded with numerous mucosal projections, is composed of four layers: the mucosa, the submucosa, the muscularis, and the serosa (Fig. 1). The mucosa has three components: 1) a single cell epithelial layer 2) an underlying stroma
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composed of vascularized, highly cellular connective tissue (lamina propria, LP) and 3) a thin layer of smooth muscle (muscularis mucosa). The epithelial cell layer forms the interface between the external and the internal environments in the gastrointestinal tract. It is the site of digestion and absorption and also functions as the principal barrier against various harmful agents and infectious pathogens. Apart from the villous-crypt epithelium covering almost the entire inside of the intestine, there is a specialized follicle-associated epithelium (FAE) covering lymphoid follicles. The villous-crypt epithelium contains columnar absorptive cells (enterocytes), goblet cells (mucus producing cells), Paneth cells, enteroendocrine cells, and intestinal epithelial stem cells. The latter generate a proliferative zone in the mid-crypts and produce daughter cells that migrate upward where they differentiate into absorptive cells and goblet cells, and downward where they differentiate into Paneth cells, which produce antimicrobial factors such as lysozyme and defensins, and enteroendocrine cells which produce hormones and neuropeptides in response to changes in the external environment [55]. In addition, the epithelium contains intraepithelial lymphocytes (IELs) derived from lymphocyte precursors. IELs are generally located close to the basal lamina between the epithelial cells. The FAE contains specialized antigen-sampling cells, so called microfold (M) cells, that pinocytose antigens present in the lumen and transport them into the lymphoid follicle [56].

Figure 1. a) Lower gastrointestinal tract b) Cross-section of small intestinal wall. IEL: Intraepithelial Lymphocyte; LPL: Lamina Propria Lymphocyte; MQ: Macrophage; DC: Dendritic Cell
The enterocytes make up the bulk of the cells in the mucosal epithelium. The apical surface of the enterocytes contains numerous microvilli, which in turn are covered by a layer of macromolecules and membrane vesicles, termed the glycocalyx, containing various enzymes and nonenzymatic proteins necessary for digestion and absorption of nutrients and for protection [57]. On top of the glycocalyx layer is a thick mucus layer. The mucous layer characteristically contains the mucins which are highly viscous and elastic substances produced mainly by goblet cells. The glycocalyx and the mucous layer participate in the protection of the epithelium against attack from various microorganisms. Other components of these layers, such as secretory IgA, lysozyme, α and β-defensins, and lactoferrin have antimicrobial activity [55].

The LP is rich in immune cells including T and B cells, plasma cells, monocytes/macrophages, mast cells, and DCs [paper I, [58].

The submucosa is a fibrous connective tissue layer containing lymphatic vessels and glands extending from the mucosa as well as blood vessels that send finer vessels into the mucosa and muscularis. Lymphoid follicles extend from the epithelium through the mucosa all the way down to the submucosa.

The serosa is composed of loose connective tissue covered by mesothelial cells [59].

Gut Associated Lymphoid Tissue (GALT)

Gut associated lymphoid tissue (GALT) is a general term for lymphoid tissues present in the intestine. GALT includes PPs, the appendix, solitary follicles, and also the diffusely spread immune cells in the LP, the so called lamina propria lymphocytes (LPL), and the IELs. GALT contains both inductive- and effector sites. PPs and solitary follicles constitute inductive sites, where immune cells first encounter antigen and where the responses are induced. Epithelium and LP constitute effector sites, where the terminal differentiation of IELs and LPLs to effector cells (e.g. CTLs and plasma cells) and execution of lymphocyte functions occur.

PPs are groups of lymphoid follicles mainly found in the distal ileum [60]. Morphologically, PP can be separated into three major regions: FAE, the interfollicular T cell zone, and the follicular area. The M cell rich FAE lacks villi and contains few goblet cells [60]. Solitary follicles are located along the whole intestine. They do not occupy fixed positions but instead they come and go depending on the conditions at a given time [3]. The two major cell types in human intestinal lymphoid follicles are T and B lymphocytes [61]. The centre of the follicle contains B cells with few T cells, and is surrounded by a zone of both B and T cells, and areas in the periphery which contain T cells only [61].
The B cell rich center area also contains follicular dendritic cells [61].

**Intraepithelial Lymphocytes (IELs)**

IELs are located within the epithelium in close contact with epithelial cells and often also the basal lamina [21]. The majority of IELs are T lymphocytes, many of them containing cytoplasmic granules [62]. Human small intestinal IELs primarily consist of CD8$^+$ αβ T cells [21, 63, 64]. The compartment-specific retention of the CD8$^+$ IELs might be explained by their prominent expression of the integrin α$E$β$7$, that binds to E-cadherin on intestinal epithelial cells [65]. In the colon, the number of CD8$^+$ and CD4$^+$ IELs is more equal and a substantial population of αβ T cells express neither CD4 nor CD8 [21]. On average, 30% of IELs in the small intestine are γδ T cells [21]. Intestinal γδ T cells in adults seem to be oligoclonal [66, 67], preferentially utilizing the Vδ1 chain, often in combination with the Vγ8, in contrast to the periphery where utilization of Vδ2 is predominant [29, 66]. The majority of human γδ T cells are CD4$^+$CD8$^-$ (DN) both in small and large intestine, but a small fraction can express CD8 [21]. Furthermore, there are IELs with a thymocyte-like phenotype, i.e. expressing CD1a, CD2 without CD3 [21, 68], and CD4 and CD8 simultaneously [paper I]. Some IELs express NK receptors. These include IELs with mature T cell phenotype and CD3CD7$^+$ cells [21, 22, 69].

CD4$^+$ TCRαβ$^+$ IELs, CD8$^+$ TCRαβ$^+$ IELs and TCRγδ$^+$ IELs all produce IL-2 and the Th1 cytokines IFN-γ and TNF-α [70, 71]. Also the CD3CD7$^+$ NK lymphocytes produce mainly IFN-γ and TNF-α [69, 72]. IELs also encompass cytotoxic cells as demonstrated by the presence of perforin containing granules [paper I] and a cytolytic machinery that can be triggered in vitro by antibodies to the TCR/CD3 complex [71]. Cell fractionation experiment showed that CD8$^+$ TCRαβ$^+$ IELs are responsible for most of this activity [paper I]. The CD3CD7$^+$ intestinal NK lymphocytes were also shown to contain perforin granules indicating cytolytic activity [69, 72]. However, NK cell cytotoxicity, manifested as killing of the NK cell target K562, could not be demonstrated [62], paper I.

Intestinal γδ T cells posses specialized functions not shared with other lymphocyte subsets. γδ T cells have been reported to play a role in maintaining epithelial integrity by producing growth factors and by secreting chemokines such as lymphotactin and RANTES, thus recruiting peripheral αβ T cells and inflammatory cell types to sites of damage in the epithelial tissues [34, 73].
Human Vδ1+ intestinal IELs have also been shown to lyse MICA+ carcinoma cell lines, hence γδ T cells are considered to be able to eradicate infected, transformed, and malignant epithelial cells [37, 74].

In mice, γδ or αβ T cells bearing a CD8αα homodimer comprise the major intraepithelial T cell populations [75, 76]. In addition to its known function as a co-receptor, the CD8αα homodimer has been described as a modulator of IEL activation. It has been demonstrated that upon binding to the MHC related molecule TL (thymus leukemia antigen) expressed almost exclusively by epithelial cells of the murine small intestine, CD8αα enhances cytokine release by IELs but inhibits their proliferation and cytotoxicity [77]. Human IELs expressing the αα form of CD8 have also been demonstrated in adult [78] and fetal gut [79].

Murine IELs expressing CD8αα homodimer are considered to have matured extratrhapsically [80, 81] and lymphoid aggregates (cryptopatches, CP) situated along the gut mucosa have been suggested as their ontogeny sites [82, 83]. However, some data imply that CD8αα+ IELs have an early thymic origin [84-86]. As will be discussed later, our demonstration of recombination activating gene-1 (RAG1) mRNA in IELs of the T cell lineage suggests that also human IELs have the capacity for TCR rearrangement [21], paper II.

Intestinal IELs have also been suggested to be involved in the induction and maintenance of oral tolerance and suppression of autoimmune reactions [64, 87].

**Lamina Propria Lymphocytes (LPLs)**

The LP contains most cellular components of the immune system, with large numbers of B cells, plasma cells, macrophages, DCs, and T cells of both the CD4 and CD8 subsets [paper I, [58]. In addition, mucosal mast cells can be found. The vast majority of LP T cells in human express the αβ TCR and have the phenotype of memory/activated T cells being CD45R0+, presumably as a result of continuous exposure to lumen antigens [88]. In addition, a small proportion of LP T lymphocytes is CD4+CD8+ [paper I, [54]. CD56+ NK cells and T cells expressing the γδ TCR are undetected or rare in LP [paper I, [54].

