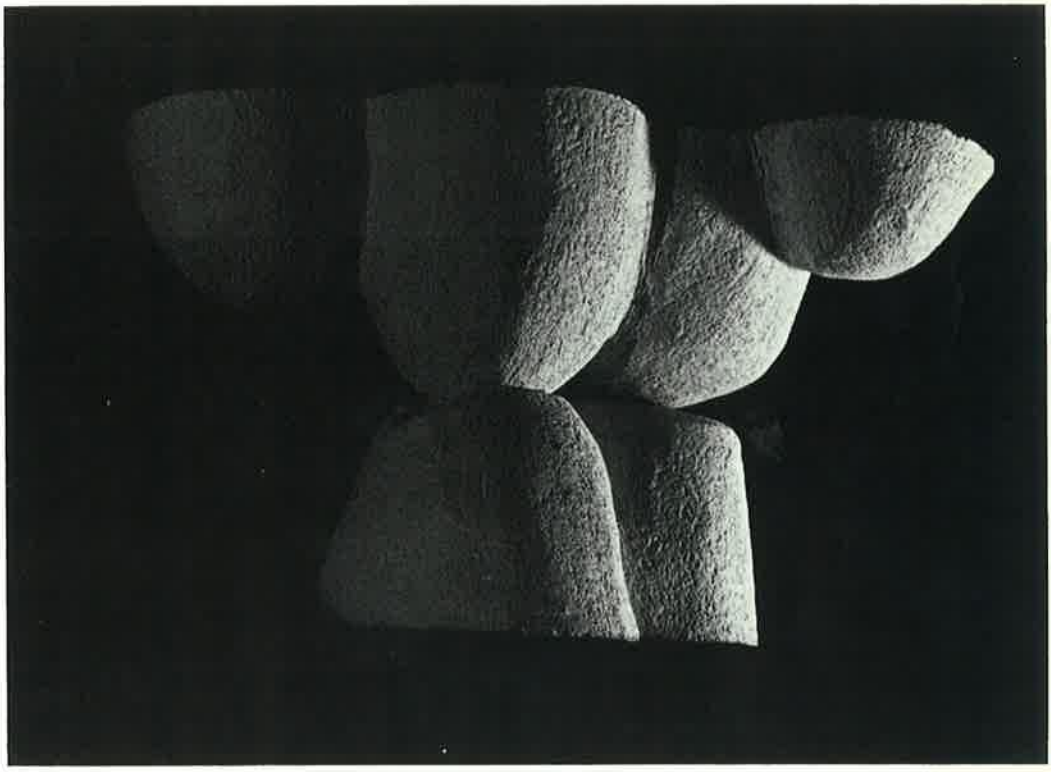
DIFFERENTIATION AND CHARACTERIZATION OF MURINE INTESTINAL INTRAEPITHELIAL LYMPHOCYTES



Bernard de Geus

DIFFERENTIATION AND CHARACTERIZATION OF MURINE INTESTINAL INTRAEPITHELIAL LYMPHOCYTES

CIP- GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Geus, Bernard de

Differentiation and characterization of murine intestinal intraepithelial lymphocytes / Bernard de Geus. - Leiden : IVVO-TNO, Gaubius Laboratorium. -Ill.
Proefschrift Leiden. - Met lit. opg.
ISBN 90-5412-004-5
Trefw.: immunologie

The printing of this thesis was financially supported by:

- TNO institute of Ageing and Vascular Research (IVVO), Leiden, The Netherlands

- FILTRON technology bv, Breda, The Netherlands

- ITK diagnostics bv, Uithoorn, The Netherlands

Cover design and photography by Marco Luiten and Bernard de Geus

STELLINGEN

1. Sommige B cellen zijn in feite T cellen.
   Dit proefschrift.

2. TcR$\gamma\delta$ positieve T cellen zijn geen evolutionair artefact.

3. Immuunhistologie ondersteunt FACS analyse, en vice versa.
   Dit proefschrift.

4. Denaturerende gradient gel electroforese (DGGE) is een bruikbare techniek om TcR of Ig gendiversiteit te bepalen.
   Fischer, S. G. en Lerman, L. S. 1983. Proc. Natl. Acad. Sci. USA. 80: 1579.

5. De conclusie van Lefrancois dat TcR$\gamma\delta^{+}$Thy-1^{+} iIEL cytolytische activiteit hebben, is niet gerechtvaardigd.
   Lefrancois, L. 1991. Immunol. Today 12: 436.
   Goodman, T. en Lefrancois, L. 1989. J. Exp. Med. 170: 1569.

6. De suggestie dat iIEL van belang zijn voor de instandhouding van het darmepitheel is onjuist.
   Dit proefschrift.

7. Het concept van contrasuppressie in het mucosa geassocieerde immuunsysteem is verwarrend en onduidelijk zolang de gepostuleerde contrasupressor cel niet geïdentificeerd is.
   Fujihashi, K. et al. 1990. J. Immunol. 145: 2010.
   Fujihashi, K. et al. 1992. J. Exp. Med. 175: 695.

8. De aanwezigheid van mRNA, coderend voor het RAG1 recombinatie eiwit, in iIEL subsets is geen argument om te stellen dat recombinatie processen plaatsvinden in het darmepitheel.
Guy-Grand, D. et al. 1991. J. Exp. Med. 173: 471.
Guy-Grand, D. et al. 1992. Eur. J. Immunol. 22: 505.

9. Gezien de grote individuele variatie van antigene belasting in de darm van de mens zijn onderzoeks resultaten naar iIEL bij laboratorium-muizen ,die onder identieke condities gehuisvest zijn, moeilijk te extrapoleren naar de mens.
Lefrancois, L. en Goodman, T. 1989. Science 243: 1716.
Bandeira, A. et al. 1990. J. Exp. Med. 172: 239.
Dit proefschrift.

10. Het 'gat' in de ozonlaag heeft als voordeel dat DNA in een agarose gel, mits aangekleurd met ethidium bromide, aangetoond kan worden door deze gel op een zonnige dag uit het raam te houden.

11. De vis, gevangen door een sportvisser, wordt duur betaald.

Stellingen behorend bij het proefschrift
"Differentiation and characterization of murine intestinal intraepithelial lymphocytes"
Bernard de Geus
Leiden 25 juni 1992

DIFFERENTIATION AND CHARACTERIZATION OF MURINE INTESTINAL INTRAEPITHELIAL LYMPHOCYTES

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR AAN DE RIJKSUNIVERSITEIT TE LEIDEN, OP GEZAG VAN DE RECTOR MAGNIFICUS DR. L. LEERTOUWER, HOOGLERAAR IN DE FACULTEIT DER GODGELEERDHEID, VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN TE VERDEDIGEN OP DONDERDAG 25 JUNI 1992 TE KLOKKE 16.15 UUR

DOOR

BERNARD DE GEUS

GEBOREN TE VLAARDINGEN IN 1960

1992
PASMANS OFFSETDRUKKERIJ BV, 's-GRAVENHAGE

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Overige leden:	Prof. dr. C. J. M. Melief	RUL
	Prof. dr. A. M. Kruisbeek	NKI

CONTENTS

1 GENERAL INTRODUCTION

1.1

Introduction

The immune system is an unique organ system that has evolved to protect the individual against invading micro-organisms, such as viruses and parasites and possibly against transformed malignant cells. To perform this function, the immune system is composed of a specific and a nonspecific limb. The nonspecific limb of the immune system includes macrophages and granulocytes. In contrast, the specific immune system is almost completely composed out of lymphocytes which can be distinguished in the humoral and the cellular limbs of the specific immune system. The humoral immune system comprises B cells, which produce antibodies. Antibodies are antigen binding proteins, generated upon encountering and subsequent recognition of antigen. The response of the humoral immune system is regulated by T helper cells. Together with cytotoxic T cells and natural killer (NK) cells, T helper cells form the cellular immune system. Important characteristics for proper function of the specific immune system are immunologic memory and the ability to make a distinction between 'self' and 'nonself'. Memory is a feature of both the humoral and in the cellular immune system. In contrast, discrimination between 'self' and 'nonself' is a property of T cells which are largely dependent on the thymus for differentiation. These T cells are educated in the thymus to recognize foreign antigens in the context of 'self' molecules encoded by the major histocompatibility complex. T cells can be divided into two populations of cells, based on expression of their antigen specific T cell receptor (TcR), one major population expressing the TcR$\alpha\beta$ and one minor population expressing the TcR$\gamma\delta$. In contrast to the peripheral immune system, such TcR$\gamma\delta$ expressing cells form a predominant fraction of the epithelium- associated T cells of the mucosa-associated lymphoid tissue. Especially the epithelium of the small intestine contains high numbers of T cells expressing the TcR$\gamma\delta$. Despite the abundant presence of T cells in the intestinal epithelium, the function and origin of these intestinal intraepithelial lymphocytes (iIEL) remains largely unclear. This thesis describes an analysis of the phenotypical and differentiational

characteristics of this fascinating population of iIEL in the mouse.

1.2

The T cell receptor

Two types of T lymphocytes expressing a monospecific T cell receptor (TcR) have been identified in mouse and man: one expressing the TcR$\alpha\beta$ [1-3] and another, expressing the TcR$\gamma\delta$ [4-6].

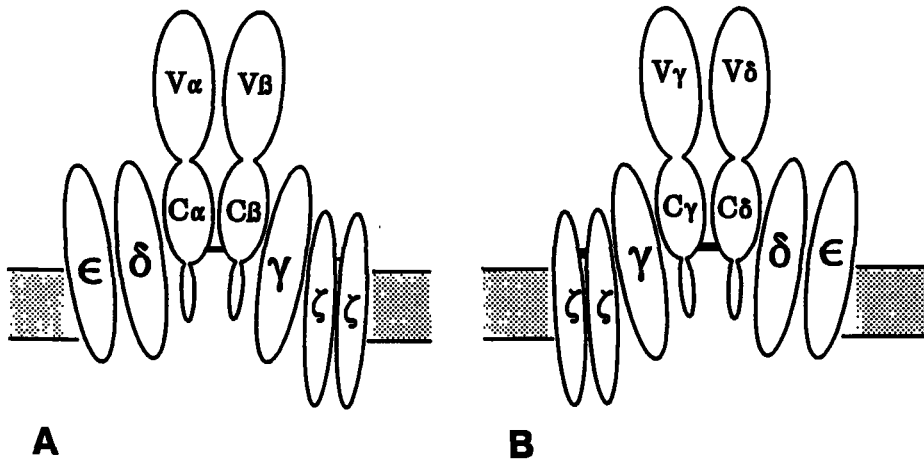

Figure 1. Figure A and B respectively represent the TcR$\alpha\beta$/CD3 complex and the TcR$\gamma\delta$/CD3 complex. The individual TcR chains can be subdivided into various domains: The Constant (C) domain contains a hydrophobic transmembrane segment which anchors the TcR chain in the cell membrane. The Variable (V), Joining (J) and Diversity (only present in the β and δ TcR chains) domains contribute to TcR chain diversity, antigen specificity and MHC interaction.

T cell receptors are coordinately expressed on the cell membrane in association with proteins encoded by the CD3 locus, forming the TcR/CD3 complex [7,8] as shown in figure 1. The individual TcR chains display a strong structural similarity when compared with immunoglobulin chains, with a constant (C) domain, a joining (J) and/or diversity (D) and a variable (V) domain. The TcR chains form disulfide linked heterodimers. The TcRα chain normally pairs with the TcRβ chain, as do the TcRγ and TcRδ chains. In general, T lymphocytes display either a TcR$\alpha\beta$, or a TcR$\gamma\delta$ on their surface. There are, however, exceptions to this rule. A human T cell line has been isolated, which expresses a heterodimeric receptor of the β and δ TcR chains [9]. Furthermore, from the murine intestinal lamina propria and epithelium T cells have been isolated which express both the TcR$\alpha\beta$ and the TcR$\gamma\delta$ [10, appendix paper 4].

The TcR$\alpha\beta$ is expressed by T cells which mediate cytolytic or helper/inducer activity. They specifically recognize processed peptides in the context of MHC class I molecules for TcR$\alpha\beta^+$ T cells coexpressing CD8 [11,12]. T cells expressing the TcR$\alpha\beta$ and CD4 recognize processed peptides in the context of MHC class II molecules [11,12]. Upon correct recognition of a peptide in association with MHC, the TcR mediates the transfer of a signal, via CD3, into the cell. This initiates a cascade of intracellular reactions resulting in T cell activation and the ability to perform its specific function [13].

Antigenic specificities for TcR$\gamma\delta^+$ T cells in the mouse have recently been described [14-17]. Also reactivity to antigens in relation to MHC class I encoded gene products has been reported [18-20]. It remains ,however, still unclear how the response of TcR$\gamma\delta^+$ T cells is restricted.

1.3

Genome organization of murine TcR genes

The genomic organization of the genes, encoding the α, β, γ and δ TcR chains in man and mice is largely similar to the genomic organization of the loci encoding the immunoglobulins [3,21,22]. For each TcR chain the encoding genes in the mouse have multiple V, J and/or D gene segments and one to three C genes as shown in figure 2.

The V, J and/or D gene segments are separate in the germline and are brought together during T cell differentiation by DNA rearrangement

[21,22]. At those sites where V and J segments, for functional TcRα and TcRγ genes, or V, D and J segments, for functional TcRβ and TcRδ genes, combine, additional diversity is generated by nucleotide addition and deletion.

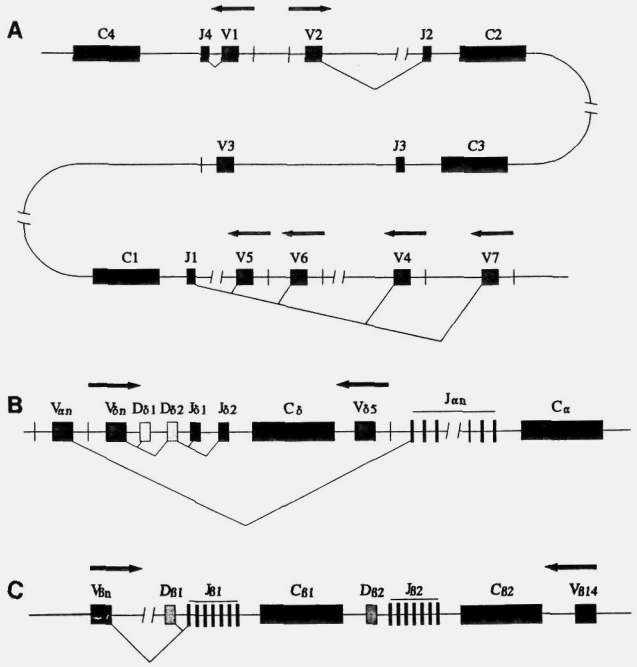

Figure 2.

The organization of the murine TcR gene families.(A) The TcRγ genes are shown as gene clusters. The nomenclature used is based on that suggested by Heilig and Tonegawa [23]. Relative positions along the chromosome are shown. Breaks in the lines represent unmapped distances or uncharacterized regions. Arrows indicate the direction of transcription. Typical rearrangements are represented by lines connecting V-J or V-D-J gene segments. The Cγ3 gene is a pseudogene [24]. (B) Representation of the δ- gene locus which is located between the Vα and Jα gene segments of the TcRα chain locus [25]. The precise number of Vα and Vδ gene segments has not been determined. The structure of the TcRα and TcRδ chain loci implies that the TcRδ locus is deleted from the chromosome upon rearrangement of the TcRα chain genes [25,26]. (C) Representation of the TcRβ chain locus, the precise number of Vβ gene segments has not been determined in detail yet. The figure is adapted after Carding et. al. [27].

The DNA rearrangements are mediated by signals located 3′ to the V gene segments, 5′ to the J gene segments and 3′ and 5′ to the D gene segments. Comparison of the nucleotide sequences of these rearrangement signals of the α, β, δ and γ TcR genes and the immunoglobulin genes shows a high degree of similarity. These sequences are composed of a conserved heptamer, 5′CACAGTG3′ and a conserved A/T rich nonamer, separated by a non conserved sequence of either 12 or 23 base pairs. DNA rearrangement is believed to occur only when one segment has a rearrangement signal with a 12 base pair spacer and the other segment has a rearrangement signal sequence with a 23 base pair spacer. In this case the two signal sequences are inverse complementair and can pair with each other.

All α, β, δ and γ V gene segments have a rearrangement signal with a 23 base pair spacer directly adjacent to their 3′ ends. As a consequence of this, the joining elements of the TcR chain loci have a rearrangement signal with a 12-base pair spacer adjacent to the 5′ ends. The diversity gene elements in the TcRβ and TcRδ chain loci are located between the V and J gene segments. To ensure correct rearrangement these D elements contain at their 5′ ends a rearrangement signal with a 12-base pair spacer and at their 3′ ends a rearrangement signal with a 23-base pair spacer.

1.4

Gut-associated lymphoid tissue

The gut-associated lymphoid tissue (GALT) consists of the so-called solitary lymphoid nodules, the Peyer's patches (PP) and the lymphocytes in the lamina propria and epithelium of the intestinal villi. The GALT is part of the common mucosal immune system (MALT) [28-31]. The basis for the concept of the MALT is that exposure of one mucosal surface to antigen results in dissemination of the antigen response to other mucosal surfaces [31-33]. The tissues which provide the functional environment of the MALT do not only form a local immune system but are also linked to the systemic immune system by the blood and lymph circulation [34]. The concept of the common mucosal immune system applies predominantly to the immunoglobulins, and in particular those of the IgA isotype, produced at mucosal sites.

The GALT can be divided into afferent and efferent limbs [35]. The

afferent limb is involved in antigen-specific responses against pathogens from the intestinal lumen and is mainly located in the Peyer's patches. These responses result in the generation of antigen specific IgA and IgM expressing B cells [32,33,35-37] and antigen specific T cells [38,39]. These cells migrate subsequently to the mesenteric lymph nodes and recirculate through the thoracic duct and the peripheral blood stream [33,35,38,39]. The efferent limb of the GALT consists of the lymphocytes in the intestinal epithelium and lamina propria. Especially the IgA and IgM lymphoblasts and a subset of the T cells in the intestinal epithelium mature in the afferent limb [33,35,38,39] and specifically home to their specific intestinal locations to perform their effector function [40].

The cellular component of the MALT, which is localized in the epithelia of mucosa associated organs, appears to be less disseminative than lamina propria lymphocytes since especially TcR$\gamma\delta^+$ cells at various epithelial mucosal sites display distinct phenotypes and express site-specific TcR types [41-43] This argues against the notion that such cells are generated in the afferent limb.

1.5

Localization of $CD3^+$ IEL

The localization of the major $CD3^+$ intra-epithelial lymphocyte (IEL) populations in the mouse is restricted to epithelia of organs, facing the internal or external body surfaces. These tissues are the skin, the digestive and the reproductive system [41]. In the skin, the tongue and vagina the $CD3^+$ IEL population is embedded in the basal cell layer of stratified squamous epithelium [41,42]. In the uterus the $CD3^+$ IEL are associated with simple columnar luminal epithelium [41], while in the stomach, small and large intestines the $CD3^+$ IEL are attached to or embedded in simple columnar epithelium [35,41]. The IEL populations will further be denoted according to Lafaille et al. [43] as iIEL for intestinal IEL, sIEL for skin IEL, rIEL for IEL present in reproductive organs and tIEL for IEL in the tongue.