The CD8+ T cells in human LP have cytolytic effector functions [paper I] and are thought to contribute to the control of viral infections [58]. LP T cells also secrete IL-2 and effector cytokines such as IFN-γ and TNF-α [70, 89, 90]. LP contains a large number of plasma cells of which 70-90% secrete antibodies of the IgA isotype [91]. The precursors of LP plasma cells arise in
the PP, migrate through the lymph and blood circulation, and eventually home to the LP where CD4⁺ T cells produce cytokines that regulate the differentiation of B cells into IgA producing plasma cells [92]. All plasma cells in the LP, irrespective of their Ig isotype, express the J chain [93].

Cryptopatches and Lymphocyte-Filled Villi

Cryptopatches (CPs) are lymphoid aggregates located along the murine gut mucosa between the crypts in the LP and are in immediate contact with the epithelium [94]. These structures have been suggested as the sites for progenitor cells in extrathymic T cell maturation (ETCM). CPs contain small lymphocytes that express Thy1, the receptor for stem cell factor (c-kit), and receptor for IL-7 (IL-7R), but do not express RAG, apparently representing an early immature step in the T cell lineage as suggested by the presence of CD3ε and germ-line TCRγ- and β-chain gene transcripts [83]. The precursor T cells that develop in CPs then migrate directly into the overlying epithelium where TCR rearrangement subsequently occurs [83, 95]. This would seem consistent with the previous description of the expression of RAG detected in a small population of CD3⁻ IELs, which were suggested to be the precursors of the CD3⁺CD8αα⁺ IELs [75, 96]. No CP like structures has been identified in human intestine.

Other form of lymphoid aggregations that have recently been described in the murine, rat, and human small intestine are lymphocyte filled villi (LFV) [97-99]. These specialized villi are distinguished by containing a LP that is filled with closely packed lymphocytes. The main population of lymphocytes in murine LFV expresses Thy1, but is negative for B, T and NK cell markers [97]. In both mouse and rat small intestine, the LFV contain mainly immature cells that do not express surface-Ig, CD3 or TCR. In contrast, human LFV contain mature CD3⁺CD4⁺ lymphocytes and have an activated/memory cell phenotype (CD45R0) [99]. Furthermore, lymphocytes in human LFV do not express c-kit or CD1 and do not stain for RAG1 (own unpublished data) [99]. This indicates that these structures are not sites for extrathymic T cell maturation in man. Instead, since LFV were shown to contain large numbers of MHC class II⁺ cells, human LFV have been suggested to be sites for T cell activation and may possibly represent an early stage of solitary lymphoid follicle development.
Mucosal Tolerance

It is essential that the intestinal mucosa is protected from invasion by pathogens. However, it is also important that immunological responses are not induced against harmless or potentially beneficial antigens such as food antigens and commensal bacteria. While oral tolerance refers to peripheral unresponsiveness to orally administered antigens, mucosal tolerance refers to a similar phenomenon at the mucosal surfaces.

The first cells in the small intestine that orally administered antigens are likely to encounter are the epithelial cells. These cells are believed to participate in the tolerance induction by presenting antigens to TCRαβ⁺CD8⁺ intraepithelial T cells or subepithelial TCRαβ⁺CD4⁺ T lymphocytes. Because the resting epithelial cells have inadequate co-stimulatory capacity, the epithelial cell-T cell interaction will result in tolerance through unresponsiveness or induction of active down-regulatory responses [56]. The observation that a significant fraction of LP T cells undergoes apoptosis in normal mucosa as compared to inflamed mucosa, where apoptosis in LP T cells is greatly reduced, suggests that apoptosis might be another mechanism that contributes to the maintenance of unresponsiveness to normal luminal antigens [100]. LP T cell responses may also be kept in check by prostaglandin E₂ that is released by the epithelium or LP macrophages [56]. The prostaglandins are induced in response to environmental stimuli, such as lipopolysaccharide, and they have been shown to orient T cell mediated responses toward immunosuppression by inhibition of pro-inflammatory cytokines, increase of IL-10 production, or down-regulation of T cell activation [101]. Furthermore, immunosuppressive cytokines such as IL-10 and TGF-β produced by regulatory T cells and APCs play an important role in immune suppression in the gut [102].

Other sites at which antigens are likely to encounter APCs and T cells and where tolerance may be induced are the PPs. In PPs, distinct populations of DCs have been identified and proposed to play specific roles in tolerance [103-105]. Kelsall et al [104] have demonstrated that DCs in PPs produce IL-10 and also prime naïve T cells to produce this cytokine. It was also shown that upon antigen encounter, the DCs in PPs may undergo two differentiation pathways [103-105]. If the antigen is a harmless protein, DCs generate Th3 responses via secretion of IL-10 and TGF-β. In contrast, if an antigen is encountered in the context of some form of microbial stimulus/inflammatory signal, DCs mature and migrate to the interfollicular region where they promote the production of IFN-γ in the naïve T cells they prime.
Celiac Disease

Celiac disease (CD) is an inflammatory intestinal disorder characterized by a permanent intolerance to wheat gliadins and related prolamines in rye (secalins) and barley (hordeins). CD is a common disease with a worldwide prevalence of 1 in 266 as estimated by serologic screening [106]. In Sweden, CD is one of the most commonly diagnosed chronic diseases in children with an incidence of at least 4 per 1000 live births [107]. The age of onset is variable; it can range from infancy to late in adulthood. In young children the disease is typically characterized by malabsorption, diarrhea, weight loss, and retarded growth. In older children and adults the symptoms are vague. Symptoms cease upon withdrawal of gluten from the diet and the present treatment is a gluten-free diet. CD is strongly associated with the MHC class II alleles HLA-DQ2 and HLA-DQ8 [108] which are carried by more than 95% of patients with CD compared to 20%-30% in the general population [109]. Even though the expression of HLA-DQ2 or HLA-DQ8 is a predisposing factor for CD, it is not sufficient to develop the disease, suggesting additional genetic and/or environmental factors.

The inflammation in active disease results in flattening of the mucosa with parallel villous atrophy and crypt cell hyperplasia. Characteristics of active CD also include increased numbers of intestinal lymphocytes and production of anti-gliadin, and anti-endomysium antibodies of the IgA class [110, 111]. In the intestinal lesion, elevated numbers of $\alpha\beta$ T cells can be noted both intraepithelially and in LP [109]. While TCR$\alpha\beta^+$ IELs and LP lymphocytes return to normal levels when gluten is removed from the diet, the level of TCR$\gamma\delta^+$ IELs remains elevated [109]. Presence of anti-endomysium antibodies of IgA class in serum is considered a specific marker for CD [110]. Anti-endomysium antibodies were reported to recognize tissue transglutaminase [112], the level of which is increased in active CD [110, 113]. The mechanism of the intestinal immune-mediated response is not fully understood, but it is known that it involves gliadin specific, HLA-DQ2 or HLA-DQ8 restricted T cells in the LP. Gliadin is a substrate for tissue transglutaminase, which transforms positively charged glutamines to negatively charged glutamic acid residues by deamidation [112]. Deamidation allows binding of gliadin peptides to the HLA-DQ2 or HLA-DQ8 groves that have positively charged binding pockets. Intestinal LP CD4$^+$ $\alpha\beta$ T cells of CD patients that recognize deamidated peptides presented by these HLA molecules have been demonstrated and were shown to produce IFN-$\gamma$ upon in vitro challenge with gliadin peptides, suggesting that these cells could provoke inflammation and villous atrophy in active disease [111].
IELs of both the \( \alpha \beta \) TCR (mostly CD4\(^{+}\)CD8\(^{+}\)) and the \( \gamma \delta \) TCR lineage are expanded in active CD [109, 114-116]. These cells are thought to be involved in lesion formation but there are currently no reports of IELs that recognize gliadin in CD. However, a recent report by Forsberg et al [70] showed that major changes related to the activity of CD take place within the epithelium, with a marked increase of IFN-\( \gamma \) and IL-10 production by intraepithelial T cells. The pronounced increase of IFN-\( \gamma \) may be an effect of IEL activation. The majority of the activated IFN-\( \gamma ^{+} \) IELs were found to be CD3\(^{+}\)CD8\(^{+}\) [117]. It has been suggested that T cell mediated immune responses are not only directed against gliadin peptides but may also be aimed at MICA and MIBA which are expressed on stressed epithelial cells [37].

NASOPHARYNGEAL- AND PALATINE TONSILS

The nasopharyngeal and the palatine tonsils are lymphoid tissues of the upper aerodigestive tract. They are members of the Waldayer’s ring and participate in the first line of defense against inhaled, exogenous pathogens, and function as inductive sites for humoral and cell mediated immune responses. Histologically, the nasopharyngeal and palatine tonsils consist of four compartments which all participate in the immune response: the epithelium, the follicular germinal centers (GCs), the mantle zone, and the tissue between the lymphoid follicles, known as the interfollicular area [118] (Fig. 2). GCs develop by the proliferation of mature, antigen-primed B cells, in a T cell-dependent process in secondary lymphoid organs. The follicles can be divided into a light and a dark zone of the GC, and a mantle zone. The dark zone harbors highly proliferating B-cells (centroblasts), whereas the light zone primarily contains smaller, more mature B cells (centrocytes) [3, 119]. The mantle zone harbors naïve B cells [120]. GCs contain a T cell population that has a helper-inducer memory phenotype, expresses \( \alpha \beta \) TCR, CD4, and CD45R0 and also molecules associated with early T cell activation, such as CD69 [121]. In addition to B and T cells, GCs also contain follicular dendritic cells that trap and retain intact antigens and participate in the selection of antigen reactive B cells upon hypermutation [119]. A population of antigen presenting cells, termed germinal center DCs, is also present within human tonsillar GC. Macrophages that mainly seem to be involved in phagocytosis of B cells that have died during the selection process [122] are also present. Virtually all T cells in the GC are \( \alpha \beta \) T cells and the majority of them are CD4\(^{+}\), supporting the theory of local T helper cell activity [123].
Most of the studies of the GC reaction in humans have been performed on palatine tonsils. However, immunomorphometry analysis of palatine and pharyngeal tonsils demonstrated striking similarity in cellular composition and distribution between GCs in the two organs [123, 124].