1.6

Expression of functional Vγ genes in different IEL populations

The TcRγδ IEL populations display an organ specific distribution pattern of Vγ gene usage. Functional, rearranged, Vγ7 genes are abundantly expressed in iIEL [43,44]. The junctional diversity of these functional Vγ7 genes is high [43,44]. In contrast to Vγ7, however, the junctional diversity and expression of functionally rearranged Vγ6 genes, is less abundant within the iIEL population [44]. For functional Vγ6 genes a similar limited junctional diversity has been shown in rIEL and tIEL [41]. In these latter IEL populations the majority of cells express Vγ6 genes [41]. In frame Vγ5 rearrangements are predominantly expressed by sIEL and display a limited diversity [43-46]. Expression of other, functionally rearranged Vγ genes, such as Vγ4 and Vγ1, is more variable in the various IEL populations and may even depend on the mouse strain examined [47].

The epithelial homing of TcRγδ$^+$ T cells expressing a transgenic TcRγ, originally isolated from TcRγδ$^+$ cell lines of non-epithelial origin, demonstrates that localization of TcRγδ$^+$ cells to intestinal epithelial tissue does not depend on a site specific usage of TcR V genes [48,49].

1.7

Phenotype of IEL

Based on TcR expression, two major subpopulations of iIEL can be distinguished: TcRαβ$^+$ iIEL and TcRγδ$^+$ iIEL, table 1 [48,50-53, appendix papers 1 and 2]. TcR expressing iIEL have in common that they express CD8 on the majority of the cells [48,50-53, appendix papers 1 and 2]. In 1983 Parrot et al. [54] reported that a significant percentage of the iIEL expresses the CD8α chain only. Recently it was shown that the majority of the TcRγδ$^+$ iIEL express CD8 in a, probably, homodimeric form of the CD8α chain [55,56]. Also a significant percentage of the TcRαβ$^+$ iIEL express CD8α only [55-58]. The remaining part of the TcRαβ iIEL population expresses in majority CD8 as an heterodimer of the CD8β chain in combination with the CD8α chain [55-58]. This CD8 phenotype is common to peripheral T cells [59]. Only one other population of T cells, described sofar, expresses a CD8α homodimer. These are activated thymocytes

expressing the TcR$\gamma\delta$ [60-62].

In contrast to Thy-1 expression by T cells at other locations than the intestine, Thy-1 expression by iIEL is variable and largely restricted to a subpopulation of TCR$\alpha\beta^+$ iIEL as shown in table 1 [48,53, appendix paper 2]. These Thy-1$^+$ iIEL express CD5 and the CD8$\alpha\beta$ heterodimer [55-57]. Another subset of TCR$\alpha\beta^+$ iIEL and the majority of TcR$\gamma\delta^+$ iIEL does not express Thy-1 and CD5 [56]. When present on iIEL, Thy-1 expression is highly variable [48,53,56,57, appendix paper 2]. Furthermore, a small TcR$\gamma\delta^+$ iIEL population can be detected expressing Thy-1 and no CD8 or CD4 [B. de Geus, unpublished results]. TcR$\gamma\delta^+$ T cells with this phenotype form the majority of the peripheral TcR$\gamma\delta$ cells [63] and of IEL populations, located at other anatomical sites such as the skin (sIEL) [46] and IEL in the reproductive tract (rIEL) [64].

Besides the two major TcR$^+$ iIEL populations also a small iIEL population can be detected expressing both the TcR$\gamma\delta$ and the TcR$\alpha\beta$ [10, appendix paper 4]. This remarkable cell type can also be shown in the intestinal lamina propria [appendix paper 4]. These cells express Thy-1 in low membrane density and are negative for CD4 and CD8 [table 1 and appendix paper 4].

Initial reports suggested that the majority of iIEL are largely CD4 negative [56,50-55, appendix papers 1 and 2]. However, Mosley et al. described the presence of iIEL expressing CD4, CD8, Thy-1 and the TcR$\alpha\beta$ [65,66]. This observation is confirmed by others and extended in that these cells express CD5 and that CD8 expression is restricted to expression of the CD8α chains [56,57]. In our laboratory we observed variation in expression of this iIEL population between individual animals: In 20% of the examined mice CD4 and CD8 positive cells constitute significant numbers of the CD3 positive iIEL, but in the majority of the mice examined, less than 5% of the iIEL display this phenotype. When we studied aging mice, up to the age of 2 years, the frequency of such cells appeared to increase in older mice [appendix paper 7].

Finally, one other population of CD8$^+$ cells, present in the intestinal epithelium, should be mentioned. This population does not express a TcR or CD3, but expresses the CD8α homodimer [55,67, appendix papers 2 and 3]. Cells with this phenotype can be detected in all mouse strains, including

mutant strains as athymic nude nu/nu [55, appendix papers 2 and 3], viable motheaten me/me [B. de Geus, unpublished results] and CB17 scid/-scid mice [55,67, appendix paper 3]. Although these cells are probably bone-marrow derived [67], their relationship to the lymphocyte lineage is uncertain. In this respect it should be noted that the $CD8^+$ phenotype of these cells suggests that they are the murine equivalent of $CD8^+$ rat and human NK cells. However, this option remains unclear [appendix paper 3].

TcR$\gamma\delta^+$	**Presence**
Thy-1$^-$**CD8α^+**CD8β^-CD4$^-$CD5$^-$	+ + +
Thy-1$^+$CD8α^-CD8β^-CD4$^-$CD5?**	+\-
TcR$\alpha\beta^+$	
Thy-1$^+$CD8α^+CD8β^+CD4$^-$CD5$^+$	+ + +
Thy-1$^+$CD8α^-CD8β^-CD4$^+$CD5$^+$**	+\-
Thy-1$^+$CD8α^+CD8α^-CD4$^+$CD5$^+$**	+\-
Thy-1$^-$**CD8α^+**CD8β^-CD4$^-$CD5$^-$	+ +
Thy-1$^+$CD8α^+CD8β^-CD4$^-$CD5$^-$**	+\-
TcR$\alpha\beta^+$TcR$\gamma\delta^+$	
Thy-1^{low+}CD8α^-CD8β^-CD4$^-$CD5?**	+\-
TcR$^-$	
Thy-1$^-$**CD8α^+**CD8β^-CD4$^-$CD5$^-$**	+ +

Table 1. Phenotypes of iIEL in Balb/c mice. The score for the presence of iIEL is a relative measure since it is likely that the composition of the iIEL is influenced by antigenic exposure (see chapter 1.9). Also, the composition of the iIEL may be strain dependent. Uncertainty about CD5 expression by several iIEL populations is denoted with a question mark.

1.8

Differentiation of iIEL

1.8.1

Thymus dependent differentiation of iIEL

The majority of the T cells in lymphoid organs and blood as well as a subpopulation of TcR$\alpha\beta^+$ iIEL depend on the thymus for their differentiation. This iIEL population can be defined by the TcR$\alpha\beta^+$CD8$\alpha\beta^+$Thy-1$^+$ phenotype [55,57]. During T cell differentiation in the thymus the repertoire of T lymphocytes is generated by negative and positive selection events. Negative selection is the process associated with interaction of immature thymocytes with self-antigens, resulting in the deletion of self-reactive cells [68-70]. Positive selection requires the interaction of the TcR with MHC molecules expressed by the thymus epithelium and induces the differentiation of CD4$^+$CD8$^+$ double-positive thymocytes into mature single positive cells expressing CD4 or CD8 [71,72]. Positive and negative selection processes have also been described for thymus dependent TcR$\gamma\delta^+$ T cells [73-75].

1.8.2

Thymus independent differentiation of T cell populations other than iIEL.

The existence of an extrathymic pathway of T cell differentiation has been a topic for discussion for a long time. Most results which would indicate such an extrathymic differentiation pathway, were obtained in athymic, nude mice [76]. The major point of criticism on such data has been that the T cells in these animals can be maternally derived or have matured in the thymus rudiment, present in these animals. However, a number of studies showed that these options can not explain the presence of T cells in athymic, nude mice since in the offspring of mice, both homozygous for the 'nude' mutation, T cells are present [27]. Also in organ cultures, containing thymus rudiment, [77] and in the thymus rudiment in adult athymic, nude mice [78] no lymphocytes have been detected. Finally TcR$\gamma\delta^+$ IEL in murine skin have been shown to be dependent upon

maturation in fetal thymus [79,80]. Such cells have been shown to be absent in athymic, nude mice [80,81], indicating that the fetal thymus rudiment in these animals is not functional in early T lymphocyte differentiation. The first clear indications for extrathymic generation of T cells came with the availability of monoclonal antibodies directed to T cell markers and the introduction of molecular biological tools for immunological studies. This resulted in observations that TcR$\alpha\beta$ cells were present in spleens and lymph nodes of adult athymic, nude mice [82-85]. Such a TcR$\alpha\beta^+$ T cell population is likely to differentiate independently of the thymus since TcR$\alpha\beta^+$ T cells can be detected in athymic, nude mice expressing Vβ region segments for which the thymus negatively selects [86-88]. Others studies indicated that TCR$\gamma\delta$ cells could differentiate in the absence of a thymus in athymic nude mice [88,90-93]. Furthermore, in vitro studies on bone marrow cultures showed that it is possible to induce rearrangements in the γ, β and δ TcR loci in the absence of thymic epithelium suggesting that rearrangements can be induced outside a thymic micro-environment [94,95].

1.8.3

Thymus independent differentiation of iIEL.

iIEL are not only present in euthymic animals, but can also be detected in athymic rats and mice [96-99]. The availability of mAb and cDNA probes specific for CD3 and TcR chains, indicated that iIEL in athymic nude mice express an CD3 associated TcR$\gamma\delta$ [48,52, appendix papers 1 and 2]. The phenotype of these TcR$\gamma\delta^+$CD8$\alpha\alpha^+$ iIEL in athymic mice is similar to the phenotype of their counterparts in euthymic animals [48,52,55,57, appendix papers 1 and 2]. These observations indicate that TcR$\gamma\delta^+$ iIEL can mature in the absence of the thymus via an extrathymic differentiation pathway.

Proof has accumulated that another iIEL population, the TcR$\alpha\beta^+$CD8$\alpha\alpha^+$ iIEL, can also differentiate in the absence of the thymus [55,66,100,101]. However, the true extrathymic nature of these cells remains unclear since the presence of this population varies to a great extent in adult nude mice [56,57 and B. de Geus unpublished results].

The existence of an extrathymic differentiation pathway for a subset of iIEL has gained further experimental support from ontogenetic studies of

iIEL in athymic and euthymic mice [appendix paper 4]. These studies showed that the growth kinetics of $CD3^+$ iIEL are similar in both athymic and euthymic animals [appendix paper 4]. Furthermore, transfer studies of allogeneic bone marrow cells or fetal liver cells into thymectomized and lethally irradiated recipients [55,66,96,98,100-102 and B. de Geus unpublished observations] clearly indicate that at least a substantial part of the TcR$\alpha\beta^+$CD8$\alpha\alpha^+$ [55,66,100,101] and TcR$\gamma\delta^+$CD8$\alpha\alpha^+$ [55,66,100-102] iIEL are bone marrow derived and can develop outside the thymus.

The TcR$\alpha\beta^+$ iIEL population, detected in the mentioned bone marrow transfer studies [55,66,100,101] contains significant numbers of TcR$\alpha\beta^+$CD4$^+$CD8$\alpha\alpha^+$Thy-1$^+$ iIEL. It is tempting to speculate that such cells are immature differentiation intermediates, comparable to CD4$^+$CD8$^+$Thy-1$^+$ cortical thymocytes. However, some clear differences between the double positive iIEL and cortical thymocytes exist: Double positive cortical thymocytes express the heterodimeric form of the CD8 α and β chains [103] and express their TcR and CD5 in low membrane density [103-105]. In contrast, the double positive iIEL express the homodimeric form of the CD8α chains only [56] and express high levels of TcR and CD5 on the cell membrane [56]. Thus, although the double positive iIEL population may differentiate outside the thymus, the role of these cells in an extrathymic differentiation pathway remains unclear.

Recently another intestinal cell type was described which may be an intermediate during iIEL differentiation. Individual cells within this population express, most likely, both the TcR$\gamma\delta$ and the TcR$\alpha\beta$ [10, appendix paper 4]. These double TcR expressing cells are present in relative high numbers in the lamina propria of newborn athymic and euthymic mice [appendix paper 4]. These cells remain present as a significant population in the lamina propria of athymic mice and form a small subset of the LPL in euthymic mice [appendix paper 4].

1.8.4

Extrathymic selection of iIEL

In analogy to thymus dependent T cell differentiation, recent reports indicate that processes of positive and negative selection may be involved in extrathymic generation of iIEL [49,102]. Positive selection of Vδ4,

TcR$\gamma\delta^+$ iIEL, has been described to be dependent on expression of the MHC class II molecule I-E [102], although other element(s) appear to be involved in the selection process as well [57]. Evidence for negative selection through elimination of self-reactive iIEL by clonal anergy of TcR$\gamma\delta^+$ iIEL has also been reported [49]. Furthermore, TCR$\alpha\beta^+$ iIEL, which differentiate independently of the thymus are not deleted by self-antigens and are positively selected by recognition of antigen in the context of self-MHC [10].

The nature of the element(s) responsible for processes resulting in the extrathymic generation of TcR$^+$ iIEL is not clear yet. However, the murine intestinal epithelium expresses MHC class II encoded proteins [106,107] which may serve as elements in extrathymic processes of selection and/or deletion [102]. Furthermore, the intestinal epithelium expresses various MHC class I encoded proteins, such as Tla antigens [108,109] and CD1 [110]. These antigens have been reported to be recognized by mature TcR$\gamma\delta^+$ T cells [74,111,112]. However, TcR$\gamma\delta^+$ iIEL are present in normal numbers in the intestinal epithelium of mice which do not express MHC class I encoded antigens on their cell membrane [113]. These MHC class I deficient animals lack a functional β2-microglobulin gene on both alleles [114]. It is, therefore unlikely that the presence of these antigens influences the development of TcR$\gamma\delta^+$ iIEL. In contrast, it is likely that both TcR$\alpha\beta^+$CD8$\alpha\alpha^+$ and the TcR$\alpha\beta^+$CD8$\alpha\beta^+$ iIEL require expression of MHC class I encoded antigens during development since these iIEL are absent in β2-microglobulin deficient mice [113].

Selective processes involved in the extrathymic generation of TcR$\alpha\beta^+$ expressing T cells may be fundamentally different from those governing thymus dependent differentiation of these cells. In euthymic mice 'forbidden' Vβ gene segments, specific for self-antigens in association with, in particular, class II MHC molecules are deleted in the thymus [70]. However, such 'forbidden' Vβ segments are expressed by T cell populations which are considered to differentiate independently of the thymus as peripheral TcR$\alpha\beta^+$ T cells in athymic nude, mice [86-88] and TcR$\alpha\beta^+$CD8$\alpha\alpha^+$ iIEL [115].

1.8

Function of iIEL

Although **in vitro** T cell responses have given detailed insight in the function of other populations of T lymphocytes, this approach has not been very helpful in the study of the function of iIEL. The murine TcR^+ iIEL populations display **in vitro** cytolytic activity in various, iIEL subset dependent, anti-CD3 or anti-TcR redirected cytolysis assay systems [50,55,116-118]. Other **in vitro** activities as mixed lymphocyte reaction and mitogenic responses of iIEL subsets have also been shown [38,119,120], but these responses are in general low. Furthermore, iIEL are unresponsive to proliferative signals mediated through the CD3/TcR$\alpha\beta$ or the CD3/TcR$\gamma\delta$ complex [121]. **In vitro** production of several cytokines such as Il-2, Il-5 and IFNγ by iIEL has been reported [122-124].

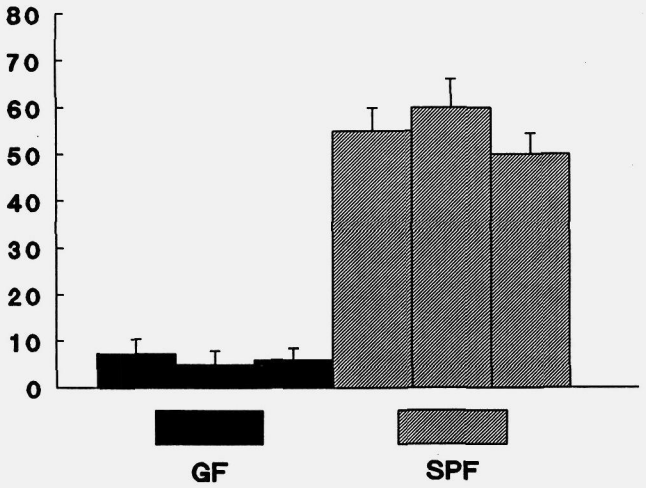

Figure 4. The presence of $CD3^+Thy\text{-}1^+$ iIEL is dependent upon presence of viable antigens. iIEL were isolated from germ free Balb/c mice and from Balb/c mice associated with a colonization resistent flora (CRF). After isolation cells were double stained with anti-CD3 [130] and anti-Thy-1 [131] mAb and analyzed by double fluorescence flow cytometry. Values are expressed as a percentage of total CD3 expression (100%). Mean and standard deviation of three independent experiments, including 3 animals each, are shown.

The major problem in defining the function of these cells is that iIEL are poorly defined for antigen specificity and MHC restriction. Only one iIEL subset has been shown to be reactive with a defined antigen in a MHC restricted way. This TcR$\alpha\beta^+$CD8$\alpha\beta^+$Thy-1$^+$ iIEL population has been shown to contain virus specific cytotoxic T lymphocytes after challenge of mice with enteropathogenic virus [Dr. J. J. Cebra, personal communication, and 125-127]. These iIEL originate in the Peyers patches after contact with antigen and subsequently migrate to the intestinal epithelium [38,128]. Figure 4 shows that the presence in the intestinal epithelium of these TcR$\alpha\beta^+$Thy-1$^+$ iIEL is largely dependent on the presence of viable microbial antigens in the intestinal lumen [118,129].

TcR$\gamma\delta^+$ iIEL may be involved in the reversal of oral tolerance in antigen primed mice upon adoptive transfer [132,133]. This indicates that such cells may display so-called contra-suppressor activity [134-136]. However, no antigen specificity of this iIEL subset has been described yet. Only the observed, aspecific, **in vitro** cytolytic activity of TcR$\gamma\delta^+$ iIEL [116-118] suggests that these cells are **in vivo** activated and are functional upon transfer.