The interfollicular region of palatine and pharyngeal tonsils is primarily populated by CD4+ αβ T cells [119, 123, 124]. This region also contains DCs, plasma cells, and macrophages [120]. The crypt epithelium of palatine tonsils plays a key role in the initiation of immune responses by uptake of luminal antigens by M cells [120]. The luminal antigens are taken up and transported to intra- and subepithelial spaces where they are presented to lymphoid cells. T cells constitute the major intraepithelial immune cell population in the surface epithelium of nasopharyngeal and palatine tonsils. Both αβTCR+ and γδTCR+ cells are present [123, 124]. Analysis of V-gene usage in intraepithelial γδ T cells in palatine tonsils of patients with the diagnoses of recurrent tonsillitis and idiopathic tonsillar hypertrophy, revealed a specific γδ T cell subtype utilizing the rare combination of Vδ1/Vγ9. This tonsil specific γδ T cell subset was suggested to be reactive to antigens specific to the tonsillar milieu and to contribute to the maintenance of the epithelium integrity [124].

The adenoid and palatine tonsils are largest in early childhood but atrophy thereafter to become anatomically minor structures in normal adults [125], and personal communication with Professor Sten Hellström, Department of Otorhinolaryngology, Umeå University. Analyses of age-associated changes in frequencies of lymphocyte populations in adenoids have revealed an age dependent decrease in B lymphocyte proportion and an increase of T cells.
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[126]. However, CD3+ cells with the HLA-DR marker were shown to significantly decrease with age [127]. Since 30-40% of T cells in the mantle zone are stained by anti-HLA-DR+ antibodies, a decrease in the proportion of CD3+ HLA-DR+ cells may implicate diminished T cell activation resulting in a cessation of B cell stimulation [128].

Hypertrophic Obstructive Adenoids and Chronically Infected Adenoids

The two main indications for adenoidectomy, i.e. surgical removal of the nasopharyngeal tonsil, are upper airway obstruction due to recurrent chronic tonsillitis (chronically infected adenoids, CIA) and tonsillar hypertrophy (hypertrophic obstructive adenoids, HOA) [129]. CIAs are associated with viral and bacterial infection often leading to middle ear infection, otitis media, which is one of the most common causes of hearing loss in children [130-132]. The bacteria that cause infections in the middle ear, predominantly Streptococcus pneumoniae, Haemophilus influenzae, and Moraxella catarrhalis, are also found in the nasopharyngeal tonsil [132], leading to the assumption that the nasopharyngeal tonsil serves as a reservoir for pathogenic bacteria which subsequently colonize the middle ear.

The cause of adenoid hypertrophy, which is a common feature of childhood, is less investigated and more controversial. It has been proposed that it is caused by an imbalance of the system of the intraepithelial cytotoxic lymphocytes, causing an epithelial destruction and uncontrolled penetration of foreign antigens e.g. viral and bacterial microbes to the B cell area. This in turn may lead to abnormally intense B cell response to antigen stimulation [133-135]. Adenoidectomy of the enlarged adenoid is mainly performed due to obstruction to breathing, which causes snoring and disturbed sleep.

It has been found that while αβ TCR+ are the dominating intraepithelial T cell population in CIA [123] as well as in normal nasopharyngeal tonsils [133], γδ TCR+ cells constitute the major T cell population in the surface epithelium of HOA [123].
T CELL DEVELOPMENT AND ANTIGEN RECEPTOR GENE REARRANGEMENT

INTRATHYMIC T CELL MATURATION

Common lymphoid progenitors are generated from hematopoietic stem cells in the bone marrow. Whereas some undergo B cell commitment, other common lymphoid progenitors migrate to the thymus where they become T cells. The signals involved in the induction of T cell commitment are provided by the interaction between Notch-1 and its ligand Delta1 [136]. Notch family members are transmembrane receptors that contain epidermal growth factor (EGF) repeats in their extracellular domain, which are implicated in ligand binding. The cytoplasmic region contains a RAM23 domain, a PEST sequence, and 6 ankyrin repeats, and is involved in intracellular signaling [136]. Four mammalian Notch receptors (Notch 1-4) have been identified which interact with two Notch ligand families, Delta and Jagged [137]. It has recently been reported that Delta1 but not Jagged1 can induce Notch activation and subsequent T cell commitment, which suggests that Notch signaling can be differently regulated by Notch ligands [138].

The thymus contains three compartments: the subcapsular zone, the cortex, and the medulla. The subcapsular zone is where bone-marrow derived pro-thymocytes begin to differentiate, proliferate, and rearrange their TCR β-chain gene segments [139]. The earliest thymocytes lack CD4 and CD8 and are referred to as DN cells. A model of T lymphocyte differentiation and marker expression is illustrated in Figure 3. The DN thymocytes differentiate along one of two developmental pathways: they either become DN CD3⁺ γδ T cells or DN CD3⁺ αβ T cells. Only about 0.5-1% of thymocytes develop into γδ T cells while the majority of DN thymocytes of a young individual progress along the αβ T cell pathway and start to rearrange the TCR β-chain genes. T cell receptor genes are assembled from their component variable (V), diversity (D), and joining (J) gene segments in a lymphocyte specific process referred to as V(D)J recombination. Gene segments undergoing recombination are flanked by a recombination signal sequence (RSS). Each signal sequence is composed of conserved 7 and 9 base pair motifs separated by a spacer of 12 or 23 base pairs [140]. Efficient recombination occurs only between two signal sequences that have a different spacer length, a restriction known as the 12/23 rule. V(D)J recombination is initiated by a sequence-specific cleavage of the DNA between the coding region and the RSS. The
cleavage is catalyzed by two lymphoid-specific key proteins, RAG1 and RAG2 [140, 141]. The human RAG1 and RAG2 genes lie 8 kilobases apart on chromosome 11, they are convergently transcribed, and their coding sequence is contained in a large continuous open reading frame within a single exon [141, 142]. The molecular weights of RAG1 and RAG2 are 119 and 58 kilo Daltons (kDa) respectively [141, 143]. The central portion of RAG1 contains a region termed the nonamer binding domain which is involved in recognition of the signal sequence [144]. This interaction is facilitated by the high-mobility-group proteins HMG1 and HMG2 which enhance the binding of RAG1 to the nonamer through an interaction with the nonamer binding domain [145]. Even though RAG1 alone can recognize the signal, cleavage of the RSS requires participation of RAG2, which forms a stable complex with RAG1 [141]. Upon DNA cleavage, covalently sealed hairpin coding ends are generated and are then cleaved by the action of RAG1 and RAG2 [146, 147].

**Figure 3.** A model for the early stages of T-lymphocyte development in thymus. DN, double negative; DP, double positive

A hallmark of V(D)J recombination is generation of receptor diversity. Many coding elements can be joined in various combinations and joining is imprecise due to nucleotide deletion and insertion. Terminal deoxynucleotidyl transferase (TdT) is a lymphoid cell specific polymerase that adds untemplated nucleotides to DNA ends. However, its presence is not required for a proper
gene rearrangement. The human TdT gene is located on chromosome 10 and contains 11 exons which encode a protein that has a molecular weight of 58 kDa [148-150]. Another source of nucleotide addition is asymmetric nicking of coding-end hairpins to generate palindromic overhangs termed P nucleotides [140]. Joining of the coding ends requires the presence of the DNA-dependent protein kinase, composed of the catalytic subunit DNAPKcs and the Ku70 and Ku80 proteins, as well as the XRCC4 protein and DNA Ligase IV [148]. When the gene segments of the β-chain have been assembled, the β-chain is expressed on the cell surface together with an invariant pre-TCR-chain (pTα), and associates with CD3 subunits to form the pre-TCR complex. [151]. The pTα chain is expressed in two forms, a long form (pTαa) encoded by all four exons and thus containing an extracellular domain that associates with the TCR β-chain during TCR α–chain gene rearrangement forming a preTCR, and a short form which lacks the extracellular domain (pTαb) encoded by exon 2 [152, 153]. The human pTα gene is located on chromosome 6 and the molecular mass of pTαa and pTαb is 33 and 18.4 kDa, respectively [151, 153]. The pre-TCR functions to rescue the DN thymocytes from apoptosis, to induce cell proliferation, and to promote maturation of DN thymocytes to the double positive (DP) stage [151]. Next, the cells move to the thymic cortex where the TCR α-chain gene-segments rearrange, forming a functional αβTCR. During the recombination, there are two waves of RAG1 and RAG2 expression [154]. The first wave peaks early at the CD25+CD4-CD8-CD3- stage and coincides with the initial expression (and hence rearrangement) of TCR β-chain. The second wave occurs at the DP stage coincident with initial expression of TCR α-chain genes [154]. Active down-regulation of RAG1 and RAG2 proteins and/or mRNA expression appears to occur between the two peaks of recombinase activity [154, 155]. Following expression of the mature αβTCR, the T cells test whether their receptors have sufficient affinity for self-MHC molecules. Those T cells that are unable to recognize self-MHC or have very low avidity for self-MHC die from lack of the TCR signal (death by neglect). T cells which recognize self-MHC survive and expand (positive selection). Subsequently, in the thymic medulla, T cells that have very high avidity for self-peptides (autoreactive T cells) are removed by apoptosis (negative selection). Finally, cells that have survived the negative selection are exported to the circulation.