The **in vivo** acquired state of activation of iIEL to perform **in vitro** cytolytic activity is not in agreement with the observed iIEL phenotype of the Ly6A and Ly6C antigens [appendix paper 6]. These antigens are not expressed on iIEL, whereas these antigens are expressed on activated peripheral CTL [137,138]. Ly6A and Ly6C both belong to the family of glycosyl phosphatidyl inositol (GPI) linked membrane proteins. Members of this family of proteins are believed to play a role in interactions between T cells and accesory cells [139-141] and to be involved in cell activation and signal transduction processes [142]. Also Thy-1, another GPI-linked protein, displays a rather unusual expression on iIEL [appendix paper 6]. The absence of GPI-linked proteins is not the result of a deficiency of iIEL to synthesize such proteins, since Qa2, a membrane protein also linked to the cell membrane via a GPI-anchor, is expressed by iIEL [appendix paper 6]

Besides the unusual expression pattern of GPI linked membrane proteins, the majority of iIEL is also unique in the expression of CD8 in a homodimeric form of the CD8α chains. The expression of the CD8α chain is sufficient for interaction with a target cell and signal transduction via p56^{LCK} [143,144]. However, it remains unclear whether all types of MHC class I encoded gene products interact efficiently with a CD8 molecule,

composed of the α chain only.

The unusual phenotype of several iIEL subsets fits the notion that these cells require different ligands than classic T cells to perform their, yet unclear, function **in vivo** and **in vitro**.

1.10

iIEL and disease

The possible involvement of iIEL in intestinal pathology is unclear due to our lack of knowledge about the function of these cells. The only intestinal pathological condition in the mouse in which iIEL have been shown to be involved is acute graft-versus-host disease (GVHD) [38,106,145-150]. The major manifestations of acute GVHD in the intestine are infiltration of the intestinal mucosa by donor lymphocytes, villus atrophy and destruction, and crypt hyperplasia [145]. Furthermore it has been shown that during acute GVHD the number of iIEL increases [147] and that the majority of the iIEL are of donor origin and display a $CD3^+Thy\text{-}1^+$ phenotype [38,106]. This is in contrast with observations during chronic GVHD in the small intestine [151,152]. In this latter condition no major pathology in the small intestine is observed.

However, more knowledge on functional aspects of iIEL may result in a better understanding of the mechanisms- and more practical animal models of human enteropathy. For example in coeliac disease an increase in the number of $TcR\gamma\delta^+$ iIEL has been observed [153,154]. Coeliac disease is characterized by sensitivity to gluten and wheat gliadins in particular [155,156]. Furthermore serum antibodies to gliadins can be detected in most coeliac patients [157,158], suggesting that in these patients a state of oral tolerance against gliadins has been reversed. Murine $TcR\gamma\delta^+$ iIEL have recently been shown to be involved in the reversal of oral tolerance in immunized animals when adoptively transferred [132,133]. This may indicate that $TcR\gamma\delta^+$ iIEL abrogate the normal state of oral tolerance against gluten in coeliac disease in man.

2 INTRODUCTION TO THE EXPERIMENTAL WORK

The studies, described in **appendix papers 1 and 2** were carried out to determine the phenotype of $CD3^+$ intestinal intraepithelial lymphocytes (iIEL) in euthymic and athymic, nude mice. The predominant phenotype of iIEL is $CD3^+CD8^+$. Moreover, this $CD8^+$ iIEL population can be subdivided, based on Thy-1 expression, into two sub-populations: One $CD3^+$ population expressing Thy-1 and another negative for Thy-1. Flowcytometric analysis and Northern blotting indicate that these $CD3^+CD8^+$ iIEL populations are different in TcR expression: $TcR\gamma\delta^+$ iIEL express no Thy-1, or Thy-1 in low membrane density. In contrast to this, Thy-1 expression in high membrane density by $CD3^+$ iIEL is associated with expression of the $TcR\alpha\beta$. This $TcR\alpha\beta^+Thy\text{-}1^+$ population is almost completely absent in athymic, nude mice. In these animals expression of CD3 and mRNA, encoding the $TcR\delta$ chain, indicates that the predominant TcR expressed is the $TcR\gamma\delta$. The data of **appendix papers 1 and 2** imply that the $TcR\gamma\delta^+$ iIEL differentiate extrathymically. However, also a subpopulation of $TcR\alpha\beta^+$ iIEL has been reported to differentiate extrathymically. Extrathymically differentiating iIEL have expression of CD8 in the homodimeric form of $CD8\alpha$ chains in common.

The data described in **appendix paper 3**, show that CD8 expression by iIEL is not restricted to $CD3^+$ iIEL. A cell population expressing CD8, but not CD3 is present in the intestinal epithelium. These cells are also present in athymic, nude, mice and in Scid/Scid mice. The $CD3^-CD8^+$ phenotype of this population is similar to the phenotype of a natural killer cell subset in rat and man.

Not only the intestinal $CD8^+$ cell populations, described in **appendix papers 1,2 and 3** are independent of the thymus for their differentiation. **Appendix paper 4** describes that high numbers of $CD3^+$ cells are found in the gut of fetal and young, newborn mice. At birth and during early neonatal life all gut-associated $CD4^-CD8^-CD3^{+low}Thy\text{-}1^{+low}$ cells are exclusively located in the lamina propria. At this age no $CD3^+$ iIEL can be detected. In striking contrast to the $CD3^+$ T cell population found in the intestinal epithelium, or any other peripheral lymphoid organ in adult mice, these newborn lamina propria $CD3^+$ cells express the $TcR\beta$ and the $TcR\delta$ chains simultaneously on their cell membrane. The most likely explanation for this observation is the existence of cells co-expressing $TcR\alpha\beta$ and

TcR$\gamma\delta$ on their surface. Subsequent studies showed that such cells can also be detected in low numbers in the intestinal lamina propria of adult mice. Since these cells, expressing the TcR$\alpha\beta$ and the TcR$\gamma\delta$, are also found in athymic mice one has to conclude that such cells do not depend on the thymus for their differentiation. It is proposed that the TcR double positive cell is a differentiation intermediate in the extrathymical differentation pathway of the TcR$\alpha\beta^+$CD8$\alpha\alpha^+$ iIEL.

This latter subset has been described to display an unusual Vβ usage. This observation is extended in **appendix paper 5** in which is shown that TcR$\alpha\beta^+$ iIEL display a TcR Vβ family usage which is individually determined. This is in contrast to Vβ usage of T cells in peripheral lymphoid organs which is shown to be similar when individuals are compared. This difference may be related to individual differences in the composition of the intestinal microflora, resulting in an individual pattern of TcR Vβ family expression.

A major part of the iIEL population displays in vitro cytolytic activity. The data in **appendix paper 6** describe a phenotypical analysis of iIEL in the context of this activity and the unusual Thy-1 expression of these cells. In contrast to the TcR$\alpha\beta^+$ T cell population in peripheral lymphoid organs, the TcR$\alpha\beta^+$ iIEL contain high numbers of Thy-1$^-$ cells. In order to determine whether the absence of Thy-1 on TcR$\gamma\delta^+$ and a subset of TcR$\alpha\beta^+$ iIEL is caused by a defect in the processing of GPI-linked proteins the expression of the GPI-linked proteins Ly6A, Ly6C and Qa2 on iIEL was examined. TcR$\alpha\beta^+$ and TcR$\gamma\delta^+$ iIEL are virtually negative for Ly6A and Ly6C but they do express Qa2. This indicates that the lack of Thy-1 and Ly6 expression on iIEL is not caused by downregulation of the synthesis of GPI-linked proteins. Furthermore, the lack of Ly6A and Ly6C expression by iIEL is not in line with the presumed activated and cytolytic nature of these cells since activated T cells from other locations, such as those in lymphoid organs, all express Ly6A. Furthermore, expression of Ly6C in lymphoid organs is related to cytolytic T cells.

Finally iIEL were investigated during aging in mice, as described in **appendix paper 7**. Many studies have addressed the changes that occur with age in T cells of the systemic immune system. Relatively few data have been reported on the T lymphocytes of the mucosal immune system during aging. This paper describes a phenotypical analysis of the composition of the intestinal epithelial lymphocyte (iIEL) compartment in C57Bl/Ka mice of various ages. From these experiments it can be

concluded that the $CD3^+$ iIEL fraction remains quantitatively rather stabile throughout life. A striking shift, however, is noted with age within the $CD3^+$ iIEL T cell population from presumably thymus-dependent $TcR\alpha\beta^+Thy\text{-}1^+CD8\alpha\beta^+$ cells towards thymus-independent $TcR\gamma\delta^+CD8\alpha\alpha^+$ and $TcR\alpha\beta^+CD8\alpha\alpha^+$ cells. Furthermore, the number of $CD4^+$ and $CD4^+CD8^+$ iIEL increase significantly with age. Also the percentage of $CD3^-CD8^+$ iIEL, cells with phenotypical similarities to NK cells, increases with age.

3 CONCLUDING REMARKS ON iIEL

From literature and our own data we have to conclude that a large population of $CD3^+$ lymphocytes exists in the epithelia of the body of which we do not yet know the precise physiological function, nor their involvement in certain disorders. Recently major progress has been made in the identification of subpopulations of iIEL and their phenotypes. The iIEL can be divided, on basis of their phenotypes, in many subsets (Table 1). A striking similarity between most of these subsets is their shared expression of CD8. Only a minor proportion of iIEL expresses CD4, although this proportion seems to increase with age. The composition of the CD8 molecule on iIEL, moreover, is not identical for all iIEL subsets. Part of the iIEL express CD8 in its regular form, i.e. as a CD8$\alpha\beta$ heterodimer. The majority of $CD8^+$ iIEL, however, only expresses the CD8α chain, probably in a homodimeric form. These differences in CD8 composition allow the distinction between thymus dependent and thymus independent iIEL subsets.

Combination of the various phenotypical markers results in the definition of three major subpopulations of iIEL: TcR$\alpha\beta^+$Thy-1^+CD8$\alpha\beta^+$, TcR$\alpha\beta^+$CD8$\alpha\alpha^+$ and TcR$\gamma\delta^+$CD8$\alpha\alpha^+$. The latter two are considered thymus independent, whereas the first is dependent on the thymus for its generation. Most of the data supporting this viewpoint come from studies in which the generation of the various iIEL subpopulations were studied in absence of the thymus. Thymus dependency for the TcR$^+$CD8$\alpha\alpha^+$ iIEL populations in the presence of a thymus cannot be ruled out. This leaves us with two possible models of thymus involvement in the generation of iIEL, as shown in figure 5.

Recent experimental evidence indicates that indeed a significant part of the iIEL population differentiates independently of the thymus. This statement is based on several observations: (i) In adult athymic, nude, mice the peripheral TcR$\alpha\beta^+$ T cell pool contains significant, but variable, numbers of T cells expressing TcR Vβ families that are normally depleted in the thymus of euthymic animals [86-88]. For example, the Vβ6 expressing thymocytes are deleted in the thymus of DBA/2 mice upon interaction with the Mls^a antigen [70]. However, the TcR$\alpha\beta^+$CD8$\alpha\alpha^+$ iIEL still contains significant numbers of Vβ6 expressing T cells in adult euthymic DBA/2 mice [115]. (ii) In contrast to T cells in peripheral lymphoid organs, the

TcR$\alpha\beta^+$ iIEL display an individually determined Vβ gene usage [appendix paper 5]. (iii) TcR$\gamma\delta^+$ iIEL have a similar Vγ gene expressional pattern at mRNA level as their counterparts in euthymic mice [B. de Geus, unpublished results]. Together, these data support the hypothesis that iIEL differentiate extrathymically even in the presence of the thymus, as illustrated in figure 5b.

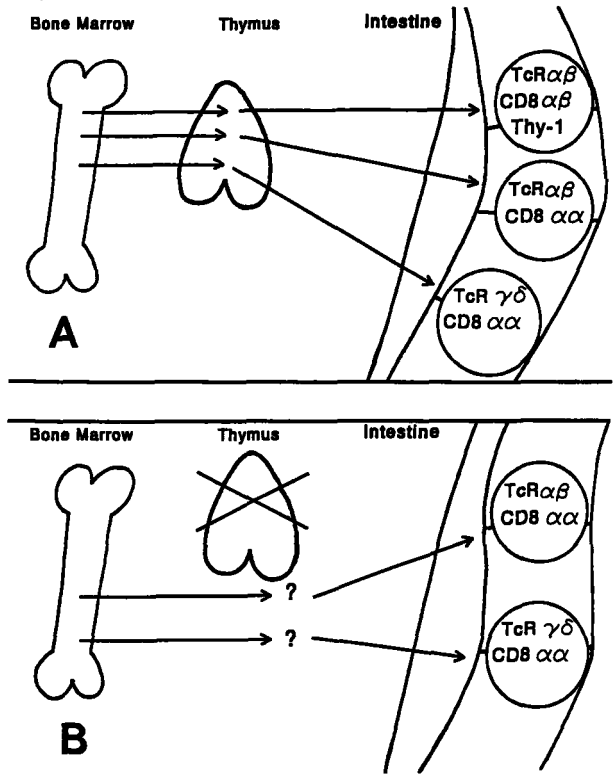

Figure 5. **Models for differentiation of various iIEL subpopulations.**
Figure 5a illustrates the situation in which all iIEL populations differentiate in the thymus. However, in absence of the thymus some iIEL subsets are still able to differentiate. In contrast to this, figure 5b shows the situation in which extrathymic differentiation occurs in both presence and absence of the thymus.

The current ideas on extrathymic differentiation of iIEL subsets focus on the possible role of the intestinal epithelium in selectional and maturation processes, involved in the extrathymic differentiation of

TcR$^+$CD8$\alpha\alpha^+$ iIEL. The evidence that the intestinal epithelium is indeed involved in such processes is circumstantial and mainly based upon: (i) Expression by the intestinal epithelium of MHC class I and II encoded gene products [106-110] and (ii) findings that iIEL express mRNA encoding the RAG 1 protein [55,159], which is involved in T and B cell gene rearrangements [160]. Both observations are interesting, but may be not relevant to the issue of extrathymic differentiation, since no antigen has yet been identified which restricts or interacts with TcR$^+$CD8$\alpha\alpha^+$ iIEL. Furthermore, the expression of mRNA encoding RAG-1 in iIEL does not necessarily indicate that RAG-1 is active in these cells or that rearrangements occur in the intestinal epithelium. In this respect it should be noted that stromal cells of the bone-marrow provides a micro-environment which efficiently supports in vitro T cell receptor gene rearrangements [94,95]. No such rearrangement inducing activity has yet been described for epithelial cells from the intestine.

In the discussions concerning T cell differentiation in the mouse one other intestinal compartment is usually not included: The intestinal lamina propria. In the rat, the intestinal lamina propria contains, at all life stages investigated, reticulum cells with a phenotype similar to reticulum cells in peripheral lymphoid organs [161]. In the thymus, the reticulum cell is a cell type which forms a part of the thymus microenvironment and which supports T cell differentiation in this organ [162]. It has been hypothesized that the reticulum cells in the intestine are involved in the development of the gut lymphoid microenvironment [161]. In line with this hypothesis are findings that the intestinal lamina propria in newborn mice has been shown to be populated with CD3 and TcR expressing cells [appendix paper 4]. In these animals iIEL are not detectable. The phenotype of these CD3$^+$ cells is highly unusual since they express both the TcR$\gamma\delta$ and the TcR$\alpha\beta$ simultaneously. Such double TcR expressing cells are not only detectable in newborn mice but also in the lamina propria of adult mice and form in these animals a small but consistent iIEL population [10,appendix paper 4]. These TcR$\alpha\beta$ *and* TcR$\gamma\delta$ expressing cells are not only present in euthymic mice but can also be shown in athymic, nude mice, indicating that these cells have a thymus independent origin [appendix paper 4].

The overall immature phenotype, i.e. CD3$^+$Thy-1^{low+}CD8$^-$CD4$^-$, of the TcR double positive cell population points to a function as a differentiation intermediate, rather than an effector cell. When we restrict ourselves to the intestine, where the TcR$\alpha\beta$ *and* TcR$\gamma\delta$ positive cell is

almost exclusively located, this cell type can well be an intermediate for all three major TcR$^+$ cell populations found in this organ: TcR$\alpha\beta^+$CD8$\alpha\beta^+$Thy-1$^+$ cells, TcR$\gamma\delta^+$CD8$\alpha\alpha^+$ cells and TcR$\alpha\beta^+$CD8$\alpha\alpha^+$ cells (fig.6). However, numerous experiments have shown that TcR$\alpha\beta^+$CD8$\alpha\beta^+$Thy-1$^+$ cells are thymus dependent for their generation. This leaves the TcR double positive cell as a candidate precursor cell type for both the TcR$\alpha\beta^+$CD8$\alpha\alpha^+$ and TcR$\gamma\delta^+$CD8$\alpha\alpha^+$ populations, which are thymus independent [55,66,100-102]. Recently reported experiments using mice treated with an anti-TcRβ chain mAb shows that such treatment depletes all TcR$\alpha\beta^+$ lymphocytes from the intestinal epithelium and lamina propria but leaves the TcR$\gamma\delta^+$ population intact [163,164]. Although these results do not exclude a role for the TcR double positive cells in the differentiation pathway of TcR$\gamma\delta^+$CD8$\alpha\alpha^+$ cells, it can be concluded nevertheless that a major extrathymic differentiation route for this latter cell type must exist in which the TcR double positive cell is not involved. It seems thus, that, if the TcR double expressing cell is indeed a differentiation intermediate, it is part of the differentiation pathway of thymus independent TcR$\alpha\beta^+$CD8$\alpha\alpha^+$ cells (fig.6).

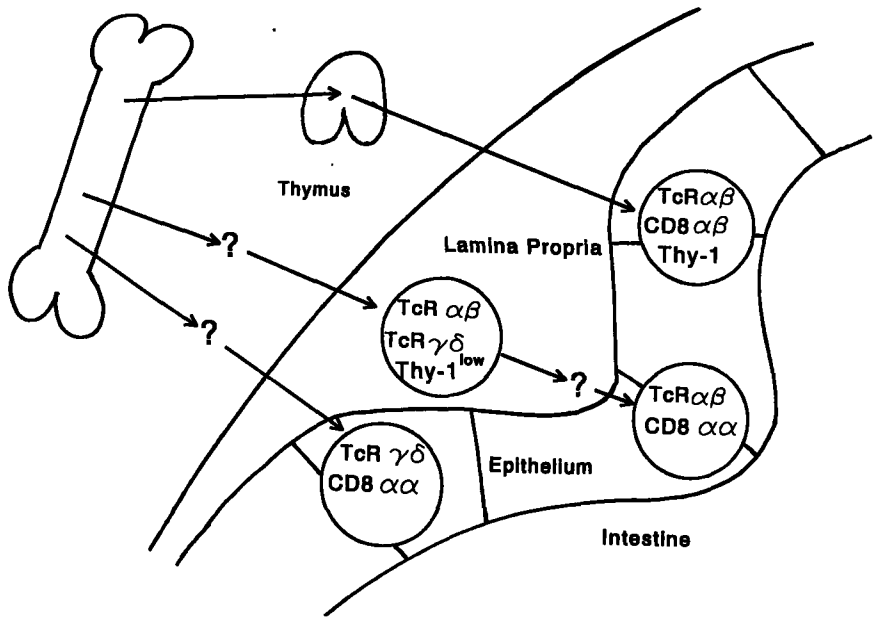

Figure 6. **Proposed differentiation scheme of bone-marrow derived cells, located in the intestinal epithelium.**

One other population of cells, located in the murine intestinal epithelium, has been suggested to be precursor cells in extrathymic ilEL differentiation [55]. These $CD3^{-}CD8\alpha\alpha^{+}$ cells have been shown to be bone marrow derived and to be present in the intestinal epithelium, irrespective of the presence or absence of the thymus [55,67, appendix paper 3]. The presence of this cell population in Scid/Scid mice, however, strongly argues against an precursor role of such cells for ilEL [55,67, appendix paper 3].