During intrathymic T cell maturation, several signals produced by the microenvironment of the thymus are important for proper T lymphocyte development. IL-7, a cytokine produced by thymic stromal cells, is particularly
T Cell Development and Antigen Receptor Gene Rearrangement

important since it triggers the recombination process and also promotes survival in progenitor thymocytes, most likely by upregulation of the anti-apoptotic gene Bcl-2 [156, 157]. While IL-7 has been shown to be required for the generation and survival of γδ T cells, the development of pre-T cells committed to the αβ T cell lineage does not seem to strictly depend on IL-7 mediated signaling [157].

Death in the thymus

Apoptosis plays a central role in the generation of T lymphocytes in the thymus. Developing T cells are destined to die unless a functional TCR is produced to trigger a rescue signal. Cells that fail to generate a functional antigen receptor or fail to receive any signals die from “death by neglect”. Thymocytes bearing self-reactive receptors are eliminated by apoptosis during negative selection.

The precise mechanisms of death by neglect are unclear. One of the possible explanations is sensitivity to glucocorticoids in the absence of TCR stimulation [158]. Since signals from the TCR can antagonize the apoptotic effect of glucocorticoids it is believed that the combined signals from corticosteroids and TCR determine the fate of the thymocyte [159]. Other proposed factors involved in death by neglect are the Bcl-2 family members, which consist of apoptosis-regulatory gene products [160]. Whereas over-expression of Bcl-2 or Bcl-xL, two death antagonists, protects DP thymocytes from dying, abrogation of Bax and Bak, two death agonists, results in prolonged survival of thymocytes in culture [158, 161]. Furthermore, Bcl-2 expression was shown to be upregulated by the Notch intracellular domain, suggesting that Notch-1 also plays a role in thymocyte survival [162].

Engagement of the TCR induces intracellular events that, depending on the strength of the ligation, lead to different outcomes. Induction of a “strong” signal generated by self-antigen commits the cell to apoptosis [158]. During negative selection, TCR cross-linking alone does not seem to be enough to cause the death of immature self-reactive thymocytes. Instead apoptosis in these cells is dependent on co-stimulatory signals through surface molecules such as CD28 [163, 164]. The situation seems to be quite different in the semi-mature HSA hi CD4+CD8- thymocytes. In these cells, TCR ligation alone can induce Fas-mediated death, even though the apoptosis is augmented upon a combined TCR/CD28 ligation [163, 165]. However, with a high concentration of antigen, the CD28 mediated pathway fails to induce apoptosis in HSA hi CD4+CD8- thymocytes and the elimination of autoreactive cells is dependent on Fas-FasL mediated killing [163, 166].
EXTRATHYMIC T CELL MATURATION

In humans, the thymus atrophies at puberty while T cells continue to be present throughout life. Extrathymic T cell maturation (ETCM) has been proposed to occur in human liver [167], pregnant uterus mucosa [168], and the small intestine [21, 169, 170], paper II. T cell maturation outside thymus was first observed in mice where primarily small intestinal mucosa [75, 80, 81, 171] and liver [172, 173] were suggested sites. The evidence for thymus independent T cell differentiation in intestine comes originally from studies in athymic (nude) mice. The T cell population in these mice differs from T lymphocytes in conventional mice with respect to $\alpha\beta$ TCR+/$\gamma\delta$ TCR cell composition and expression of CD4 and CD8. Whereas the majority of thymus derived T cells are $\alpha\beta$ TCR+, Thy1+, CD4+CD8-, or CD4-CD8+$\alpha\beta$ TCR+, the predominant T cell population among gut IELs of nude mice is Thy1- and $\gamma\delta$ TCR+, of which a high percentage express CD8$\alpha\alpha$ homodimers [75, 81, 171, 174]. The latter cell population can also be found in euthymic (normal) mice. The CD8$\alpha\alpha$ homodimer is considered to be a marker for extrathymically matured T cells and can also be found on the $\alpha\beta$ TCR+ cells [174, 175]. Recently, IELs expressing the stem cell markers CD34, c-kit, IL-7R, CD122, CD16, and CD44 were identified in murine small intestine [176]. The presence of IELs with the phenotype of pluripotent precursors further supports the notion that the small intestinal epithelium is a site for ETCM.

Ongoing ETCM in the small intestinal mucosa of man was previously suggested by Lundqvist el al [21] who detected RAG1 mRNA expression in IELs of the T cell lineage and by Lynch et al who demonstrated that RAG1 and RAG2 mRNA was present in RNA extracted from crude preparations of small intestinal epithelium and LP [169]. In addition, the expression of $\gamma$T$\alpha$ mRNA has been demonstrated in IELs and LPLs of fetal intestine [170]. Furthermore, IELs expressing immature thymocyte markers such as CD1a, and CD2 and/or CD7 without expression of the CD3/TCR complex have been identified in human small intestine [21, 68]. The human small intestinal epithelium has been shown to produce IL-7, which could promote the development of intestinal lymphocytes [177], and to express MHC class II molecules which may participate in local selection processes as well as in antigen presentation [21]. This suggests that intestinal epithelial cells share differentiation-inducing capacities with thymic epithelial cells, supporting the possibility of TCR gene rearrangement and generation of extrathymically derived T cells.
SECONDARY V(D)J RECOMBINATION

B and T cells are able to change receptor specificity through subsequent receptor gene rearrangement. *Receptor editing* is referred to rearrangement among maturing lymphocytes occurring in central lymphoid organs while the term *receptor revision* is used for secondary rearrangement occurring among mature antigen reactive cells in the periphery [178]. In mouse studies, it has been shown that B cells involved in the GC reaction re-express the RAG enzymes, thus permitting a secondary V(D)J rearrangement of the Ig genes [179-182]. The secondary receptor rearrangement might be a mechanism to rescue failing B cells whose antigen receptor avidity has been decreased or lost as a result of somatic hypermutation or those debilitated by mutation [182]. Induction of RAG1 and RAG2 genes in GC lymphocytes may also reflect a mechanism to remove autoreactive cells that arise by mutation [180]. The receptor revision hypothesis has been challenged by studies showing that the B cells expressing RAG are newly formed cells rather than GC cells [183-185]. Thus the RAG1 expressing B cells could represent mature B lymphocytes reverting to an “immature-like” phenotype or immature B cells that are recruited in an ongoing immune response. In immature B lymphocytes in the bone marrow, a high BCR reactivity to self-antigen promotes receptor editing which often alters BCR specificity and salvages the self-reactive B cells from deletion [186, 187]. Receptor editing can also be provoked in bone marrow B cells by inadequate signaling due to underexpressed non-self reactive BCR. [188]. Secondary receptor rearrangement has also been shown in mouse thymocytes where receptor editing has been suggested to rescue self-reactive cells from deletion [187, 189]. Additionally, RAG re-expression and DNA recombination in peripheral CD4+ T cells has been described in a murine TCR β-chain transgenic model [190]. In this model, it was observed that self-reactive transgenic Vβ5+ T cells underwent extrathymic recombination altering their receptor specificity by endogenous Vβ-genes. Recently, RAG1 and RAG2 expression has also been detected in T lymphocytes in murine PPs [191]. Even though these cells expressed CD4 and CD8, e-kit, IL-7R, and pTα, i.e. markers characteristic of immature lymphocytes, the T cells were considered to be mature due to their high expression levels of Thy-1.2, CD3ε, and TCR β-chain; therefore, secondary rearrangement rather than de novo synthesis, was suggested [191].

In humans, the re-expression of RAG1 and RAG2 mRNA in tonsillar B cell subsets has also been described [192, 193]. In addition, expression of TdT, Igκ, RSS breaks and expression of the light-chain surrogate (ψ)-chain has
been found in human GC reactions [192] suggesting ongoing V(D)J recombination. V(D)J recombination and RAG1 and RAG2 expression has further been detected in human peripheral and tonsillar CD4⁺ T lymphocytes [194, 195].