In conclusion, the current data on extrathymic T cell differentiation fit well in the hypothesis, that intestinal tissue plays an important role in the generation of the ilEL T cell repertoire during early (and adult) life. Furthermore, the TcR double positive cell is proposed to be an intermediate in the extrathymic differentiation pathway of intestinal $TcR\alpha\beta^{+}CD8\alpha\alpha^{+}$ cells. Although the function of ilEL remains unclear, the extrathymic generation of a substantial fraction of these cells might not only apply to the ilEL only, but also to T cell subsets in other organs than the intestine. Such an extrathymic T cell differentiation pathway would be important in the maintenance of the T cell repertoire in a condition in which the thymus looses its T cell generating capacity. One of the best examples of such a situation is the aging process, during which a natural involution of the thymus occurs.

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5 SUMMARY

This thesis describes several aspects of intestinal intraepithelial lymphocytes (iIEL) in the mouse.

In **chapter 1** the present knowledge on murine iIEL is updated in an overview of the recent literature: Together with IEL at other mucosa associated epithelial locations, such as the skin, the lungs and the urogenital tract, iIEL represent a local immune system of the same magnitude as, but clearly different from the systemic immune system as found in other lymphoid organs. Interestingly IEL at various mucosal surfaces are enriched for T cells expressing the TcR$\gamma\delta$. In this, IEL are different from T cells at other locations than mucosa associated epithelia since such cells express predominantly the TcR$\alpha\beta$.

Within IEL in general, iIEL are unique on themselves since, in contrast to IEL at other locations, iIEL form an extremely heterogeneous population. This is reflected by the phenotype of the major iIEL populations identified: A TcR$\alpha\beta^+$CD8αß$^+$Thy-1$^+$, a TcR$\alpha\beta^+$CD8$\alpha\alpha^+$ and a TcR$\gamma\delta^+$CD8$\alpha\alpha^+$ population. Not only in phenotype iIEL are unique among IEL but also in their pattern of differentiation these cells are a distinctive population: The CD8$\alpha\beta$ and Thy-1 expressing iIEL depends on the thymus for differentiation. In contrast to these iIEL the two, CD8$\alpha\alpha$ expressing, iIEL populations have been shown to be thymus independent for their differentiation.

The localization of iIEL suggests that these T cells are important in a local defensive system. However, neither the precise function of iIEL at local or systemic level, nor their role in local disorders is clear at present. Further research on functional and differentiational aspects of this intriguing T cell population will clarify this in the near future.

Chapter 2 summarizes the investigations on differentiation and phenotypes of iIEL in various mouse mutant strains and in aging mice as described in this thesis.

In **chapter 3** recent findings on iIEL are brought together in a differentiation model of iIEL. It is proposed that iIEL, which can differentiate extrathymically in the absence of the thymus, also follow this differentiation pathway in the presence of this organ. Secondly, the role of

the intestinal epithelium in the differentiation of iIEL is discussed. It is suggested that the role of the intestinal epithelium in extrathymic differentiation processes is overestimated. In contrast to this, it is proposed that the intestinal lamina propria may be important in the extrathymic differentiation process of iIEL.

In **appendix papers 1 and 2** the phenotype of iIEL in athymic nude mice is investigated. The data imply that TcR$\gamma\delta^+$CD8$^+$ iIEL differentiate extrathymically. Not all CD8 expressing iIEL express a T cell receptor. In both normal and mutant mouse strains a CD3$^-$CD8$^+$ cell population, which is also thymus independent, is present in te intestinal epithelium as described in **appendix paper 3**. The phenotype of these cells suggests that they might belong to the NK cell lineage.

Appendix paper 4 describes a population of lymphocytes, present in the lamina propria of euthymic and athymic mice, co-expressing both the TcRβ and TcRδ chains. It is postulated that cells with this unusual phenotype are intermediates in the extrathymic differentiation of TcR$\alpha\beta^+$CD8$\alpha\alpha^+$ iIEL. The TcR$\alpha\beta^+$
iIEL display variation in Vβ usage when individual mice are compared. It is suggested, in **appendix paper 5**, that local factors influence the composition of such TcR$\alpha\beta^+$ iIEL.

iIEL are extremely heterogeneous with respect to TcR, CD8, CD4 and Thy-1 expression. In contrast to this expression of GPI-linked membrane proteins Qa2, Ly6C and Ly6A is similar for all iIEL populations investigated (**appendix paper 6**). iIEL display an unusual phenotype of GPI-linked proteins, especially when compared to their suspected in vivo state of activation.

Finally, **appendix paper 7** describes iIEL in aging mice. The results imply that the iIEL population remains constant in size during aging. Minor changes in the composition of the iIEL were detected, indicating that extrathymic differentiation of a subset of iIEL is important in the maintenance of the iIEL population during aging.

6 SAMENVATTING

In dit proefschrift zijn verschillende aspecten van de intraepitheliale lymphocyten populatie (IEL) in de darm van de muis beschreven.

Samen met IEL in andere met mucosa geassocieerde epithelia zoals in de longen, huid en blaas vormen IEL in de darm een onderdeel van het lokale immuunsysteem. Dit lokale immuunsysteem is op basis van een aantal punten verschillend van het immuunsysteem in lymphoïde organen het zogenaamde systemische immuunsysteem. Zo zijn bijvoorbeeld de IEL zoals aanwezig in verschillende mucosale epithelia sterk verrijkt voor T cellen die een T cel receptor (TcR) $\gamma\delta$ tot expressie brengen. Dit in tegenstelling tot T cellen die deel uitmaken van het systemische immunsysteem. Deze cellen brengen voornamelijk de TcR$\alpha\beta$ tot expressie.

In tegenstelling tot andere IEL populaties, vormen IEL in de darm een bijzonder heterogene celpopulatie waarin een drietal fenotypes het sterkst vertegenwoordigd zijn: Een TcR$\alpha\beta^+$CD8$\alpha\beta^+$Thy-1$^+$, een TcR$\alpha\beta^+$CD8$\alpha\alpha^+$ en tenslotte een TcR$\gamma\delta^+$CD8$\alpha\alpha^+$ populatie. Niet alleen in fenotype onderscheiden IEL uit de darm zich van andere IEL populaties, maar ook op basis van hun differentiatiepatroon vormen deze cellen een unieke T cel populatie. IEL die CD8$\alpha\beta$ en Thy-1 op hun celmembraan dragen, blijken afhankelijk te zijn van de thymus voor hun vorming. Echter de twee IEL populaties die, naast een TcR, alleen CD8$\alpha\alpha$ op hun oppervlak dragen blijken onafhankelijk van de thymus te differentiëren.

Hoewel de lokalisatie van IEL in de darm een belangrijke rol voor deze cellen in het lokale afweersysteem suggereert, is de rol van deze cellen zowel op lokaal als op systemisch niveau onduidelijk. Onderzoek naar differentiatie en functionele aspecten van deze unieke T cel populatie zal in de nabije toekomst duidelijkheid verschaffen in de rol van deze celpopulatie in het afweersysteem.

In hoofdstuk 2 worden de onderzoekingen naar het differentiatie patroon en fenotype van IEL in de darm van normale en mutant muizen samengevat. Ook is in hoofdstuk 2 een beschrijving te vinden van de samenstelling van de intestinale IEL populatie gedurende veroudering.

De verschillende bevindingen op IEL uit de darm worden in hoofdstuk 3 samengebracht in een differentiatiemodel van deze cellen. Voorgesteld

wordt dat IEL uit de darm, die onafhankelijk differentiëren in afwezigheid van de thymus, zoals in athymische muizen, dit ook doen in aanwezigheid van dit orgaan. Verder wordt de rol van het darmepitheel in de differentiatie van intestinale IEL bediscussieerd: Gesuggereerd wordt dat de rol van het darmepitheel in thymus-onafhankelijke T cel differentiatie overschat wordt. Als alternatief wordt aangegeven dat de lamina propria in de darm van belang kan zijn als micro-omgeving voor extrathymaire T cel differentiatie.

In appendices 1 en 2 is het fenotype van IEL in athymische, naakte, muizen onderzocht. De resultaten impliceren dat TcR$\gamma\delta^+$CD8$^+$ IEL extrathymair differentiëren. Niet alle IEL in de darm die CD8 tot expressie brengen dragen een T cel receptor op hun cel-oppervlak. In appendix 3 is beschreven dat in zowel normale als mutant muizenstammen een CD3$^-$CD8$^+$ celpopulatie, die ook onafhankelijk van de thymus differentieert, aanwezig is in het darmepitheel. Deze cellen kunnen, op basis van hun phenotype, deel uitmaken van de NK cel populatie.

In appendix 4 wordt een celpopulatie beschreven die aangetoond en geïsoleerd kan worden uit de lamina propria van normale en athymische, naakte muizen. Deze unieke celpopulatie brengt zowel de TcRβ en de TcRδ ketens tot expressie op het celoppervlak van individuele cellen. Gesteld wordt dat deze celpopulatie een intermediair is in de extrathymaire ontwikkeling van TcR$\alpha\beta^+$CD8$\alpha\alpha^+$ intestinale IEL. Bovendien is aangetoond (appendix 5) dat het Vβ gen gebruik in de intestinale TcR$\alpha\beta^+$ IEL populatie varieert op een individuele basis.

IEL in de darm zijn heterogeen wanneer zij geanalyseerd worden op basis van TcR, CD8, CD4 en Thy-1 expressie. Echter expressie van, via een glycosyl-phosphatidyl-inositol residu verankerde, membraan eiwitten zoals Qa2, Ly6A en Ly6C blijkt identiek te zijn voor alle intestinale IEL populaties die onderzocht zijn (appendix 6). Het expressie-patroon van deze eiwitten door intestinale IEL is ongewoon, vooral wanneer de beschreven in vivo geactiveerde toestand van deze cellen in aanmerking wordt genomen.

Tenslotte is in appendix 8 beschreven dat gedurende veroudering de IEL populatie constant blijft in grootte. Kleine veranderingen in de samenstelling van de intestinale IEL populatie werden waargenomen tijdens het verouderingsproces hetgeen erop wijst dat thymus-onafhankelijke differentiatie van een subpopulatie van belang is in het instandhouden van de IEL populatie in de darm.

7 APPENDIX

APPENDIX PAPER 1

LOCALIZATION AND PHENOTYPE OF CD3 ASSOCIATED $\gamma\delta$ RECEPTOR EXPRESSING INTRAEPITHELIAL LYMPHOCYTES

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Published in: **Thymus** 1989. 14: 31.

Summary

This study was carried out to determine the exact phenotype and localization of intestinal intra epithelial lymphocytes (iIEL). iIEL reside in the small intestine in the epithelial cell layer which covers the lamina propria. These $CD3^+$ cells were found in equal numbers at this location both in the villi as well as in the crypts. The majority of these cells express a CD3 associated TcR$\gamma\delta$. Cell surface marker analysis of isolated iIEL reveals high percentages of cells expressing CD8 and/or Thy-1. No $CD4^+$ cells could be detected in significant numbers. Equal quantities of iIEL could be isolated from the intestine of athymic, nude mice. Although the percentage of $CD3^+$ iIEL from athymic, nude mice was lower than the number obtained from euthymic mice, Northern blot analysis revealed a strong expression of $\gamma\delta$ message in the iIEL of athymic, nude mice. In the $CD3^+$ iIEL fraction of these animals the relative contribution of the $CD8^+$ cells remained unchanged, whereas the percentage of Thy-1^+ cells had decreased. Still significant numbers of such cells could be demonstrated in the iIEL of athymic, nude mice. Altogether these results show that, at least, a significant part of the TcR$\gamma\delta^+$ iIEL is independent of the thymus for their differentiation.

Introduction

As previously reported by us [1] and others [2,3] iIEL express predominantly the CD3 associated TcR$\gamma\delta$. This type of receptor has recently been identified [4]. The locus encoding the TcRδ chain is located within the TcRα chain locus [4]. The TcR$\gamma\delta$ in mice is composed of two glycosylated and disulfide linked protein chains, the γ and δ chain [5-7]. Since the δ chain locus is located between the J-α and V-α gene segments [4] rearrangement of the α locus results in the complete deletion of C-δ, J-δ and D-δ sequences [8,9]. Cultured, activated, cells which express the TcR$\gamma\delta$ perform a MHC non restricted lysis in vitro [10-12]. However, the in vivo function and antigen specificity of these cells is at the moment still unclear. A recent study by Goodman and Lefrancois [2] demonstrated that iIEL with a $CD3^+CD8^+$Thy-1^- phenotype could perform in vitro cell lysis in a non-MHC-restricted fashion directly after isolation. This finding suggests that iIEL with such a phenotype are probably already activated in vivo.

The predominantly reported phenotype of iIEL is $CD3^+CD8^+$Thy-1^-

[2,3], but also iIEL with phenotypes as $CD3^+CD8^+Thy\text{-}1^+$ and $CD3^+CD8^-Thy\text{-}1^{+/-}$ have been identified [this report,2,3]. No significant numbers of iIEL have been observed with a $CD4^+$ phenotype. It is still debated wether cells which express the CD3 associated TcR$\gamma\delta$ are able to differentiate in the absence of the thymus [13,14]. Small numbers of TcR$\gamma\delta^+$ cells can be isolated from blood and spleen from athymic mice [13,14]. These cells have a $CD3^+CD4^-CD8^-$ $Thy\text{-}1^+$ phenotype [10,14]. There are indications, however, that the major differentiation pathway of the TcR$\gamma\delta^+CD3^+Thy\text{-}1^+$ cells is thymus dependent [14].

In this report we show that in the intestinal epithelium from both euthymic and athymic, nude mice large numbers of iIEL are present. Phenotypical analysis of these iIEL revealed both expression of CD8 and/or Thy-1 on a high proportion of the cells in this population in euthymic mice. In athymic mice the percentage of $Thy\text{-}1^+$ cells was decreased, but still significant numbers of $Thy\text{-}1^+$ cells could be demonstrated in the iIEL fraction. iIEL obtained from both euthymic and athymic, nude mice expressed high levels of mRNA for the γ and δ receptor chains, while virtually no mRNA for the T cell receptor α and β chains could be detected. The implications of these findings will be discussed.

Materials and methods

Animals

Inbred Balb/c +/+ and nu/nu specific pathogen free mice of 8-10 weeks age were used for iIEL isolation. They were obtained from the central animal facility of the TNO Radiobiological Institute, Rijswijk, The Netherlands.

Isolation of iIEL

iIEL were isolated as reported previously [15] with minor modifications. After flushing with PBS (NBPI BV, Emmer-Compascuum, The Netherlands) of the intestine the Peyer's patches were dissected and the intestine was cut longitudinally. Subsequently the intestine was cut in pieces of 1-2 mm and this material was incubated in HBSS medium without Ca^{2+} and Mg^{2+} (Seromed, Berlin, Germany), supplemented with 1mM EDTA (Fluka AG, Buchs, Switzerland) at 37°C for 20 minutes in a

waterbath, shaking at a frequency of 100 strokes per minute. After incubation the debris was removed and the supernatant was processed further by addition of FCS (Sera-lab, West Sussex, UK) to a final concentration of 10% v/v. After pelleting the cells for 10 minutes at 400g the cells were washed once in RPMI 1640 (Flow Laboratories, Irvine, Scotland), supplemented with 10 % FCS. After centrifugation of the cells the pellet was resuspended in RPMI 1640. The resulting suspension was passed over a 50 μm nylon gauze. Percoll (Seromed, Berlin, Germany) was added at a density of 1.037 g/ml. This 1.037 g/ml fraction was layered onto a discontinuous percoll gradient of 1.086 g/ml and 1.054 g/ml in PBS. Gradients were centrifuged at 600g for 30 minutes at 4°C. After centrifugation the cell-layer at the 1.086-1.054 g/ml interphase was processed further by washing twice in RPMI 1640, supplemented with 10% FCS. Cells in this fraction are further referred to as iIEL.

FACS analysis and staining

Monoclonal antibody (mAb) 145-2C11, directed against mouse CD3 was a gift of Dr.J.A.Bluestone [16]. mAb 145-2C11 was used directly conjugated with FITC or in combination with a FITC-conjugated polyclonal Rabbit anti-Rat-immunoglobulin antibody (Miles Laboratories Inc., Elkhart, USA). A directly FITC conjugated mAb (59AD2.2) (Becton Dickinson, Sunnyvale, USA) was used for Thy-1 staining. mAb GK1.5 conjugated with biotin, directed against mouse CD4, was used in combination FITC-Avidine (Sigma Chemical Company, St.Louis, USA). mAb 53-6.7 (Becton Dickinson, Sunnyvale USA) directly conjugated with FITC was used for CD8 staining. All monoclonal antibodies were purified, conjugated and stored in PBS/ 1%BSA (Sigma Chemical Company, St.Louis, USA) at -20°C. For FACS analysis cells were washed once in PBS/ 1%BSA and incubated with a mAb at the appropriate dilution for 30 minutes at 4°C. For the biotine conjugates this first step was followed after two rounds of washing in PBS/ 1%BSA by a second incubation with FITC-Avidine. After the final incubation the cells were washed twice in PBS / 1%BSA and resuspended in this medium at a final concentration of $1x10^6$ cells/ ml. Labelled cells were analysed using a FACScan equipped with the Consort 30 program and Paint-a-Gate Software (Becton Dickinson, Sunnyvale, USA).

Northern blot analysis

RNA was isolated by the guanidinium isothiocyanate method [17]. Total RNA (15 μg) was blotted on Biotrans nylon membranes (ICN Biochemicals Inc., Irvine, USA) after electrophoresis on formaldehyde agarose gels. Blots were hybridised for 15 hours at 42°C with a VJC-α T cell receptor (TcR) probe (a gift of Dr. H Hengartner) [18], and/or a C-δ TcR probe (provided by Dr. T. Mak through Dr. R. de Weger), labelled with P^{32}-α-dCTP (Radiochemical Centre Amersham, UK) by random priming (Gibco BRL, Breda, The Netherlands) in 50% formamide (Aldrich Chemical Company, Milwaukee, USA), 5xDenhardt's solution [17], 5xSSC, 50mM Na_3PO_4, 0.1% SDS (all Merck, Darmstadt, Germany) and 250 μg/ml salmon sperm DNA. After hybridisation blots were washed in 0.5xSSC 0.1%SDS at 50°C. When the same blot was hybridised two times, the blot was stripped between the subsequent hybridisations by incubation for 20 minutes at 60°C in 50% formamide, 0.1% SDS and 50mM Na_3PO_4. After hybridisation blots were exposed for 24 hours to Kodak XAR films in cassettes with Kodak lanex fine amplification screens (Kodak Company, Rochester, USA).