**HOUSEKEEPING GENES**

Housekeeping genes are those genes that code for the many proteins which are essential for cell viability and structure, and are therefore expressed in most cells. To this end, levels of housekeeping gene mRNAs have been used as internal standards. The most commonly used housekeeping genes in this regard are glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, and 18S rRNA [196]. The GAPDH enzyme participates in the degradation of glucose in a process known as glycolysis. β-actin is one of the components of the cell cytoskeleton and is present in non-muscle cells. 18S ribosomal RNA (rRNA) is a part of the small ribosomal subunit that, together with the large subunit, is required in protein synthesis.

One prerequisite for usefulness in normalization is that the expression level of the housekeeping gene does not vary markedly throughout the cell cycle or in response to experimental conditions. The recent development of real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR), which allows determination of low concentrations of mRNA copies and thereby analyses of small samples where cell counting is not possible [90, 197, 198], papers II, III, IV], even more strongly necessitates reliable housekeeping genes for normalization. Since no single housekeeping gene always manifests stable expression levels under all experimental conditions or in all cells and tissues, it is necessary to characterize the suitability of internal RNA controls for the cell or tissue type in which transcription levels are being analyzed.
PART II – THE PRESENT STUDIES

AIMS OF THE THESIS

Thymus involutes at puberty and yet T cells exist in large numbers throughout life. In humans the site for T cell development later in life has not been satisfactory determined. The overall scope of this work was to examine the occurrence of extrathymic T cell maturation in man. The small intestinal mucosa and the nasopharyngeal tonsil were analyzed. Expression of recombination activating gene 1 (RAG1) and RAG2, two proteins exclusively associated with V(D)J recombination, was used as an indicator of ongoing gene-segment rearrangement in the process of generating functional antigen receptor genes. This is an obligatory step in T and B lymphocyte development. Expression of the preTα-chain (pTα), a vital component in the immature TCR-complex, was used as an indicator of ongoing V(D)J recombination of antigen receptor genes in lymphocytes of the T cell lineage. The specific aims were to:

▪ Evaluate the possibility that the small intestinal mucosa is a site for extrathymic T cell maturation in man by:
  o Estimation of ongoing TCR-gene segment recombination through analyses of RAG1, RAG2, and pTα expression in intestinal lymphocytes of the T cell lineage.
  o Examination of the presence of immature progenitor cells.
  o Exploring the possibility of ongoing negative selection by investigating the capacity of intestinal T lymphocytes to execute Fas/FasL mediated cytotoxicity.

▪ Explore the possibility that aberrant extrathymic T cell maturation and/or secondary T cell receptor gene rearrangement in the small intestinal mucosa is a contributing factor to the adverse T lymphocyte reactions in celiac disease.

▪ Investigate whether the nasopharyngeal tonsil with its strategic position in the upper aero-digestive tract is an additional site for extrathymic TCR-gene segment rearrangement in T cell maturation and/or antigen-driven TCR revision.

▪ Evaluate the suitability of frequently used housekeeping genes for normalization of mRNA expression levels in T lymphocytes.
SUMMARY OF INCLUDED PAPERS

Paper I


This study documented that IELs as well as LPLs of the human small intestine can act as effector cells in TCR/CD3-dependent cytotoxicity and demonstrated for the first time that a TCR/CD3-independent, spontaneous cytotoxicity is effectuated by the same cell populations. The TCR/CD3-dependent killing was demonstrated in an anti-CD3 mediated redirected cytotoxicity assay. CD8⁺TCRαβ⁺ lymphocytes accounted for virtually all the cytotoxicity, which was Ca²⁺ dependent suggesting that it was executed via the perforin/granzyme pathway. The TCR/CD3-independent killing was demonstrated in an assay where a Fas-expressing human T cell line was used as target. It was inhibited by antibodies interfering with the Fas/FasL interaction suggesting that it was executed via the Fas/FasL pathway. Perforin and FasL expressing lymphocytes were found both within the epithelium and in LP. FasL mRNA expression was shown in the CD8⁺ and TCRγδ⁺ subpopulations. Characterization of T cell subsets revealed a minor population of CD4⁺CD8⁺ double-positive cells in both IELs and LPLs. We speculated that cytotoxic CD8⁺TCRαβ⁺ T cells acting via the Ca²⁺ dependent TCR/CD3-mediated cytotoxicity are important in the clearance of virus-infected epithelial cells through perforin/granzyme exocytosis. The Fas/FasL-mediated cytotoxicity may reflect ongoing down-regulation of local immune responses, but could also play a role in the selection processes of extrathymically maturated T cells.

Paper II


Two new 5'-untranslated region (5'UTR) exons were identified in the human RAG1 gene. These 5'UTR exons were localized 5' to the previously described 5'UTR exon of the RAG1 gene and were therefore named 1A and 1B. The previously described 5'UTR exon was renamed 1C. The new 5'UTR exons 1A and 1B could be expressed in three different splice forms, none of which included the previously described 1C 5'UTR exon. All three mRNA
splice forms were expressed in jejunal lymphocytes of the T cell lineage with the highest expression levels occurring in immature CD2⁺CD7⁺CD3⁻ cells. This cell population also expressed high levels of mRNA for the preT α-chain. One of the new 5’UTR exons, the RAG1 1A, was present in two splice forms. None of these were expressed in thymocytes. In contrast, jejunal lymphocytes did not express the RAG1 splice form utilizing the previously described 5’ UTR exon 1C. The RAG1 and pTα expressing cells were located both intraepithelially and in LP of the jejunal mucosa. Cells expressing c-kit were present and were most frequent in the LP, indicating the presence of pluripotent precursors. Lymphocytes expressing markers associated with early stages of T cell development, e.g. CD1a and IL-7R were also identified in the small intestinal mucosa both intraepithelially and in the LP. These results strongly indicated ongoing T cell receptor gene rearrangement in human small intestinal mucosa and are compatible with the notion that the small intestinal mucosa is indeed a site for extrathymic T cell maturation in man. However, a parallel antigen-driven TCR revision cannot be excluded.

**Paper III**

Anna Bas, Göte Forsberg, Olle Hernell, Sten Hammarström, and Marie-Louise Hammarström. Changes in T cell receptor gene rearrangement in the small intestinal mucosa of children with celiac disease. *Manuscript*

RAG1 and pTα mRNA expression levels in intestinal lymphocytes of the T cell lineage were compared between children with untreated celiac disease (CD) and controls. TCRγδ⁺, TCRαβ⁺, and CD2⁺CD7⁻TCR⁻ lymphocytes were retrieved by sequential positive selection and expression levels for the 1A/2, 1A/1B/2, and 1B/2 mRNA splice forms of RAG1 were determined by qRT-PCR.

The expression level of RAG1 1A/2 was significantly lower in intraepithelial TCRγδ⁺, TCRαβ⁺, and CD2⁺CD7⁻TCR⁻ cells in CD patients as compared to controls. For the other splice forms, however, there was no significant difference between the two groups. Interestingly, the highest levels of RAG1 1A/2 mRNA were seen in the TCRγδ⁺ cells in both patients and controls. No age related changes in RAG1 mRNA levels were detected in this relatively small group of children (age 1-18 years; n=16). The implications of these findings are unclear. The high RAG1 levels in TCRγδ⁺ cells and relatively low levels in CD2⁺CD7⁻TCR⁻ cells may indicate that revision dominates over maturation in IELs of children and that the revision is impaired in the disease.
Summary of Included Papers

Paper VI

Anna Bas, Sten Hammarström, Sten Hellström, and Marie-Louise Hammarström. The nasopharyngeal tonsil - a site for T cell receptor gene rearrangement in man. Submitted

It was demonstrated that the nasopharyngeal tonsil/adenoid harbor a population of immature T cells with high expression levels of mRNA for proteins required for TCR-gene segment rearrangement, suggesting that the nasopharyngeal tonsil is a site for T cell development. Indications for ongoing antigen-driven receptor gene revision in both T and B cells were also obtained. Lymphocytes were isolated from chronically infected adenoids (CIA) and hypertrophic obstructive adenoids (HOA). Cells of the T and B cell lineages were isolated by sequential positive selection of CD3+, CD2−CD7−CD3−, and CD19+ cells, and analyzed for expression levels of RAG1, RAG2, pTαa, and TdT mRNA. mRNA for the four genes could be detected in all three cell populations analyzed and the CD2−CD7+CD3− cells exhibited the highest expression levels. Although the RAG1 mRNA 1C/2 splice form, expressed in thymocytes but not in intestinal T cells, showed the highest expression, two of the three new splice-forms, i.e. 1A/2 and 1B/2, were also expressed. Significant amounts of the third new RAG1 mRNA splice form (1A/1B/2) were only detected in CD3+ cells. In situ hybridization experiments showed that RAG1 and pTαa mRNA expressing cells co-localize and can be seen in the GC, the outer rim of the mantle zone, the interfollicular area, and adjacent to the surface epithelium. Interestingly, the expression levels of the new RAG1 mRNA splice forms 1A/2 and 1B/2 were significantly higher in the total lymphocyte population of HOA compared to that of CIA, suggesting different needs for peripheral antigen receptor gene rearrangement in the two groups. These results showed that tonsillar lymphocytes of T cell lineage have a fully competent molecular machinery for V(D)J recombination and thereby strongly suggested that the nasopharyngeal tonsil is a site of T lymphocyte ontogeny and most likely also a secondary TCR revision.