Histology

After removal of the intestine this organ was processed in so-called "Swiss rolls" and frozen in liquid nitrogen. 6 μm thick cryostate sections were cut and incubated for 30 minutes at room temperature with mAb 145-2C11, directed against mouse CD3 at the appropriate dilution. After washing the sections twice with PBS/ 1%BSA a second incubation was done with a FITC-conjugated Goat anti-Rat immunoglobulin, crossreacting with the hamster mAb 145-2C11. In order to check for possible crossreactivity of the FITC-conjugate on mouse B cells and plasmacells a two-colour staining procedure was followed with a TRITC-conjugated Goat anti-Mouse immunoglobulin antibody as reagent. A Zeiss microscope equipped for selective visualization of fluorescein and rhodamine was used to read the slides.

Results

Localization of iIEL in the small intestine

In order to determine the exact localization of the $CD3^+$ lymphocytes in the intestinal cell layers cryostat sections of the intestine of euthymic Balb/c mice were incubated with mAb 145-2C11. As can be seen from figure 1A cells displaying a typical membrane staining pattern for CD3 (arrows) are present between in the epithelial cell layer which covers the lamina propria. This is true for both the epithelia aligning the villi (fig.1AB) as well as that in the crypts (data not shown).

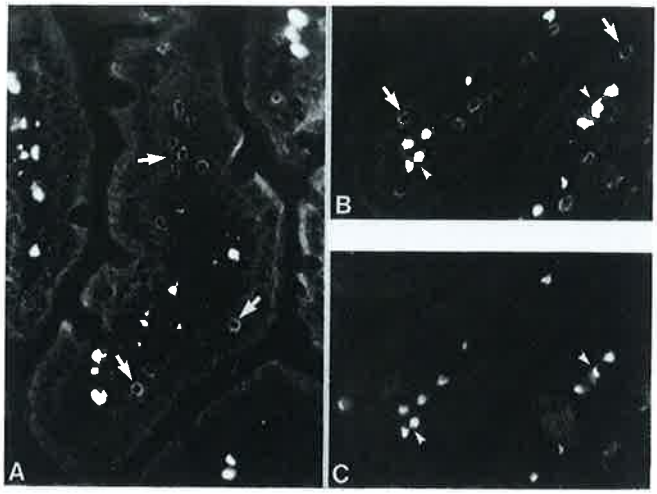

Figure 1. Fluorescence staining of cryostat sections of intestine of euthymic Balb/c mice with an anti-CD3 mAb (1A & 1B) or an anti-mouse immunoglobulin antibody (1C). 1B and 1C are double exposures of the same section after two-colour flurescence staining. Arrow = CD3 membrane positive lymphocyte, arrowhead = immunoglobulin containing plasma cell.

Examination of the whole intestine reveals that most iIEL are present in the duodenum with decreasing amounds in the direction of the jejunum (data not shown). The strong intracellular FITC-staining of cells in the lamina propria (asterix) is due to a cross-reactivity of the Goat anti-Rat antibody with plasma cells in that area, since these cells can be defined as such by double staining with a TRITC-Goat anti-Mouse antibody (fig.1C).

Phenotyping

After isolation and staining FACS analysis was performed on the iIEL. Such a cell suspension consisted reproducibly of cells with a lymphoid morphology (approximately 50-60%) and a remainder fraction of epithelial and other cell types. For further analysis cells with a lymphoid appearance as defined by combined forward and perpendicular light scatter patterns were selected using Paint-a-Gate Software (Becton and Dickinson) in combination with the Consort 30 program. Subsequently the fluorescence profiles of the various mAb's were measured and analysed.

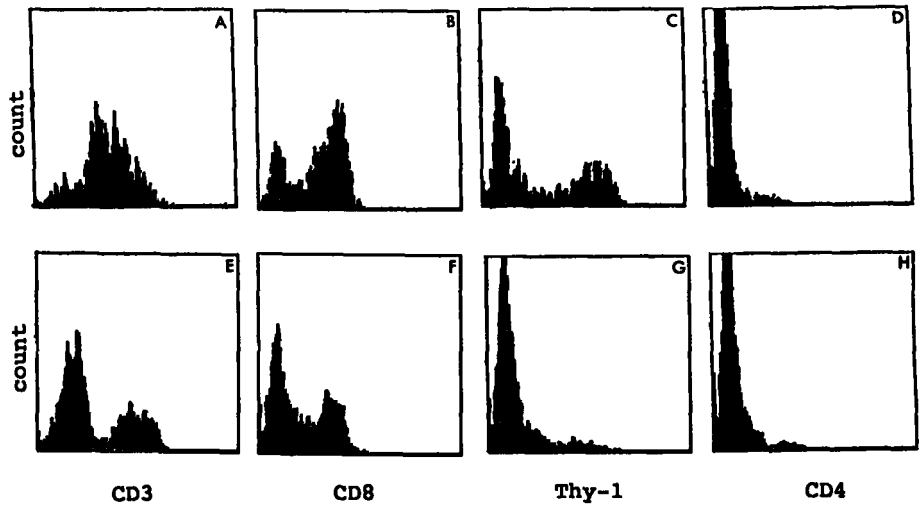

Figure 2. Histograms of FACS analysis of iIEL obtained from euthymic Balb/c mice (2A -2D) and athymic, nude Balb/c mice (2E - 2H) stained with mAb's against CD3 (2A & E), CD8 (2B & F), Thy-1 (2C & G) and CD4 (2D & H). On the x-axis the respective fluorescence intensities for the various markers are presented and on the y-axis the number of events per channel. Viable lymphoid cells were selected on basis of forward and perpendicular light scatter gate settings.

As can be seen in figure 2 a high proportion of iIEL express the CD3 (fig.2A&E), CD8 (fig.2B&F) and Thy-1 (fig.2C&G) markers. Hardly any

staining is observed for CD4 (fig.2D&H). This staining pattern is found both in euthymic mice (fig.2A-D) and in athymic, nude mice (fig.2E-H), although the overall expression of the markers in nude mice is slightly lower. When quantified the number of $CD3^+$ cells in iIEL normally range from 60-90%.

In table 1 the results are given of a representative eperiment showing a regular pattern of iIEL analysis. In this experiment 85% of iIEL obtained from euthymic Balb/c mice were $CD3^+$, 70% $CD8^+$, 47% Thy-1^+ and 6% $CD4^+$. Quantitative analysis of iIEL of athymic, nude mice resulted in 62% $CD3^+$ cells, 42% $CD8^+$ cells, 16% Thy-1^+ cells and 7% $CD4^+$ cells.

Table 1. FACS analysis[1] of iIEL from euthymic and athymic, nude Balb/c mice

Marker	Balb/c euthymic[2]	Balb/c athymic (nude)[3]
Thy-1	47%[4]	16%
CD3	85%	62%
CD4	6%	7%
CD8	70%	42%
control[5]	2%	4%

[1]Data of one representative experiment are given

[2]iIEL of 2 individual euthymic Balb/c mice were pooled and used for analysis

[3]iIEL of 2 individual athymic, nude Balb/c mice were pooled and used for analysis

[4]Percentage of positive cells is expressed as percentage of viable cells with a lymphoid morphology according to forward and perpendicular light scatter criteria.

[5]Cells were stained with an irrelevant anti-human mAb as control

Nucleic acid hybridisation

For hybridisation of blots probes for the α and δ TcR sequences were used because of the unique chromosomal localization of the δ locus [4,9]. DNA rearrangements of TcR genes usually occur in both loci. Therefore

rearrangement of the α locus will lead to the deletion of the δ locus [4,9]. Thus a cell which expresses the TcR$\alpha\beta$ can not produce mRNA for the δ chain, whereas the opposite is also true: a cell which expresses the TcR$\gamma\delta$ does not contain any mRNA coding for the TcRα chain. As can be seen in fig.3. hybridisation of the blots with TcRα and TcRδ chain probes reveals a high expression of mRNA for the TcRδ chain in total RNA that has been isolated from iIEL obtained from the intestine of euthymic Balb/c mice. A similar high expression of TcRδ chain mRNA can be observed in the RNA derived from iIEL from athymic, nude mice. Almost undetectable amounts of mRNA for the TcRδ chain can be detected in total RNA isolated from the MLN of either euthymic or athymic, nude Balb/c mice.

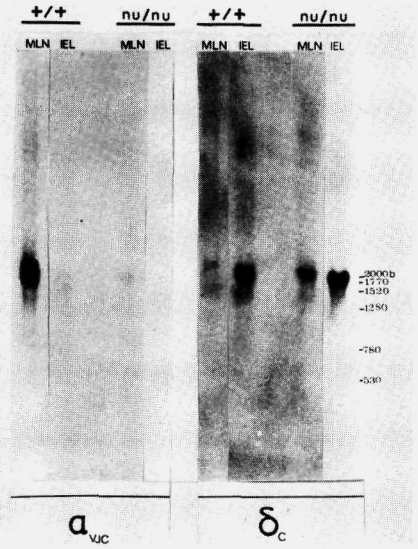

Figure 3. Northern blot analysis of total RNA obtained from mesenteric lymph nodes (MLN) and intraepithelial lymphocytes (iIEL) of euthymic Balb/c mice (+/+) and athymic, nude Balb/c mice (nu/nu). mRNA coding for TcR α and δ chains was determined by hybridisation with probes for TcR α-VJC (left lanes) or δ-C (right lanes) on the same blot, after stripping.

Hybridisation of the same blot with the T cell receptor α chain probe shows just the opposite: Virtually no TcRα chain mRNA expression in the iIEL fraction of both the euthymic and athymic, nude Balb/c mice and the MLN of athymic, nude mice, whereas the TcRα chain mRNA expression in the MLN of euthymic Balb/c mice is expectedly high.

Discussion

Since iIEL are located at the borderline of the internal and external milieu it is likely to speculate that these cells are involved in the primary immune defense mechanisms towards invading foreign material. This is in line with the hypothesis put forward by Janeway et al [12] who suggested that such cells survey the epithelial surfaces of the body. Relatively little is known, however, about the true function of the iIEL. Klein [19] and Goodman [2] described a Thy-1$^+$CD8$^+$ cell type in murine intestine with a cytotoxic potential. Such a function would fit in with the speculations on a cytotoxic activity of iIEL against infected or transformed epithelial cells [12]. Also a role in the allogeneic lymphocyte cytotoxicity response, as suggested by Bell [20] would be in line with these observations. Nevertheless another type of activity for the iIEL should be considered too. Local responses to cell bound antigens display a biphasic pattern in which the antigen specific CD3$^+$CD4$^+$CD8$^-$Thy-1$^+$ T cell mediated second phase is preceeded by an essential series of initial events [reviewed in 21]. In a variety of assay systems, including contact hypersensitivity [22-24], infection models [25] and allogeneic and syngeneic tumour systems [22,26], a key role has been appointed in the first phase of the response to the so-called initiator lymphocytes [21,27]. This initiator lymphocyte is supposed to produce specific factors which arm mast cells and macrophages [28]. Recent evidence has been obtained that the presumable phenotype of the initiator lymphocytes is CD3$^+$CD4$^-$CD8$^-$Thy-1$^+$ [21]. Cells with a similar phenotype are most likely also found in the iIEL fraction in mice [1-3,19]. As shown above the majority of murine iIEL display CD3 in combination with CD8 and/or Thy-1. Preliminary results from double-marker studies suggest that iIEL can at least be divided in three subsets: CD3$^+$CD4$^-$Thy-1$^+$CD8$^+$, CD3+CD4$^-$Thy-1$^+$CD8$^-$ and CD3$^+$CD4$^-$Thy1$^-$ CD8$^+$ cells. Of these the Thy-1$^+$ cells might be further divided into a Thy-1^{high} and a Thy-1^{low} population. The subset of Thy-1$^+$CD8$^+$ iIEL is similar as the one described by Goodman and Lefrancois [2] and may indeed have cytotoxic potential. The subset of Thy-1$^+$CD8$^-$ iIEL ,however, seems to be identical to the earlier described initiator type of cell in responses to cell bound antigens. It is tempting, therefore, to speculate a similar function for these cells in the intestinal epithelium.

From Northern blot analysis using specific probes for C-δ and VJC-α TcR sequences on total RNA obtained from iIEL of normal mice it could be

demonstrated that iIEL express almost exclusively TcR$\gamma\delta$. No $\alpha\beta$ message could be determined in iIEL. TcR$\gamma\delta$ expression on iIEL has recently also been reported by us [1] and others [2,3]. TcR$\gamma\delta$ bearing cells supposedly recognize antigens in a non-MHC restricted way [10-12,29,30] and with a limited specificity repertoire [11]. These criteria are considered to be essential for initiator lymphocytes [21]. Thus not only the phenotype of the cells but also the type of receptor would be well in line with the above hypothesised initiator function for (at least part of) the iIEL population.

iIEL obtained from athymic, nude mice express the TcR$\gamma\delta$ at a similar if not higher level than the iIEL of euthymic mice do. TcR$\gamma\delta^+$ has also been reported recently in peripheral lymphoid cells in athymic, nude mice [13-14]. Obviously cells bearing the TcR$\gamma\delta$ can differentiate independently from the thymus, although a thymus dependent route also has been proposed [14]. When comparing the iIEL from euthymic and athymic mice phenotypically the most striking observation is the relative decrease in Thy-1^+ cells in nude animals. Preliminary data from more detailed analysis of such cells furthermore suggest a shift in the ratio between Thy-1^{high} and Thy-1^{low} cells in the iIEL of athymic, nude mice. Whereas the balance in euthymic mice is in favour for Thy-1^{high} cells, the majority of Thy-1^+ cells in nude mice seem to be Thy-1^{low}. Especially the population of Thy-$1^{high}CD8^+$ appears to be lost entirely in the iIEL fraction of athymic, nude mice.

In conclusion it can be stated that according to their localization, their phenotype(s) and their receptor usage intraepithelial lymhocytes in the intestine of mice are well suited to function as initiator cells for secondary cellular responses against a variety of antigens. Although changes in composition of the iIEL fraction were noted in athymic animals the major population of such cells appears to be largely independent from the thymus for their differentiation.

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APPENDIX PAPER 2

PHENOTYPE OF INTRAEPITHELIAL LYMPHOCYTES IN EUTHYMIC AND ATHYMIC MICE: IMPLICATIONS FOR DIFFERENTIATION OF CELLS BEARING A CD3 ASSOCIATED $\gamma\delta$ T CELL RECEPTOR

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Published in : **European Journal of Immunology** 1990: 20: 291.

Summary

This study was carried out to determine the exact phenotype of intestinal intraepithelial lymphocytes (iIEL) in euthymic and athymic nude mice. The phenotype of iIEL in euthymic and athymic mice is mainly $CD3^+CD8^+$. However, based on Thy-1^- and CD3-associated T cell receptor (TcR) expression we can subdivide the $CD3^+CD8^+$ population into different subpopulations in euthymic and athymic, nude mice. In euthymic and athymic, nude mice several $CD3^+CD8^+$ populations can be defined. One population expressing and the TcR$\alpha\beta$. This population is absent in athymic, nude mice. Two other $CD3^+CD8^+$ populations can be detected in euthymic and athymic, nude mice. Based on Northern blot and flow cytometric analysis we have to conclude that these populations express the CD3 associated TcR$\gamma\delta$. One of the TcR$\gamma\delta^+$ populations also expresses at low surface density. This is in contrast to the TcR$\alpha\beta^+CD3^+CD8^+$ population. Which expresses at high surface density. There are also, however, especially in athymic, nude mice, significant numbers of $CD3^-CD8^+$ cells present with the same localization as iIEL. The function of these cells is yet unknown. Using a probe, specific, for TcRδ chain encoding mRNA we have shown that iIEL preferentially express 2 kb TcRδ chain mRNA, while nearly no TcRδ chain 1.7 kb mRNA is expressed by these cells. This is in contrast to TcRδ chain mRNA in thymocytes. Equal quantities of the 1.7- and 2.0-kb TcRδ chain mRNA species were found in RNA isolated from thymocytes.

The results imply that $CD3^+CD8^+$ intestinal iIEL expressing the CD3-associated TcR$\gamma\delta$ can differentiate in absence of the thymus and represent a thymus-independent lineage of cells bearing this receptor.

Introduction

Cells expressing the CD3-associated TcR$\gamma\delta$ [1-5] represent a subset of lymphocytes with an as yet unidentified function. Recently, some evidence has been obtained indicating a possible role for these cells. The first observation on these cells was that cloned TcR$\gamma\delta^+$ cell lines frequently exhibit nonspecific cytotoxicity [6-7]. Their activity as killer cells implied a role for TcR$\gamma\delta^+$ cells in immune surveillance as killers of virally infected or tumorigenic cells. A second observation is that a large percentage of lymphoid cells in the murine skin and small intestinal epithelium expresses

the TcR$\gamma\delta$ [8-13]. At these particular epithelial sites cells are present which apparently express V, J and C sequences derived from the same TcR β chain gene [9,14]. Such site specificity of receptors may indicate that these cells do not circulate to perform their effector function, but instead remain fixed and are predisposed to recognize an antigen common to that particular site of localization [15]. Recent experiments have been reported [16-18] which showed that at least a significant proportion of TcR$\gamma\delta^+$ cells with the $CD3^+CD4^-CD8^-$ phenotype appear to recognize epitopes on a class of phylogenetically conserved stress proteins. These results are well in line with the suspected role for these cells in immune surveillance against virus-infected cells, since such cells usually start to express stress proteins shortly after infection.

At this moment, it is not clear whether TcR$\gamma\delta^+$ cells recognize antigen in a MHC restricted fashion. Murine cell lines have been generated, of which the activity appears to be restricted to MHC class I or a gene product related to class I [19,20]. However, such a restriction could not be demonstrated in other cloned lines from human origin [6,7], although this might be due to nonspecific activation of cells during the cloning and testing procedures.

In this report we present the phenotype of intraepithelial lymphocytes iIEL in euthymic and athymic, nude mice. The phenotype of iIEL in euthymic animals is complex and appears to include two subsets bearing a CD3 associated receptor. One subset with the $CD3^+CD8^+$ phenotype is thymus dependent and expresses the TcR$\alpha\beta$. This subset is not detectable in athymic, nude mice. The other subset includes two populations expressing the CD3-associated TcR$\gamma\delta$. The phenotypes of TcR$\gamma\delta^+$ populations are $CD3^+CD8^+$ with one population negative for and the other positive for . The subset expressing TcR$\gamma\delta$ is thymus independent for differentiation since this one can be shown in euthymic as well in athymic, nude animals.

Materials and methods

Animals

Balb/c +/+ and nu/nu specific pathogen free mice of 8-10 weeks old were obtained from the Central Animal Facilities of the TNO Radiobiological Institute, Rijswijk, The Netherlands.