Paper V

Anna Bas, Göte Forsberg, Sten Hammarström, and Marie-Louise Hammarström. Utility of the housekeeping genes 18S rRNA, β-actin, and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) for normalisation in real-time quantitative RT-PCR analysis of gene expression in human T lymphocytes. Submitted

This study showed that 18S rRNA is a suitable housekeeping gene for normalization of gene expression analyses estimated as mRNA expression
levels in human T lymphocytes. Using qRT-PCR, the levels of 18S rRNA, β-actin and GAPDH mRNA were analyzed in freshly isolated peripheral blood mononuclear cells (PBMC) and in PBMCs subjected to polyclonal T cell activation for up to 96 hours. Careful cell counting was performed at each time-point. IL-2, IL-4, IL-10, IFN-γ, TNF-α and TGF-β1 were selected for quantitative analysis of mRNA levels in PBMCs before and after activation. While the GAPDH and β-actin mRNA expression level per cell fluctuated markedly upon activation, the 18S rRNA level per cell remained almost unchanged. Comparison of cytokine mRNA levels, expressed as mRNA copies per cell or as mRNA copies per unit housekeeping gene, showed that normalization to 18S rRNA was consistent with the cytokine expression level per cell while normalization to the other two genes was not. We concluded that 18S rRNA was the most stable housekeeping gene and hence superior for normalization in comparative analyses of gene mRNA expression levels in human T lymphocytes.
GENERAL DISCUSSION

In order to rearrange the TCR genes, developing T lymphocytes have to receive proper signals from their microenvironment and express molecules necessary for induction and progression of V(D)J recombination. One of factors that drives the V(D)J recombination in thymus is IL-7 \[199\]. Thus it should be expressed in the intestinal milieu in order to support TCR rearrangement. Indeed, we demonstrated that both IELs and LPLs should be responsive to IL-7 since as many as 40% expressed its receptor IL-7R \[paper II\] and small intestinal epithelial cells were previously shown to produce IL-7 \[177\]. In addition, the presence of pluripotent cells that could be recruited into TCR-gene recombination was indicated by the fact that \(\sim 15\%\) of the LPLs and \(\sim 3\%\) of the IELs expressed c-kit \[paper II\]. The presence of immature T cells was suggested by the presence of both IELs and LPLs that were DP for CD4 and CD8, expressed CD1a, and CD2 and/or CD7 without expressing the CD3/TCR complex \[papers I and II\].

The next criterion that has to be fulfilled is the expression of RAG1 and RAG2, two molecules essential for the initiation and execution of TCR and BCR gene rearrangement \[140\]. Previously, the expression of RAG1 mRNA has been demonstrated in jejunal IELs of the T cell lineage \[21\] and mRNA for both genes in RNA extracted from crude preparations of small intestinal epithelium and LP \[169\]. In these studies, DNase treated RNA and primers placed in exon 2, were used in the RT-PCR analyses. In paper II, we constructed a qRT-PCR assay for RAG1 mRNA on the basis of the more recently published organization of the RAG1 gene \[143\], i.e. the 5’- and 3’-primers were placed in the 5’UTR exon 1 (now termed 1C, paper II) and exon 2, respectively. Unexpectedly, no signal was seen in the samples previously demonstrated to be positive for RAG1 mRNA expression in the experimental setup where primers were placed in exon 2. Furthermore, no signal was detected even when these samples were reanalyzed in a more sensitive, nested qRT-PCR assay, with the 5’-primer placed in exon 1. Based on the contradicting results, a 5’-RACE was performed to investigate whether the results could be explained by the existence of alternative 5’UTR RAG1 exon(s) that were used by jejunal T lymphocytes. Two new 5’UTR exons spliced into three new splice variants were identified in RNA extracted from jejunal T cells. In accordance with our observation, the previously published 5’UTR exon 1 was not present in the sequence obtained from the 5’RACE. The new 5’UTR exons were named 1A and 1B according to their order relative to exon 2 and the previously described 5’UTR exon 1. The latter was located between the new exons and exon 2 and was renamed 1C. qRT-PCR
assays with RNA copy standards were constructed to analyze the expression levels of the new RAG1 mRNA splice forms. The assays were designed to discriminate between splice forms having exon 1A or 1B spliced to exon 2 and to estimate the frequency of mRNA containing the long splice form with the 1A exon spliced to the 1B exon. All three new splice variants were expressed in mature CD3⁺ and immature CD2⁺CD7⁺CD3⁻ intraepithelial- and lamina propria cells. There was a tendency for higher expression levels of the 1A/2 and 1B/2 RAG1 mRNA forms in CD2⁺CD7⁺CD3⁻ cells compared to CD3⁺ cells. RAG1 mRNA expression in jejunal IELs and LPLs was confirmed by *in situ* hybridization with a probe binding to the RAG1 1B/2 splice form.

Expression of RAG1 mRNA in murine IELs was demonstrated already in 1991 [75]. However, in a recent report from the same group only minute amounts of RAG1 mRNA expressing IELs could be detected [200]. The main difference between the studies is implementation of the RT-PCR assay. In the first study both primers were placed in exon 2, which in mice also contains the entire coding sequence, while RT-PCR with the 5’-primer placed in the 5’UTR exon 1 and the 3’-primer in exon 2 was used in the second study. Given the similar gene organization and the high sequence homology between the human and the murine RAG1 exon 2, i.e. 80% at the nucleotide level and as much as 96% at the protein level, it seems likely that the murine RAG1 gene also contains additional 5’UTR exons. Thus, possible differential expression of RAG1 mRNA splice forms utilizing yet unidentified 5’UTR exons could explain their difficulties in demonstrating significant amounts of RAG1 mRNA expressing cells in murine small intestine in the later study.

Analysis of the expression of the different RAG1 mRNA splice forms in thymus revealed that 5’UTR exon 1A, and thus splice forms 1A/2 and 1A/1B/2, was not utilized in thymocytes. This finding, together with the fact that exon 1C is not utilized by jejunal T cells, suggested tissue specific expression of the three RAG1 5’UTR exons. Although the four splice forms of RAG1 mRNA differ in their 5’UTRs, they splice into the same site upstream of the translation start site in exon 2. Thus, they all encode the same protein. Relatively little is known about the role(s) of selective expression of mRNAs differing only in the 5’UTR. In analogy with RAG1, *Drosophila* ferritin mRNA was shown to have four different mRNA splice forms generated by alternative splicing of 5’UTR exons [201]. Only one of them contained the iron-responsive element and exhibited regulation of translation activity dependent on iron concentration. Alternative use of 5’UTR exons has also been shown to correlate with regulation of developmental and tissue specific expression of the human growth hormone receptor [202]. Thus the different 5’UTR splice forms of RAG1 mRNA may regulate both tissue specific
expression and the amounts of protein produced.

All three new splice forms of RAG1 mRNA were also expressed in the small intestinal mucosa in young children [paper II and III]. When the total T cell population was analyzed, RAG1 mRNA was detected in IELs and in occasional LPL samples [paper II]. RAG1 mRNA expression was further seen when cells of the T cell lineage were enriched by sequential retrieval of γδTCR+, αβTCR+, and CD2+CD7+TCR− cells from IEL samples [paper III]. In all three cell fractions, RAG1 1A/2 was the splice form with the highest expression level although 1B/2 was also present. 1A/1B/2 was detected in γδTCR+ and αβTCR+ cells but not in CD2+CD7+TCR− cells. Expression of RAG1 mRNA in TCR+ cells suggests ongoing TCR editing or revision, while RAG1 expression in CD2+CD7+TCR− cells suggests \textit{de novo} generation of TCR in locally maturing T cells. As demonstrated in paper II, the majority of CD2+CD7+CD3− cell samples of adults expressed RAG1 mRNA both from the epithelium and from the LP. It is tempting to speculate that the extent of extrathymic T cell maturation increases with age with significant involvement of cells residing in the LP later in life.

In papers II and III, we identified for the first time in the human jejunal mucosa the presence of lymphocytes of the T cell lineage expressing mRNA for pTα+, a vital molecule in the rearrangement of TCR genes of αβ T cells. Expression of pTα+ mRNA was demonstrated in jejunal IELs and LPLs of both children and adults. pTα+ mRNA expressing cells were present both within the epithelium and in LP, as shown by \textit{in situ} hybridization. Interestingly, the expression level of pTα+ mRNA was particularly high in immature T cells (CD2+CD7+CD3− and CD2+CD7+TCR− cells in adults and children, respectively). Detection of pTα+ mRNA required an enhanced nested qRT-PCR, which also allowed detection of pTα− mRNA in the Jurkat T cell leukemia cell line. Previously, pTα− mRNA could not be detected in this cell line, most likely due to lower sensitivity of the assay used [153].

Celiac disease (CD) is a small intestinal enteropathy characterized by permanent intolerance to gluten and can be seen as a consequence of failure to generate and/or maintain tolerance to this common food antigen. Previous studies point toward a central role of T cells in the disease process. Hence aberrant extrathymic TCR gene rearrangement could be a contributing factor for contraction of CD. Comparative analyses between expression levels of mRNA for the three new RAG1 splice forms and pTα+ in γδTCR+, αβTCR+, and CD2+CD7+TCR− IELs of children with CD and controls was therefore performed [paper III]. Expression levels of the RAG1 1A/2 splice form were
decreased in CD in all three studied cell fractions. This supports the notion that impaired TCR gene rearrangement could be a contributing factor to CD. Recently, Forsberg et al [70] have shown that production of IL-2 is significantly reduced in newly diagnosed CD patients with active disease. An impairment of intraintestinal T lymphopoiesis due to IL-2 deficiency in mice has been reported to associate with intestinal inflammation and histological changes resembling those seen in CD, i.e. crypt hyperplasia and an increased number of lymphocytes [203]. Additionally, it was established that the intestinal pathology was dependent on the presence of thymus-derived T cells. Thus, the intestine-derived T lymphocytes seem to play an important role in regulating the function of thymus-derived T cells within the intestinal mucosa, and consequently, a balance of thymus and gut derived T lymphocytes in the intestinal mucosa could be important for the maintenance of gut integrity.

IELs and LPLs in normal human small intestine exhibit cytolytic activity upon cross-linkage of the TCR/CD3 complex [71], paper I]. We found that the killing was mediated via perforin/granzyme exocytosis, and the main cell population responsible for the cytotoxicity was shown to be CD8⁺ αβ T cells [paper I]. In accordance with this we demonstrated that mRNA for both perforin and granzyme B was present in CD8⁺ IELs and LPLs. The proportion of cells expressing the perforin protein was, however, small (~2.5% of the IELs). These cells could either represent active CTL caught in the act of their effector function or CD8⁺ memory cells programmed for cytolytic responses [12]. The perforin/granzyme mediated cytotoxicity has been suggested to eliminate virus infected cells [204] and the mucosal cytolytic CD8⁺ cells may thus be important for prevention of recurrent viral infection by removal of infected epithelial cells.

In paper I we further demonstrated that IELs and LPLs spontaneously killed Jurkat cells (Fas expressing TCRαβ⁺CD4⁺ cells) in a TCR/CD3 independent manner suggesting that cytolytic responses in small intestinal mucosa can be mediated via Fas/FasL interactions. We confirmed the expression of FasL in jejunal T lymphocytes by immunohistochemical analysis and by RT-PCR. In thymus, T cells failing the negative selection are eliminated through a CD28-mediated process and also through Fas/FasL mediated cytotoxicity [163, 164, 166]. The Fas/FasL mediated pathway in the small intestine could thus either be a triggering event in AICD [205] or may participate in maintaining local tolerance towards dietary antigens through T cell selection processes. Epithelial cells in the human small intestine have been shown to express MHC class II molecules and thus fulfill another of the conditions for local T cell selection [21].
All four RAG1 mRNA splice forms and pT\(\alpha\) mRNA were expressed in PBMC and in mononuclear cells of the bone marrow [paper II]. The finding that RAG mRNA is expressed in PBMC although at very low levels, is in good agreement with the recent reports on RAG1 and RAG2 mRNA expression and TCR-gene rearrangement in blood CD4\(^+\) T cells [194, 195]. The samples of bone marrow cells were not fractionated with respect to expression of markers for the T and B cell lineages. However, the pT\(\alpha\) mRNA was expressed in bone marrow cells [paper II], suggesting the presence of T cell precursors and thereby raising the possibility that the RAG1 mRNA signal may, at least in part, come from this cell population. Furthermore, the onset of differentiation into the T cell lineage before migration to the thymus has been suggested by Klein et al [206] who have demonstrated that pT\(\alpha\), RAG1, and RAG2 mRNAs are expressed in T cell lineage precursors in the human bone marrow. Additionally, in a transgene mouse strain with human CD25 as a pT\(\alpha\)-controlled reporter gene, it was recently shown that the bone marrow harbors CD19 lymphoid precursors that express pT\(\alpha\) mRNA [207]. These cells were, however, not committed for development into \(\alpha\)\(\beta\) T cells, and pT\(\alpha\) was even expressed before the onset of TCR gene rearrangement. Thus it is possible that most, if not all, pT\(\alpha\) mRNA detected in bone marrow mononuclear cells, and also in PBMCs, is derived from lymphocyte precursors destined for the T cell lineage that are generated in the bone marrow and migrate to different tissues (fig. 5) via the blood.

Expression of the new RAG1 mRNA splice forms was also investigated in the nasopharyngeal tonsil/adenoid [paper IV]. The rationale behind the choice of tissue was: 1) the results from analysis of jejunal T cells had suggested the possibility of antigen-driven TCR revision in man. The nasopharyngeal tonsil is considered the major inductive site for immune reactions against inhaled antigens and hence should contain numerous activated T cells in which antigen-driven TCR revision might take place. 2) Previous demonstration of RAG1 mRNA in peripheral mouse B cells was interpreted as a reflection of antigen driven receptor revision in these cells [179-182]. Similar studies suggesting antigen receptor revision in humans have been performed using tonsillar B cells [192, 193]. We therefore addressed the question of whether any of the new RAG1 mRNA splice forms is expressed in B cells during antigen receptor revision. We confirmed the expression of RAG1 1C/2 mRNA in B cells and additionally demonstrated for the first time the presence of the alternative RAG1 mRNA splice forms 1A/2 and 1B/2 in these cells at expression levels 4 to 10-fold lower than the 1C/2 splice form [paper IV]. Thus the RAG1 mRNA splice variants are not exclusive for cells
of the T cell lineage, but can also be utilized by B cells. We further demonstrated expression of RAG1 and RAG2 mRNAs in tonsillar lymphocytes of the T cell lineage. A small population of lymphocytes with the immature phenotype CD3^−CD2^+CD7^+ was identified. This population had the highest expression levels of RAG1, RAG2, pTα^α^, and TdT mRNAs. The last of these contributes to increases in diversity during receptor gene recombination and is typically expressed in thymocytes. The “classical” 1C/2 splice form had the highest expression level, but RAG1 1A/2 and 1B/2 mRNAs were also expressed at significant levels. In mature CD3^+^ T cells, all four of the RAG1 mRNA splice forms were detected and the 1B/2 splice form was expressed at levels similar to those in the immature T cells, again pointing to a role for this splice form during TCR editing or revision. In situ hybridization revealed that RAG1 1B/2, RAG1 1C/2, and pTα^α^ mRNA expressing cells were located in the proximity of the surface epithelium, inside the follicles, at the outer rim of the mantle zone, and also in the interfollicular space. According to our hypothesis of different RAG1 mRNA splice form utilization during de novo synthesis and TCR revision (see paper II and below), expression of all RAG1 mRNA splice forms in tonsillar T lymphocytes suggests that the nasopharyngeal tonsil is a site where extrathymic T cell development and antigen driven TCR revision are occurring in parallel. The location of the nasopharyngeal tonsil in the nasopharynx predisposes it to constant stimulation by antigens that enter the body through the respiratory tract. The nasopharyngeal tonsil shares at least two properties with the thymus: T cell development and regression at puberty. It is therefore quite feasible that it not only functions as an inductive organ but also as a primary lymphoid organ.

The analyses of RAG1, RAG2, and pTα^α^ mRNA expression in the nasopharyngeal tonsil/adenoid were performed by using tissue from patients belonging to two diagnostic groups; patients with chronically infected adenoids (CIA) associated with middle ear disease and patients with hypertrophic obstructive adenoids (HOA). It has been proposed that adenoid hypertrophy is caused by an antigen-dependent increase in activity of B lymphocytes [133]. We found that the expression of RAG1 1A/2 and 1B/2 mRNA splice forms was significantly higher in the total mononuclear cell fraction purified from HOAs as compared to the same cells isolated from CIAs. Because the comparative analysis was performed on the total AMC population, either T or B lymphocytes, or both, could be responsible for the elevated signals. Since B lymphocytes in HOAs have been postulated to increase their activity due to antigen stimulation, the enhanced recombination
activity in HOAs may be explained by increased B cell receptor revision in response to incoming antigens. However, elevated receptor rearrangement in HOAs may also be due to increased T cell receptor revision or to local T cell maturation. Our group has previously demonstrated that TCRαβ+ cells constitute the dominant intraepithelial T cell population in CIA, whereas TCRγδ+ cells constitute the major T cell population in the surface epithelium of HOA, suggesting distinct T cell responses in the two groups [123]. Since IL-7, which especially appears to promote the differentiation of pre-T cells into γδ T cells [208], is expressed in tonsils [209], and IL-7R was demonstrated to be present on adenoid T cells [paper IV], the γδ T cells found in the intraepithelial compartment of HOAs could originate from an extensive local rearrangement process. Thus, the difference in intraepithelial T cell composition between HOAs and CIAs could be a reflection of the elevated RAG1 mRNA expression and consequent γδ T cell development in HOAs.

**Figure 5.** Schematic drawing of the expression pattern of various RAG1 mRNA splice forms in T cell development and in T cell precursors during migration in man as suggested from the results of paper II and IV.