Isolation of iIEL and lamina propria lymphocytes (LPL)

iIEL and LPL were isolated as reported before with minor modifications [10,21]. Shortly, after removal of the small intestine this organ was flushed with PBS and the Peyer's patches were removed. After cutting the intestine longitudinally, the tissue was minced in pieces of 1-2 mm and subsequently incubated in HBSS, supplemented with 1 mM EDTA, for 20 min at 37°C. After incubation and removal of debris, the cells were washed and filtrated over a nylon wool column [22]. Nylon wool filtration does not influence the composition of the iIEL fraction (data not shown). This step was included to remove most of the epithelial cells. After filtration, the cells were centrifuged through a discontinuous Percoll (Pharmacia, Uppsala, Sweden) density gradient (= 1.086 g/ml, 1.054 g/ml and 1.037 g/ml) at 600 x g and 4°C for 30 min. Subsequently, the densest interphase was harvested. Cells in this interphase were further denoted as iIEL.

FCM analysis and staining

mAb 145-2C11, directed against mouse CD3 was a gift of Dr. J. A. Bluestone [23]. mAb 145-2C11 was used directly conjugated to FITC. A directly FITC- or biotin conjugated mAb, 59AD2.2, was used for staining [24]. mAb GK1.5 conjugated to biotin was used to detect $CD4^+$ cells [25]. mAb 53-6.7 (Becton Dickinson, Sunnyvale, CA) directly conjugated to FITC or biotin was used for CD8 staining. The mAb H57-597, a pan TcR$\alpha\beta$ recognizing mAb, was a kind gift of Dr. R. Kubo [26]. This mAb was used in combination with a second-step FITC-conjugated rabbit anti-rat polyclonal Ig antibody (Miles Inc., Naperville, IL). FITC conjugated mAb KJ-16 [27], a gift of Dr. P. Marrack was used for staining cells expressing Vβ8.1/8.2. For detection of surface Ig (sIg) a goat anti-mouse, FITC conjugated, (GAM) polyclonal Ig antibody was used (Nordic, Tilburg, The Netherlands). All biotin-conjugated mAb were used in combination with avidin-FITC (Sigma Chemical Company, St.Louis, MO) or streptavidin-PE (Becton Dickinson). mAb were purified, conjugated and stored in PBS, 1% BSA (Sigma) at -20°C. For FCM analysis cells were washed once in PBS, 1% BSA and incubated with an mAb at the appropriate dilution for 30 min at 4°C. For the biotin-conjugates, the first step was followed, after washing twice in PBS and 1% BSA, by a second incubation step with

either avidin-FITC or streptavidin-PE. After the final incubation, cells were washed twice in PBS, 1% BSA and resuspended in the same medium at a final concentration of 10^6 cells/ml. Labeled cells were analyzed using a FACScan equipped with the Consort 30 program and Paint-a-Gate software (Becton Dickinson).

Histology

After removal of the small intestine this organ was processed in so-called "Swiss rolls" [28] and snap frozen in liquid nitrogen. 6-μ-thick cryostat sections were cut and incubated for 30 min at room temperature with mAb in appropriate dilutions as described in detail previously [10]. After washing the sections twice with PBS and 1% BSA, a second incubation with streptavidin-PE was performed for two-color staining with biotin- conjugated antibodies. A Leitz (Wetzlar, FRG) microscope equipped for selective visualization of fluorescein and rhodamine was used to read the slides.

Northern blot analysis

RNA was isolated by the guanidinium isothiocyanate method [29]. After formaldehyde gel electrophoresis (1.5% agarose gel), total RNA was blotted on Biotrans nylon membranes (ICN Biochemicals Inc, Irvine, CA). Blots were hybridized for 15 h at 42°C with a VJC-α probe (a gift of Dr. H. Hengartner, Zurich, Switzerland) and/or a C-δ TcR probe (provided by Dr. T. Mak, Ontario, Canada, through Dr. R. De Weger, Utrecht, The Netherlands), labeled with ^{32}P-α-dCTP (Radiochemical Center Amersham, Amersham, GB) by random priming (Gibco BRL, Breda, The Netherlands) in 50% formamide (Aldrich Chemical Company, Milwaukee, WI), 5x Denhard's solution, 5x SSC, 50 mM Na_3PO_4, 0.1% SDS and 250 μg/ml salmon sperm DNA. After hybridization, the blots were washed in 0.5x SSC and 0.1% SDS at 50°C. When the same blot was hybridized twice, the blot was stripped (between the subsequent hybridizations) by incubation for 20 min at 60°C in 50% formamide, 0.1% SDS and 50 mM Na_3PO_4. After hybridization, the blots were exposed for 24 h to Kodak XAR films in cassettes with Kodak, lanex fine, amplification screens (Kodak Company, Rochester, NY).

Results

FCM analysis of iIEL isolated from the small intestine of euthymic and athymic nude mice.

After isolation and staining FCM analysis was performed on the iIEL fraction. An enriched cell suspension consistently contained 20%-30% cells with a lymphoid morphology. The remainder of this fraction consisted of epithelial, neuroendocrine cells and cells with a poly-morphic nucleus (data not shown). Further analysis of cells with a lymphoid appearance was performed by defining them by their forward and perpendicular light scatter. Selection and gating was done by using Paint-a-Gate software in combination with the Consort 30 program.

As can be seen in table 1 the vast majority (80%) of iIEL in euthymic mice expresses surface CD3. The remaining population consists predominantly of sIg^+ B cells. In contrast to the situation in peripheral lymphoid organs, not all $CD3^+$ cells are Thy-1^+: only 53% of iIEL express Thy-1. Also CD4 and CD8 expression seems to be in imbalance with the distribution in the periphery.

Eighteen percent of the iIEL displays a TcR$\alpha\beta$ as determined both with a pan TcR$\alpha\beta$ mAb (18%) and a Vβ family specific mAb (13%). The percentage of $CD3^+$ iIEL in athymic, nude mice (40%) was half as that in euthymic mice (table 1). Also, a lower number of Thy-1^+ cells is found (14%). Since the number of B cells approximately remains the same (18%), there must be a significant contribution by nonlymphoid cells in this fraction in athymic,nude mice. In parallel with the smaller $CD3^+$ population, the $CD8^+$ population is decreased in size. However it must be pointed out that in the iIEL fraction, derived from athymic, nude mice, the percentage $CD8^+$ cells is actually higher than that of the $CD3^+$ cells (45% vs. 40%). Neither CD4 nor the TcR$\alpha\beta$ were detected in the iIEL fraction of athymic, nude mice.

Table I. FACS analysis of iIEL from euthymic and athymic, nude mice after single staining with monoclonal antibodies.

Monoclonal antibody	Specificity	iIEL source[1] +/+	Nu/Nu
145-2C11	CD3	80%[2]	40%
GK1.5	CD4	6	1
53-6.7	CD8	70	45
59AD2.2	Thy-1	53	14
H57-597	TCR$\alpha\beta$	18	1
KJ16	Vβ8.1/8.2	13	0
GAM	sIg	17	18

[1]iIEL were obtained from euthymic (+/+) and athymic, nude (nu/nu) Balb/c mice as described in Materials and Methods. iIEL from 2 individual mice were pooled before analysis.

[2]Results are expressed as a percentage of viable cells with a lymphoid morphology as determined by forward and perpendicular light scatter gating (see Material and Methods). The mean of four independent experiments is given. Non-specific staining has been checked by staining with non-relevant mAb's and subtraction of these background values (data not shown).

To gain insight in the actual phenotypes of the cells in these iIEL fractions, double staining experiments with combinations of the mAb 145-2C11 (anti-CD3), 59AD2.2 (anti-Thy-1), 53-6.7 (anti-CD8) and KJ16 (anti-Vβ8.1/8.2) were performed. The results of a typical experiment are shown in fig. 1 and the combined quantitative data of four independent experiments are given in table 2. Two-color analysis of the iIEL fraction of euthymic mice for CD3 and Thy-1 (fig.1A) reveals that all Thy-1$^+$ cells (52%) are CD3$^+$. The intensity of CD3 expression on Thy-1$^+$ cells is, on average, lower than the CD3 expression on Thy-1$^-$ cells. Based upon a

more detailed analysis (data not shown) the Thy-1$^+$ population can be further divided into Thy-1^{high} (roughly above channel 200) and Thy-1^{low} (until channel 200) cells. In euthymic mice most of the Thy-1$^+$ cells belong to the Thy-1^{high} subset (29% high vs. 21% low). About one third of the iIEL (32%) is CD3$^+$Thy-1$^-$. Finally minor populations of CD3$^-$Thy-1$^+$ (8%) and CD3$^-$Thy-1$^-$ cells are found in the iIEL. These latter cells are probably B cells.

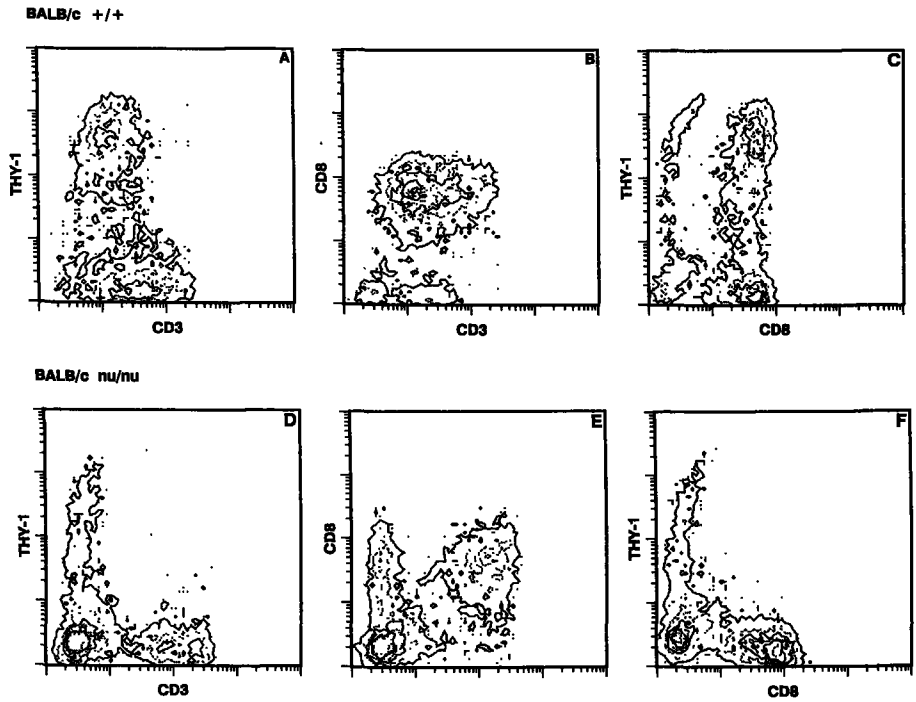

Figure 1. Contour plot after double fluorescence staining of iIEL from euthymic (+/+) (A,B & C) and athymic (nu/nu) (D,E & F) mice. Lymphoid cells were defined by forward and perpendicular light scatter and selected using Paint-a-Gate software in combination with the Consort 30 program. On the vertical axis the log red fluorescence is plotted. The horizontal axis represents log green fluorescence. Double-staining was performed for CD3 (green) and Thy-1 (red) (A&D); CD3 (green) and CD8 (red) (B&E); CD8 (green) and Thy-1 (red) (C&F). iIEL from 2 individual mice were pooled before analysis.

Table 2. FACS analysis of iIEL from euthymic and athymic, nude mice after double staining with monoclonal antibodies.

Monoclonal antibody combination		iIEL source[1]	
		+/+	nu/nu
145-2C11	**59AD2.2[2]**		
$CD3^+$	$Thy\text{-}1^H$	29%[3]	0
$CD3^+$	$Thy\text{-}1^L$	21	6
$CD3^+$	$Thy\text{-}1^-$	32	30
$CD3^-$	$Thy\text{-}1^{H+L}$	8	23
145-2C11	**53-6.7**		
$CD3^+$	$CD8^+$	72	30
$CD3^+$	$CD8^-$	12	11
$CD3^-$	$CD8^+$	4	17
53-6.7	**59AD2.2**		
$CD8^+$	$Thy\text{-}1^H$	22	0
$CD8^+$	$Thy\text{-}1^L$	19	4
$CD8^+$	$Thy\text{-}1^-$	28	43
$CD8^-$	$Thy\text{-}1^{H+L}$	15	22
KJ16	**59AD2.2**		
Vβ8.1/8.2	$Thy\text{-}1^H$	13	0

[1]iIEL were obtained from euthymic (+/+) and athymic (nu/nu) Balb/c mice as described in Materials and Methods. iIEL from 2 individual mice were pooled before analysis.

[2]Thy-1 staining intensity was considered as high from channel 200 and up (see also fig.1).

[3]Results are expressed as a percentage of viable cells with a lymphoid morphology as determined by forward and perpendicular light scatter gating. The mean of four independent experiments is given.

A different picture emerges when CD3 expression is compared with CD8 expression. As can be seen in fig. 1B, the majority of iIEL from euthymic mice are $CD3^+CD8^+$ (72%). Only a few cells remain which are either $CD3^-CD8^-$ (B cells?) or $CD3^+CD8^-$ ($CD4^+$ T cells?, 12%). Double staining with CD8 and Thy-1 shows a rather complicated pattern (fig.1C). A significant proportion of the iIEL expresses both CD8 and Thy-1 (42%). Of these cells the majority belongs as expected to the Thy-1^{high} subset (22%). Nevertheless, a rather large population of $CD8^+$Thy-1^- cells (28%) and a minor subset (15%) of $CD8^-$Thy-1^+ (both high and low) cells ($CD4^+$ T cells?) can be observed. Finally, also a small population of $CD8^-$Thy-1^- cells (B cells?) can be observed in the iIEL fraction of euthymic mice.

A completely different pattern observed when the iIEL fraction of athymic nude mice is analyzed in a similar way (fig.1D-F). Almost no $CD3^+$Thy-1^+ cells (6%) are found (fig.1D). If present at all, these cells belong entirely to the Thy-1^{low} population. iIEL from athymic, nude mice were composed of two major populations: $CD3^+$Thy-1^- iIEL and $CD3^-$Thy-1^+. The percentage of the $CD3^+$Thy-1^- population (30%) is equal to that in euthymic mice. However the percentage of the $CD3^-$Thy-1^+ population was substantially higher in athymic, nude mice.

When comparing CD3 with CD8 (fig.1E), it is obvious that the majority of the $CD3^+$ is $CD8^+$. However, a significant number of $CD3^-CD8^+$ cells (17%) also exists in the iIEL fraction of athymic, nude mice. Double staining for CD8 and Thy-1 showed a mutually exclusive expression of these markers on iIEL of athymic, nude mice: besides many $CD8^-$Thy-1^- cells a large population of $CD8^+$Thy-1^- cells (43%), a significant number of $CD8^-$Thy-1^+ cells (22%) and only a few (4%) $CD8^+$Thy-1^+ cells were found. The latter belonged to the Thy-1^{low} subset.

Finally, double staining with 59AD2.2 and KJ16, and single staining with H57-597 revealed that TcR$\alpha\beta$ are expressed on iIEL expressing Thy-1 in high membrane density (no contour plot shown, 13%). This subset is obviously absent in the iIEL isolated from athymic, nude mice.

From the various data one can calculate the relative contribution of subpopulations with a defined phenotype to the iIEL fraction. The results of these calculations are shown in table 3.

Table 3. Calculated values of subpopulations of iIEL with a defined phenotype in euthymic and athymic, nude mice.

			iIEL	
			+/+	nu/nu
$CD3^+$	$CD8^+$	$Thy\text{-}1^-$	39%[1]	87%
$CD3^+$	$CD8^+$	$Thy\text{-}1^L$	26%	13%
$CD3^+$	$CD8^+$	$Thy\text{-}1^H TCR\alpha\beta$	30%	0%

[1]Percentages have been calculated and are expressed as values of the total CD3 percentage in a given animal.

Of the three major subpopulations in the iIEL of euthymic mice the $CD3^+CD8^+Thy\text{-}1^-$ subset comprises approximately 40% of the $CD3^+$ cells. The remainder is almost equally divided between cells with a $CD3^+CD8^+Thy\text{-}1^{low}$ phenotype and cells with a $CD3^+CD8^+Thy\text{-}1^{high}$ phenotype. This latter phenotype is completely associated with $TcR\alpha\beta$ expression. In the iIEL of athymic nude mice only the first two populations are found in a ratio of approximately 6:1 (table 3), since cells in the $Thy\text{-}1^-$ and the $Thy\text{-}1^{low}$ populations do not stain with H57-597 for the $TcR\alpha\beta$. These populations express another CD3 associated receptor, probably the $TcR\gamma\delta$.

Localization of iIEL in the small intestine of euthymic and athymic mice.

Membrane $CD3^+$ iIEL are present in the small intestine of normal euthymic mice and are located between or directly beneath the epithelial cell layer which covers the lamina propria (fig.2) [10,30,31]. A similar observation is made for surface $CD3^+$ iIEL in athymic, nude mice (fig.2EF), although the total number of positive cells per villus is slightly decreased as compared to euthymic animals. Two-color staining of cryostat sections with mAb specific for CD3, CD8 and CD4 shows in the euthymic animals that CD3 expression of iIEL is predominantly associated with CD8 expression (fig.2AB). In addition some $CD8^+CD3^-$ iIEL are present (fig.2AB). Staining with anti-CD3 and anti-CD4 (fig.2EF) shows that double positive cells can only be observed in the stroma of the villi and in the lamina

propria. Therefore, these cells do not have the specific localization of iIEL. A high proportion of cells that express both CD3 and CD8 is evident in athymic nude mice (fig.2EF). However, $CD3^{-}CD8^{+}$ cells can also be detected whereas no $CD3^{+}CD4^{+}$ cells were observed in athymic, nude mice (not shown).

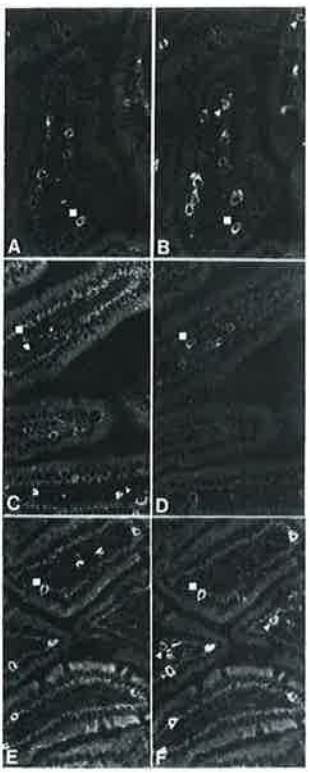

Figure 2. Double fluorescence immunohistology of small intestine of euthymic Balb/c mice, using mAb FITC-conjugated 145-2C11 (anti-CD3) (fig. 2A) and biotinilated mAb 53-6.7 (anti-CD8) (fig.2B). High numbers of $CD3^{+}CD8^{+}$ iIEL can be observed (□) as well as some $CD3^{-}CD8^{+}$ cells (∇). Double fluorescence immunohistology of the small intestine from euthymic Balb/c mice using FITC-conjugated mAb 145-2C11 (anti-CD3) (fig.2C) and biotinilated mAb GK-1.5 (anti-CD4) (fig.2D). Only a few $CD3^{+}CD4^{+}$ cells can be observed (□). Note the stromal localization of these cells. Double fluorescence immunohistology of small intestine of athymic Balb/c nude mice using the same mAb combinations as described for panels 2A and B. $CD3^{+}CD8^{+}$ iIEL (□) as well as $CD3^{-}CD8^{+}$ cells (∇) can readily be observed. Biotinilated mAb were used incombination with streptavidin-PE

Northern blot analysis of iIEL and LPL

Immunoprecipitation studies [8,9] have already shown that at least a large proportion of purified iIEL expresses TcR $\gamma\delta$. In this report we have analyzed iIEL receptor usage by Northern blot hybridization using a TcR VJC-α and a C-δ probe. Since the δ locus is located within the α locus [2] these probes allow a discrimination between cells which express TcR$\gamma\delta$ or $\alpha\beta$. Genomic rearrangement in the α locus will lead to deletion of the δ locus [2,32]. Fig. 3 shows the results of a hybridization with ^{32}P-labeled VJC-α and C-δ probes of a blot containing total RNA isolated from MLN, Peyer's patches, LPL and iIEL. Comparison of the blot after hybridization with the ^{32}P-labeled VJC-α sequence reveals abundant levels of functional 1.7 kb TcR α chain mRNA in the MLN and Peyer's patches of euthymic mice. Only low levels of TcR α chain mRNA can be detected in both LPL and iIEL.

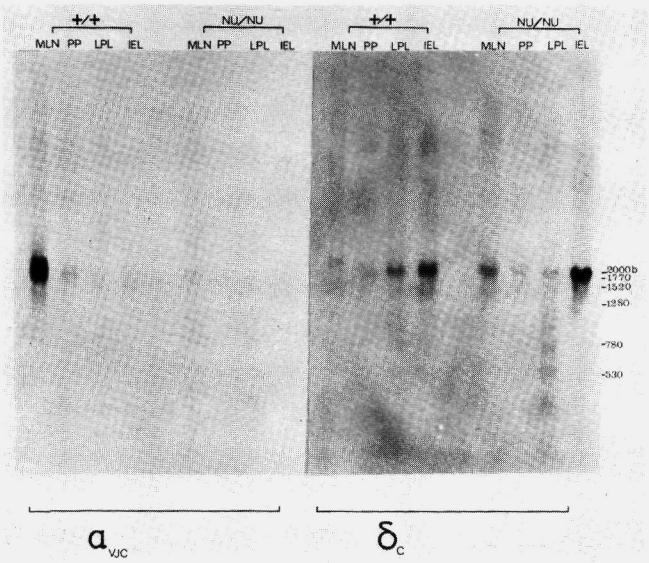

Figure 3. Northern blot analysis of total RNA, isolated from mesenteric lymph nodes (MLN), Peyer's patches (PP), Lamina Propria lymphocytes (LPL) and Intra Epithelial Lymphocytes (iIEL) of euthymic and athymic Balb/c mice. RNA was isolated and hybridization was performed as described under Material and Methods. The same blot was used twice after removal of radioactive probe between subsequent hybridizations.

In athymic, nude animals no TcRα chain mRNA can be detected in any of the RNA samples. After hybridization with the labeled C-δ probe a high level of a 2 kb transcript was detected with RNA isolated from iIEL of euthymic mice.Total RNA extracted from iIEL of athymic, nude mice contained comparable levels of functional TcRδ chain mRNA. Both in euthymic and athymic, nude mice a relatively low level of this transcript was found in the MLN. Based on these Northern blot data, one can conclude that iIEL in athymic nude mice express TcR$\gamma\delta$ but not TcR$\alpha\beta$.

Another feature of the TcRδ chain mRNA expressing iIEL appears from the hybridization with the C-δ probe. As can be seen in fig.3 the probe hybridizes in iIEL only with one 2.0-kb RNA species, which probably represents the functional mRNA for this chain. Nonfunctional TcRδ chain mRNA usually has a length of 1.7 kb [2,32]. The functional and nonfunctional mRNA species can be detected in RNA extracted from (fetal) thymocytes or clones and hybrids derived from such cells [2,32].

As can be seen in fig.4, total RNA isolated from adult thymus displays, after hybridization with the C-δ probe these two RNA species with an intensity ratio of approximately 1:1. In this particular experiment two

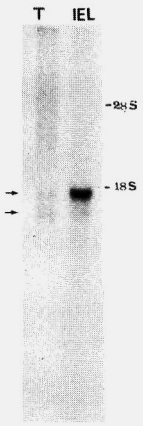

Figure 4. Northern blot analysis of total RNA from thymus (T) and iIEL. RNA was isolated from an euthymic Balb/c mouse. Bands of 18S and 28S ribosomal RNA are indicated.

bands also appeared in the total RNA from iIEL. However, in the iIEL RNA the intensity ratio is still approximately 30:1 in favor of the 2.0 kb band (fig.4).

Discussion

This report describes a detailed analysis of the phenotypes of iIEL, based on CD3, CD8, CD4, Thy-1 and TcR$\alpha\beta$ expression. As summarized in table 3 three major $CD3^+$ populations can be found in the iIEL of euthymic mice. Distinction between these subsets is done primarily through Thy-1 cell surface expression. Whereas the $CD3^+CD8^+Thy\text{-}1^-$ and $Thy\text{-}1^{low}$ cells can still be detected in the iIEL fraction of athymic nude mice the $CD3^+CD8^+Thy\text{-}1^{high}$ subset, which are most likely TcR$\alpha\beta^+$ cells, is virtually non-existent in such mice. Given the complicated isolation procedures used to obtain iIEL, one has to be aware, however, of possible technical artifacts which can influence the final results. Therefore, specific immunohistology was performed on frozen tissue. This approach revealed that the subpopulations observed by FCM analysis of isolated iIEL truly reflects the in situ situation in the epithelial cell layers of the small intestine.

Besides the above mentioned CD8- and Thy-1-expressing CD3-associated cell populations, cells with other phenotypes were also observed in the isolated iIEL fraction. Both in immunohistology and by FCM analysis, $CD3^-$ cells with a high level of CD8 expression could be determined. The nature of these cells, relatively abundant in the iIEL fraction of athymic mice, is yet unknown, but we have evidence that these cells do not belong to a lymphoid lineage, since we can detect equal numbers of cells with this phenotype by histology at the same location in the small intestine of Scid/Scid mice [33]. Also $CD3^-CD8^-Thy\text{-}1^{high}$ cells were found in the iIEL from athymic, nude mice (see fig.1F). Probably these cells are stromal cells from the villi which express Thy-1. By histological procedures we could indeed show that mAb 59AD2.2 also recognizes Thy-1 on stromal cells in the villi of the small intestine (data not shown). $CD4^+$ cells are absent from iIEL from athymic animals and present in very low numbers in euthymic mice. CD4 expression in the iIEL fraction is associated with a $Thy\text{-}1^{high}$ phenotype and with TcR$\alpha\beta$ expression. Moreover, it was obvious from immunohistology that the detected $CD4^+$ cells do not belong to the iIEL population, since they are located predominantly in the stromal compartment of the villi and the lamina propria (see fig.3).

Receptor analysis, using Northern blotting and anti-TcR$\alpha\beta$ mAb, indicated that the $CD3^+CD8^+Thy\text{-}1^-$ and $CD3^+CD8^+Thy\text{-}1^{low}$ iIEL in athymic, nude mice express a TcR$\gamma\delta$. By analogy, one would expect the same for these cell types in euthymic mice. The predominant appearance of

the 2.0 kb TcR δ chain mRNA species in total RNA, extracted from iIEL of both euthymic and athymic, nude mice, actually leaves no doubt that in most of the TcR$\gamma\delta^+$ cells in the small intestine, the only transcribed δ chain mRNA is the 2.0 kb mRNA. Since CD3$^+$CD8$^+$Thy-1$^-$ and CD3$^+$CD8$^+$Thy-1^{low} TcR$\gamma\delta^+$ cells are present iIEL from athymic, nude mice, it can be concluded that these cells do not depend on the thymus for their differentiation. This is in striking contrast with observations on TcR$\gamma\delta^+$ cells with a CD3$^+$Thy-1^{high} phenotype [34]. Such cells are present in peripheral lymphoid organs [34] and, as Thy-1$^+$ DETC cells, in large numbers in the epidermis of mice [12, 13]. We did not analyze iIEL with this phenotype in detail, but by comparing the data in table 2 we have to conclude that such cells are present in low numbers in the iIEL fraction of euthymic mice. In athymic mice this population is probably absent in the iIEL. Moreover we have observed that the CD3$^+$Thy-1^{high} or Thy-1$^+$ DETC cells are nearly absent in the epidermis of athymic, nude mice. It seems, therefore, that at least two lines of TcR$\gamma\delta^+$ cells exist: one, which is CD3$^+$Thy-1^{high} and thymus dependent [34], and another one primarily located in the epithelial layers of the gut, which is CD3$^+$CD8$^+$Thy-1$^-$ or CD3$^+$CD8$^+$Thy-1^{low} and thymus independent. These latter cells are obviously also independent of a so-called "fetal thymic wave" as proposed for Thy-1$^+$ DETC cells [14].

The absence of CD3$^+$CD8$^+$Thy-1^{high} expression in the iIEL fraction of athymic nude mice has implications for both the function and differentiation pathway of such cells. In a recent report Lefrancois and Goodman [35] described that in germ free C57BL/6J and Swiss mice only CD3$^+$CD8$^+$Thy-1$^-$ iIEL can be detected. We have made similar observations for iIEL, isolated from germ free mice, although we can also detect a small population of CD3$^+$CD8$^+$Thy-1^{low} cells. After conventionalizing the mice for 4 weeks the CD3$^+$CD8$^+$Thy-1^{high} population could also be detected, whereas the number of CD3$^+$CD8$^+$Thy-1^{low} cells had markedly increased. Thus, exogenous antigenic pressure seems to results in an increase of the population of Thy-1 expressing cells. Whether this is due to an up-regulation of the Thy-1 receptor itself or through the appearance of a new population of Thy-1$^+$ cells remains to be established.

A small percentage of iIEL in these animals express Thy-1 at a low surface density in combination with CD8 and CD3. Thus, in contrast to the situation in euthymic mice antigenic exposure of athymic, nude animals to viable bacterial food antigens does not result in a high Thy-1 expression. One has to conclude, therefore, that in euthymic animals the thymus itself

or a thymus dependent component is responsible for the appearance of $CD3^+CD8^+Thy\text{-}1^{high}$ cells. In this respect it needs to be pointed out that FCM analysis using mAb H57-597 (anti-TcR$\alpha\beta$) and KJ16 (anti-Vβ8.1/8.2) revealed that approximately 30% of the $CD3^+$ population of iIEL of euthymic mice expresses TcR$\alpha\beta$. Furthermore, expression of TcR$\alpha\beta$ is associated with the $Thy\text{-}1^{high}$ phenotype. In view of the relatively high number of TcR$\alpha\beta$ expressing cells in the iIEL of euthymic mice, it is surprising that in immunoprecipitation studies [8,9] and mRNA hybridization studies ([10] and this report) no or only low signal for the TcR$\alpha\beta$ or α chain mRNA could be found. However, an explanation for this discrepancy might be our observation that the expression of CD3 on cells expressing the TcR$\alpha\beta$ is about five times lower than the CD3 expression on cells bearing TcR$\gamma\delta$ (see also fig.1A). This will result in an approximately five times lower iodination of TcR$\alpha\beta$ as compared to iodination of TcR$\gamma\delta$ and, hence, a strongly reduced signal after electrophoresis. Furthermore the low level of mRNA for the TcRα chain indicates that the cell surface expression of TcR$\alpha\beta$ has to be low as compared to the cell surface expression of TcR$\gamma\delta$ on cells in the iIEL fraction.

Based upon the combined results, it is reasonable that the presence of the thymus and specific (local) antigenic stimuli are essential factors for the appearance of the $CD3^+Thy\text{-}1^{high}$ (TcR$\alpha\beta^+$) cells in the epithelial cell layers of the small intestine.

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APPENDIX PAPER 3

ANALYSIS OF INTESTINAL INTRAEPITHELIAL LYMPHOCYTES IN ATHYMIC (NUDE) AND SCID MICE

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Published in : **Advances in Mucosal Immunology**, T. T. Mc Donald et al. Eds. Kluwer academic publishers. Dordrecht/Boston/ London. 1990: 71.

Introduction

About 70 % of the intestinal intraepithelial lymphocytes (iIEL) in euthymic mice expresses the CD3 associated $\gamma\delta$ T cell receptor (TcR) [1-5]. The remaining 30% of the $CD3^+$ iIEL express the TcR$\alpha\beta$ [3,5]. The relative percentage of TcR$\gamma\delta^+$ and TcR$\alpha\beta^+$ cells in the iIEL fraction, however, depends upon the gnotobiotic status of the animals [6, and our unpublished results]. In athymic, nude mice the only receptor expressed on $CD3^+$ iIEL is the TcR$\gamma\delta$, as shown by Northern blot hybridisation and negative staining results with monoclonal antibody (mAb) H57-597, which recognizes the TcR$\alpha\beta$ [3,5,7]. The phenotype of $CD3^+$ iIEL in euthymic animals is $CD8^+$Thy-1^- and $CD8^+$Thy-1^{+low} for TcR$\gamma\delta^+$ cells and $CD8^+$Thy-1^{+high} for cells which carry the TcR$\alpha\beta$ [3,5]. In athymic animals the major phenotype of $CD3^+$ iIEL is $CD8^+$ Thy-1^-. The presence of $CD3^+$ iIEL in athymic, nude mice indicates that these cells can differentiate in the absence of the thymus. Besides $CD3^+CD8^+$ cells we can also detect a population of $CD3^-CD8^+$ cells in the iIEL fraction [5 and this report]. Although more dominant in the iIEL of athymic, nude mice this $CD3^-CD8^+$ population is also found in euthymic mice. Furthermore such $CD3^-CD8^+$ cells are also present in Scid/Scid mice. Both this finding and the association with asialo-GM1 staining for at least part of these cells strongly suggest a relationship with non-lymphoid cells which express natural killer (NK) activity. In that case cells with a $CD3^-CD8^+$ phenotype would represent a novel phenotype for NK cells in the mouse.

Materials and methods

Animals

8-10 weeks old specific pathogen free (SPF) euthymic and athymic (nude) Balb/c mice were obtained from the Central Animal Facilities of the Radio Biological Institute TNO, Rijswijk, The Netherlands. CB 17 Scid/Scid mice of 12 weeks of age were obtained from the Jackson Laboratory, Bar Harbor ME, U.S.A.

Cell isolation and FACS analysis.

The isolation of iIEL from the epithelial layers of the small intestine

and the preparation of single cell suspensions from mesenteric lymph nodes was done as described in detail previously [4,5]. Monoclonal antibody (mAb) 145-2C11 (a gift of Dr.J.A.Bluestone), directed against mouse CD3 was used directly conjugated with FITC. mAb 53-6.7 (Becton & Dickinson, Sunnyvale, USA), directly conjugated with FITC or biotin was used for CD8 staining. Biotinylated mAb's were used in combination with Streptavidin-Phycoerythrin (Becton & Dickinson, Sunnyvale, USA). Asialo-GM1 was detected with a polyclonal rabbit anti-mouse asialo-GM1 antibody (Wako Chemicals, Dallas, USA) in combination with a FITC conjugated horse anti-rabbit polyclonal antibody (Miles, Elkhard, USA). For FACS analysis cells were washed once in PBS, 1% BSA and incubated with a mAb at the appropriate dilution for 30 minutes at 4°C. For the biotin- conjugates, the first step was followed, after washing twice in PBS, 1% BSA, by a second incubation step with Streptavidin-Phycoerythrin. After the final incubation, cells were washed twice in PBS, 1% BSA and resuspended in the same medium at a final concentration of 10^6 cells/ml. Labelled cells were analyzed using a FACScan equipped with the Consort 30 program and Paint-a-Gate software (Becton & Dickinson, Sunnyvale, USA).

Immunofluorescence histology.

The intestine was processed in 'Swiss rolls' and snap frozen in liquid nitrogen. 6μm cryostat sections were cut and incubated with mAb's against CD3 and CD8. After washing a second incubation with streptavidin-PE was done for CD8 staining. Fluorescent cells were visualized with a Leitz microscope, equipped for two-colour immunofluorescence.

Results

iIEL from euthymic as well from athymic, nude mice, cells were isolated, stained with mAb's as indicated under materials and methods, and used for FACS analysis. Cells with a lymphoid appearance were selected, using forward and perpendicular light scatter gating.

The results of a representative double staining experiment of iIEL with mAb's, specific for CD3 and CD8 is shown in fig.1. As can be seen in fig.1A the vast majority of iIEL in euthymic animals is $CD3^+CD8^+$. In athymic animals, however, two distinct $CD8^+$ iIEL populations can be

detected: a $CD3^+CD8^+$ and $CD3^-CD8^+$ population (fig.1B).

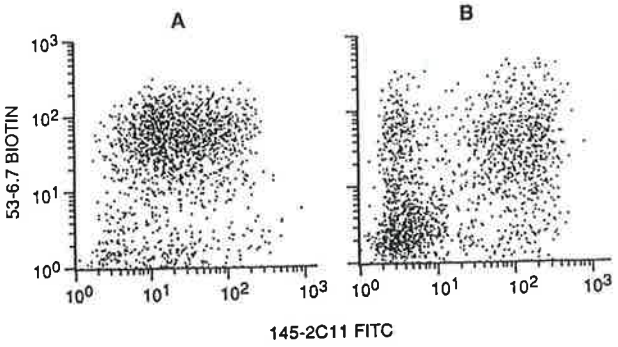

Figure 1. FACS double staining of iIEL, isolated from euthymic (A) and from athymic Balb/c mice (B). The mAb's used are directed against CD3 (145-2C11-FITC) and CD8 (53-6.7-Biotin).

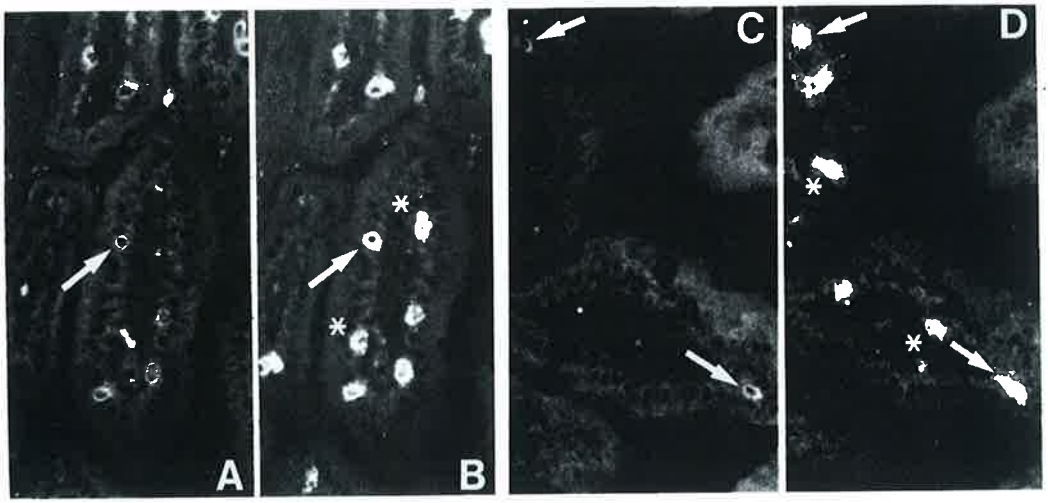

Figure 2. Immunofluorescence double staining of sections from athymic BALB/c (A&B) and CB17 Scid/Scid (C&D) mice with anti-CD3 (A&C) and anti-CD8 (B&D). Arrows: $CD3^+CD8^+$ cells; asterix: $CD3^-CD8^+$ cells.