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Based on the expression pattern of mRNA for RAG1 splice forms and pTα in the small intestine, nasopharyngeal tonsil, bone marrow, thymus, and blood, we propose a model for T cell maturation in man (schematically drawn in Figure 5). pTα mRNA expressing common lymphoid precursors develop in the bone marrow and migrate via the blood to either the small intestine, the nasopharyngeal tonsil, or the thymus where they continue differentiation and selection in the context of an appropriate cellular and molecular environment. The 1A/2 and 1A/1B/2 splice forms of RAG1 mRNA are expressed in jejunum, nasopharyngeal tonsils, and bone marrow but not in thymus (Fig. 6). We therefore suggest that T cell precursors expressing either of these two splice forms migrate directly from the bone marrow to the small intestine and nasopharyngeal tonsil and that these splice forms are utilized during de novo TCR-gene recombination at these sites. The 1C/2 splice form of RAG1 mRNA is expressed in the thymus, nasopharyngeal tonsil, and bone marrow but not in the small intestine.

![Figure 6. Expression levels of various RAG1 mRNA splice forms in lymphocytes of different tissue origin. Expression levels are shown as median copy number per 18S rRNA unit.](image)

We therefore suggest that T cell precursors expressing this splice form migrate to the thymus and to the nasopharyngeal tonsil where this splice form is used during de novo TCR-gene recombination. The 1B/2 splice form of RAG1 mRNA was expressed in the small intestine, the nasopharyngeal tonsil and in the thymus, suggesting a shared function. We propose that cells using this splice form are undergoing TCR editing/revision in these tissues. The finding that cortical thymocytes expressing an autoreactive TCR can undergo a secondary rearrangement which allows it to edits its receptor specificity,
supports the concept of TCR editing in the thymus [189]. The marked difference between the expression levels of 1B/2 and the 1C/2 splice forms in the thymus is compatible with this idea since TCR editing is supposedly much less frequent than \textit{de novo} TCR-gene rearrangement at this site. In the small intestine and nasopharyngeal tonsil, TCR receptor revision is probably induced upon antigen encounter and may involve both intra- and extrathymically matured T lymphocytes. A secondary TCR rearrangement at these sites may serve to increase the TCR affinity. Another interesting possibility is that the receptor revision serves to decrease reactivity against food antigens, thereby playing a role in maintenance of oral tolerance.

The choice of housekeeping genes as internal standards in real-time qRT-PCR is critical for the estimation and comparison of mRNA levels in gene expression studies. Since cell counting is sometimes difficult or impossible, expression levels of mRNA of interest have to be normalized to RNA for housekeeping genes. Because the levels of housekeeping genes may vary between tissues, cell types, during the cell cycle, or in response to different experimental conditions, it is crucial to evaluate the stability and reliability of the chosen normalizing gene. In paper V, three frequently used housekeeping genes, $\beta$-actin, GAPDH, and 18S rRNA, were evaluated for their accuracy as indicators of cell number when used for normalization in gene expression analysis of T lymphocytes. Quantitative real-time RT-PCR was used to determine the expression levels of the studied housekeeping genes and also of mRNAs for six cytokines in resting human PBMCs and PBMCs subjected to polyclonal T cell activation. The cytokines were chosen as model genes in analysis of mRNA expression levels. Our results demonstrated that during 96 hour activation, 18S rRNA showed very little fluctuation as compared to $\beta$-actin- and GAPDH mRNAs, the levels of which fluctuated markedly upon activation. 18S RNA was thus the most stable housekeeping gene under these conditions. When isolated $\gamma\delta$TCR$^+$, CD4$^+$, and CD8$^+$ subpopulations were studied, 18S rRNA levels remained unchanged after 21 h of activation but increased slightly after 96 h. In contrast, there was a 30 to 70-fold increase of GAPDH mRNA per cell in these cell populations upon activation. Furthermore, a great variation in expression of $\beta$-actin mRNA in lymphocytes also became evident by \textit{in situ} hybridization in tonsillar and intestinal tissues, i.e. tissues rich in activated lymphocytes. As $\beta$-actin is considered to be expressed by virtually all cells, the low frequency of $\beta$-actin mRNA positive cells was surprising (Fig. 7). Cytokine analysis revealed that only normalization to 18S rRNA gave a result that satisfactorily reflected their mRNA expression levels per cell. In addition, we found that freshly isolated
PBMC and intestinal intraepithelial and lamina propria lymphocytes all had a similar 18S rRNA content per cell. While T lymphocytes in PBMC are resting with no or low cytokine production [paper V, [90], intestinal lymphocytes contain T cells producing cytokines at levels similar to polyclonally activated blood T lymphocytes, and also exhibit cytotoxic effector functions [paper I, [71, 90, 210]. This supports the conclusion that 18S rRNA is suitable for normalization when quantifying mRNA levels of genes expressed in resting and activated human lymphocytes of blood and tissue origin. Therefore, 18S rRNA was used for normalization throughout my studies of RAG1, RAG2, and pTα mRNA expression.

**Figure 7.** *In situ* hybridization of jejunal mucosa (a) and palatine tonsil tissue (b) with a digoxigenin-labeled RNA probe specific for β-actin mRNA. Arrowheads indicate examples of positive intraepithelial lymphocytes, and arrows indicate positive lamina propria lymphocytes. E, epithelium; LP, lamina propria; CR, crypt; GC, germinal centre; IF, interfollicular area; M, mantle zone
CONCLUSIONS

I. Two new 5'UTR exons were identified in the human gene for RAG1. They are both located 5' to the previously described 5'UTR exon and named 1A and 1B according to their juxtaposition to the previously described 5'UTR exon (renamed 1C) and exon 2 which contains the entire coding sequence. The 1A 5'UTR exon was located at a considerable distance from the other exons (52.8 kb from exon 1B) while the distance between exons 1B and 1C (4.5 kb) was similar to that between exons 1C and 2 (5.2 kb).

II. The RAG1 gene can be expressed in four mRNA splice forms. Three of these include the new 5'UTR exons, i.e. 1A/2, 1B/2, and 1A/1B/2, and the fourth includes the previously described 1C 5'UTR exon (1C/2). None of the splice forms include the new 1A or 1B exons spliced to the 1C exon.

III. The different splice forms have distinct tissue expression. The 1A exon is expressed outside the thymus. The 1A/2 splice form is expressed in lymphocytes located in the small intestinal mucosa, the nasopharyngeal tonsil, the bone marrow, and the blood with similar expression levels at all four locations. The 1A/1B/2 splice form is preferentially expressed in small intestinal lymphocytes of T cell lineage. The 1B/2 splice form is expressed at similar levels in small intestinal mucosa and thymus and also expressed at the other three locations investigated, although at lower levels. The 1C/2 splice form is not expressed in the small intestinal mucosa but at all other sites investigated. Interestingly, there are marked differences in expression levels. Lymphocytes in the nasopharyngeal tonsil and the bone marrow have similar expression levels that are approximately 1000-fold lower than in the thymus but 10-fold higher than in blood. All four splice forms encode the same protein. Thus, the different splice forms may regulate both the tissue specific expression and the amounts of protein produced e.g. by different mRNA half-life and responsiveness to as yet undefined signals in the microenvironment.

IV. The demonstration of RAG1, RAG2, and pTα mRNA expression in lymphocytes of the T cell lineage in the small intestinal mucosa and the nasopharyngeal tonsil strongly suggests that these organs are sites for TCR gene rearrangement in man.
Conclusions

V. Thymocyte-like cells expressing CD2 and/or CD7 without expression of the CD3/TCR complex were identified both in small intestinal mucosa and in the nasopharyngeal tonsil. These cells had the highest expression levels of RAG1, RAG2, and pTα mRNAs. These signs of ongoing TCR gene rearrangement in immature T cells strongly indicate that both tissues are sites for T lymphocyte development. This notion is further supported by the presence of cells expressing IL-7R, and in the small intestine, CD1a+ and CD4CD8 DP cells. Unlike in the thymus, T cell development at these two sites seems to take place in parallel to adaptive immune reactions, i.e. antigen-driven, T cell dependent B cell activation in nasopharyngeal tonsils and CTL cytotoxicity and antibody production in the intestinal mucosa, and it involves only a small proportion of the lymphocytes. Furthermore, the lamina propria of the small intestinal mucosa appears to harbor pluripotent precursors as indicated by c-kit expression.

VI. The distinct preferential use of RAG1 mRNA splice forms in immature T cells of nasopharyngeal tonsils (1C/2) and small intestine (1A/2) suggests different maturation pathways or regulatory mechanisms of differentiation.

VII. The present study also indicates TCR editing both in the small intestinal mucosa and in the nasopharyngeal tonsil since RAG1 mRNA was demonstrated to be present in lymphocytes with surface expression of CD3 and/or TCR. Presently we cannot distinguish whether the TCR editing is a component driven by selection processes in the late stages of T cell maturation or an antigen-driven revision of TCR specificity.

VIII. Our results revealed an impaired utilization of the RAG1 1A/2 mRNA splice form in small intestinal intraepithelial T lymphocytes of children with celiac disease. This may implicate a deteriorated TCR gene rearrangement in this disease.

IX. Amounts of 18S rRNA accurately correlate with the number of T lymphocytes at different activation stages and are therefore suitable for normalization when measuring gene expression in comparative analyses.
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REFERENCES


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