Similar results were obtained for nude mice using CD3 and CD8 immunofluorescence double staining on frozen sections of small intestine (fig.2A&B). Moreover, cells staining both for CD3 and CD8 can also be detected in Scid/Scid mice (fig.2 C&D). The numbers of such double positive iIEL, however, are reduced in athymic, nude and Scid/Scid mice when compared to euthymic animals (results not shown). Also cells with the $CD3^{-}CD8^{+}$ phenotype are present in the epithelial cell layers both in the athymic and Scid/Scid mouse intestine (fig.2B&D). The numbers of these cells are similar in both mutant strains. These $CD3^{-}CD8^{+}$ cells can also be found in the intestinal epithelium of euthymic mice. The numbers, however, are clearly lower than in athymic and Scid/Scid mice (results not shown).
$CD8^{+}$ cells in the iIEL fraction and mesenteric lymph nodes from euthymic and athymic, nude mice were further analyzed using anti-CD8 and anti-asialo-GM1 antibodies. In fig.3 the results are shown of a typical double staining experiment with these antibodies on the iIEL fraction of athymic, nude mice (fig.3A) and mesenteric lymph node cells of euthymic mice (fig.3B).

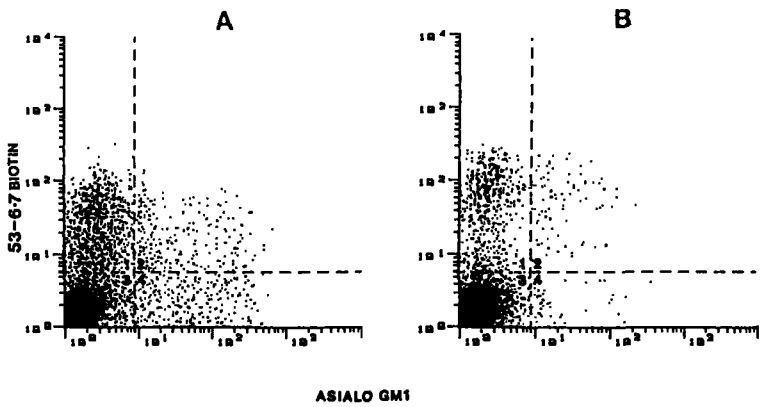

Figure 3. FACS analysis of iIEL (A), isolated from the small intestine of an athymic mouse and cells derived from the MLN (B) of an euthymic animal. The vertical axis represents CD8 staining (53-6.7-Biotin), the horizontal axis represents asialo-GM1 staining (polyclonal rabbit anti-murine asialo-GM1). Staining with second step reagents only was negative.

In the iIEL fraction of athymic mice two populations of iIEL can be recognized which are positive for asialo-GM1: one, which is positive for

CD8 and one, which is negative for this antigen (fig.3A). In the iIEL fraction of euthymic mice we can also detect these asialo-GM1$^+$CD8$^+$ and asialo-GM1$^+$CD8$^-$ populations (results not shown). However, the cell numbers of these asialo-GM1$^+$ populations are significantly increased in athymic animals when compared to euthymic mice. In mesenteric lymph nodes of euthymic animals also small numbers of asialo-GM1$^+$CD8$^+$ cells can be found (fig.3B). The asialo-GM1$^+$CD8$^-$ population at that site, however, is strongly reduced. In the mesenteric lymph nodes of athymic mice on the other hand, asialo-GM1$^+$CD8$^+$ and asialo-GM1$^+$CD8$^-$ can be detected in equal numbers (results not shown).

Discussion

The vast majority of iIEL in euthymic mice is CD3$^+$CD8$^+$ (fig.1A). We and others [1-5] have shown that these cells almost exclusively express the CD3 associated $\gamma\delta$ receptor. Such $\gamma\delta$ receptor bearing iIEL are Thy-1$^-$ or Thy-1^{+low} [4,5]. Also some TcR$\alpha\beta^+$ cells can be found in the iIEL fraction of euthymic mice [4]. The expression of the TcR$\alpha\beta$ in the iIEL fraction, however, is dependent on the presence of viable microbiological antigens in the gut, since in germfree animals only Thy-1$^-$CD8$^+$CD3$^+$ iIEL can be detected [6]. No Thy-1$^+$TcR$\alpha\beta^+$ iIEL are found in such animals. Moreover, exposure of germfree mice to a viable bacterial flora results in an increase of the percentage of $\alpha\beta$ TcR expressing cells in the iIEL fraction [De Geus et al. unpublished results]. Thus, studies in microbiologically clean animals such as germfree or SPF mice show a relative abundance of TcR$\gamma\delta^+$ cells in the iIEL, whereas TcR$\alpha\beta^+$ iIEL are found in contaminated (euthymic) mice, which are under an active microbiological pressure. Surprisingly, we and others [1,2,4] have not been able to detect α or β TcR chain expression by immunoprecipitation or RNA hybridization on iIEL, not even in mice kept under conventional conditions. This is probably due to the relatively low expression of TcR$\alpha\beta$ on iIEL [5, De Geus et al., unpublished results]. Thus, in euthymic mice virtually all CD8$^+$ iIEL do co-express CD3 and either the $\gamma\delta$ or the $\alpha\beta$ TcR. In athymic, nude mice, however, another CD8$^+$ iIEL population can be observed: a CD3$^-$CD8$^+$ population (fig.1B). Actually, cells with a similar phenotype, although not by FACS analysis, can be found in euthymic mice using immunehistological techniques (data not shown). Cells expressing this CD3$^-$CD8$^+$ phenotype represent a novel phenotype in the mouse. Cells with such a CD3$^-$CD8$^+$ phenotype have ac-

tually been reported in rat and man [8,9]. These $CD3^-CD8^+$ cells were shown to be cells with NK activity [8,9].
In order to determine whether these $CD3^-CD8^+$ iIEL are of lymphoid origin, we analysed iIEL in CB17 Scid/Scid mice by immunofluorescence using mAb's specific for CD3 and CD8. The Scid/Scid mouse has a major recombinase defect, resulting in the absence of B and T cells [10]. NK cell reactivity and cell numbers in this mouse mutant are normal or even enhanced when compared to normal animals [M.Bosma, personal communication, and 10]. As can be seen in fig.2 cells with a $CD3^-CD8^+$ phenotype can be found in the Scid/Scid mouse to a similar extent as in the athymic animals. This observation strongly suggests that the $CD3^-CD8^+$ cells in the iIEL fraction are not of lymphoid origin.

To investigate whether these cells might be NK cells, we analysed iIEL by double staining with anti-CD8 and anti-asialo-GM1 antibodies. Asialo-GM1 is known to be expressed on murine $CD3^-CD8^-$cells displaying NK activity [11,12]. In the iIEL fraction and mesenteric lymph nodes of euthymic and athymic mice we found two types of cells with a possible NK phenotype. One population, as described before [11,12], which is asialo-$GM1^+CD8^-$ and another population which is asialo-$GM1^+CD8^+$. In the athymic, nude mouse the cell numbers of the asialo-$GM1^+$ populations are enhanced in the iIEL fraction and mesenterial lymph nodes when compared to the euthymic animal. This observation is in line with other observations since in athymic nude mice and rats NK cell numbers and NK activity have been shown to be normal or even enhanced [13,14]. One explanation for the fact that asialo-$GM1^+CD8^+$ cells have not been observed before in the mouse may be the fact that most studies have been performed on splenic NK cells. These splenic NK cells are indeed asialo-$GM1^+CD8^-$ [L. Nagelkerken, personal communication, and 11,12]. Further phenotyping and functional testing of the asialo-$GM1^+CD8^+$ iIEL has to clarify whether these cells are $CD3^-$ and show NK activity.

Finally, it is also evident (fig.3) that the Scid/Scid mouse contains low but significant numbers of $CD3^+CD8^+$ cells. It has been reported that 10-25% of the Scid/Scid mice are 'leaky' for their recombinase defect [10,15]. This indicates that in such 'leaky' animals the recombinase defect has been restored to some extent. We did not yet analyse receptor usage in the intestinal iIEL fraction of Scid/Scid mice, but this may well be the first observation on the presence of $TcR\gamma\delta^+$ cells in Scid/Scid mice.

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APPENDIX PAPER 4

CO-EXPRESSION OF TcR-β AND TcR-δ CHAINS ON INTESTINAL LAMINA PROPRIA LYMPHOCYTES DURING NEONATAL DEVELOPMENT IN EUTHYMIC AND ATHYMIC MICE

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Submitted for publication.

Summary

High numbers of $CD3^+$ cells are found in the gut of fetal and young, newborn mice. At birth and during early neonatal life all of these gut-associated $CD4^-CD8^-CD3^{+low}Thy\text{-}1^{+low}$ cells are exclusively located in the lamina propria. At this life stage no $CD3^+$ intraepithelial lymphocytes can be detected. In striking contrast to the $CD3^+$ population found in the intestinal epithelium, or any other peripheral lymphoid organ in adult mice, these newborn lamina propria $CD3^+$ cells express simultaneously both TcRβ and TcRδ chains on their surface. The most likely explanation for this observation is the existence of cells co-expressing TcR$\alpha\beta$ ***and*** TcR$\gamma\delta$ on their membrane. Such cells can actually also be detected in low numbers in the intestinal lamina propria of adult mice. Since these cells, expressing TcR$\alpha\beta$ ***and*** TcR$\gamma\delta$, are also found in athymic, nude mice one has to conclude that such cells do not depend on the thymus for their differentiation. We propose that the TcR double positive cell is an intermediate in the extrathymic differentiation pathway of $TcR\alpha\beta^+CD8\alpha\alpha^+$ iIEL.

Introduction

The intestinal intraepithelial lymphocytes (iIEL) in adult mice form a heterogeneous T lymphocyte population. The majority of such cells express CD8 in combination with the TcR$\gamma\delta$ or the TcR$\alpha\beta$ [1-12]. CD8 on $TcR\alpha\beta^+$ T cells in other lymphoid organs is made up of a heterodimeric form of the CD8α (Lyt2) and CD8β (Lyt3) chains [13]. The majority of the $TcR\gamma\delta^+$, and a significant percentage of the $TcR\alpha\beta^+$ iIEL, however, express CD8 in a homodimeric form of the CD8α chains [6,8,9,10].

Recent data of Guy-Grand and Rocha [8,9] analyzing the Vβ repertoire of $TcR\alpha\beta^+$ iIEL, indicate that $TcR\alpha\beta^+CD8\alpha\alpha^+$ iIEL differentiate extrathymically. Similar conclusions can be drawn from the work of Lefrancois et al. [14] and Bandeira et al. [15] regarding $TcR\gamma\delta^+CD8\alpha\alpha^+$ iIEL. In addition cells with a $TcR^+CD8\alpha\alpha^+$ phenotype can be readily detected in adult, athymic, nude mice [3-5,8]. These data, as well those of Mosley et al [16], strongly support the notion that both $TcR\gamma\delta^+CD8\alpha\alpha^+$ and $TcR\alpha\beta^+CD8\alpha\alpha^+$ iIEL follow an extrathymical differentiation pathway.

During neonatal development iIEL can be detected from day 16-20 on after birth in athymic, nude and euthymic mice [15]. Nothing is known,

however, about the origin, the exact localization and phenotypes of gut-associated TcR^+ lymphocytes during early neonatal life. Therefore we investigated these aspects in newborn euthymic and athymic, nude mice. The results of our experiments show that directly after birth all intestinal $CD3^+$ cells are found in the lamina propria. At this time the epithelium does not contain T cells. Moreover, these $CD3^+$ lamina propria lymphocytes (LPL) co-express both TcRβ and TcRδ chains, probably reflecting the simultaneous expression of both TcR$\alpha\beta$ ***and*** TcR$\gamma\delta$ on the cell surface. Cells with this particular phenotype are present in comparable numbers in the lamina propria of both neonatal euthymic and athymic, nude mice. Such $TcR\beta^+$ $TcR\delta^+$ cells remain present during adult life in athymic animals and form a small, but stable, component of the $CD3^+$ lamina propria population in euthymic mice. It is postulated that these cells, expressing both the TcR$\alpha\beta$ ***and*** TcR$\gamma\delta$, are intermediates in the extrathymic differentiation pathway of $TcR\alpha\beta^+CD8\alpha\alpha^+$ iIEL.

Materials and Methods

Animals

Male and female euthymic (Nu/+ or +/+) and athymic, nude (Nu/Nu) specified pathogen free Balb/c mice of various ages were obtained from the Central Animal Facilities of the TNO Institute for Applied Radiobiology and Immunology, Rijswijk, The Netherlands. All nude mice used in the various experiments were carefully checked macroscopically and microscopically for thymic remnants.

Isolation of intestinal IEL and LPL

Intestinal IEL were isolated as previously reported from the small intestine of Balb/c mice [3,4]. Briefly, the small intestine was removed and flushed with PBS. Peyers patches were excised and the iIEL released by incubation in HBSS, 1mM EDTA with shaking for 20 minutes at 37°C. The supernatant was filtered through cellulose acetate columns. The filtered cells were centrifuged over a discontinuous Percoll (Seromed, Berlin, FRG) density gradient ($\rho = 1.086$ g/ml, 1.054 g/ml, and 1.037 g/ml) at 600g and 4°C for 20 minutes. After centrifugation the cells at the 1.086-1.054 g/ml interphase were harvested. Cells in this interphase are further denoted as

iIEL. The tissue debris remaining after the removal of the intestinal epithelium was further incubated, while shaking, for 1 hour in RPMI containing 20 mM HEPES, 5% fetal bovine serum, 10 mg/ml DNA'se 1 (Sigma, St.Louis MO) and 15mg/ml collagenase (Serva, Heidelberg, FRG) at 37°C. After incubation the remaining tissue was gently squeezed through nylon gauze (100 μm pore size). At this point the obtained cell suspension was filtered over a cellulose acetate column. The filtered cells were centrifuged over a discontinuous Percoll density gradient as described above. After centrifugation the population of cells at the 1.086-1.054 g/ml interface were harvested and are further denoted as LPL.

Single cell suspensions of fetal organs were made by gently squeezing the tissue through nylon gauze [100 μm pore size]. The obtained cell suspensions were washed twice.

FCM staining and analysis

Biotinylated or FITC conjugated mAb 59AD2.2 was used for Thy-1 staining [17]. mAb H57-597 (a kind gift of Dr. R.T. Kubo), a TcRβ chain constant region recognizing mAb [18,19], was used directly conjugated to FITC or biotin. TcRδ chains were detected with biotinylated mAb GL3, recognizing the constant region of the δ chain [7] (PharMingen, San Diego, CA). Mouse CD3 was detected using FITC conjugated mAb 145-2C11 (kindly provided by Dr. J.A. Bluestone) [20]. All biotin-conjugated mAb were used in combination with streptavidin-PE (Becton Dickinson, Sunnyvale, CA). Hybridoma cells were grown in vitro culture systems. mAb were concentrated and purified using the Filtron minisette membrane system® (Filtron, Northborough, MA). For double fluorescence FCM analysis cells were washed once in PBS, 1% BSA and incubated with two mAb (FITC- and biotin conjugated) at the appropriate dilution for 30 minutes at 4°C. The first incubation was followed, after washing, by a second incubation with streptavidin PE. Labelled cells were analyzed using a FACScan® (Becton Dickinson) with a life gate setting.

Immunofluorescence histology

Intestines were processed in so called "Swiss rolls" and snap frozen in liquid nitrogen: 6-μm-thick cryostat sections were cut and incubated for 30 min at room temperature with biotinylated mAb as described in detail

previously [3]. After washing the sections twice with PBS and 1% BSA, incubation with streptavidin-PE was performed. A Leitz (Wetzlar, FRG) microscope equipped for visualization of PE was used to read the slides.

Results

$CD3^+$ lymphocytes are present in the gut during fetal life and are exclusively located in the intestinal lamina propria of newborn mice.

FCM analysis of cell suspensions, made of thymus, liver and gut of euthymic Balb/c fetuses on day 18 of gestation, shows that $CD3^+$ lymphocytes are present in all organs investigated, although more pronounced in gut and liver than in the thymus (fig.1).

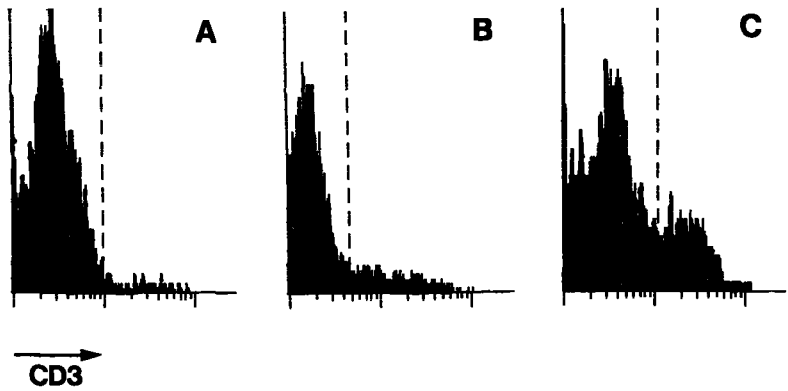

Figure 1. CD3 fluorescence histograms of fetal cell suspensions. Cell suspensions of thymus liver and gut were prepared from euthymic Balb/c mice at day 18 of gestation, stained for CD3 with FITC conjugated mAb 145-2C11 and analyzed by FCM. A: thymus, B: liver and C: gut.

The number of $CD3^+$ cells in the fetal thymus at that time of gestation is approximately 10 times lower than in the liver and gut (results not shown).

To determine the localization of cells expressing the CD3/TcR complex in the intestine during early life we performed immunofluorescence histology on intestinal tissue of newborn euthymic mice using mAb directed to the TcRβ and δ chains. As shown in figure 2, TcR^+ cells can be detected readily in the lamina propria of the gut. A similar picture emerges when analyzing the gut of 1 to 4 day old athymic mice with TcR chain specific

antibodies (data not shown). In both athymic, nude and euthymic newborn animals we could not detect any TcR expressing cells in the intestinal epithelium (fig.2). During early neonatal, and probably also fetal life TcR$\alpha\beta$ and TcR$\gamma\delta$ T cells are thus exclusively localized in the lamina propria.

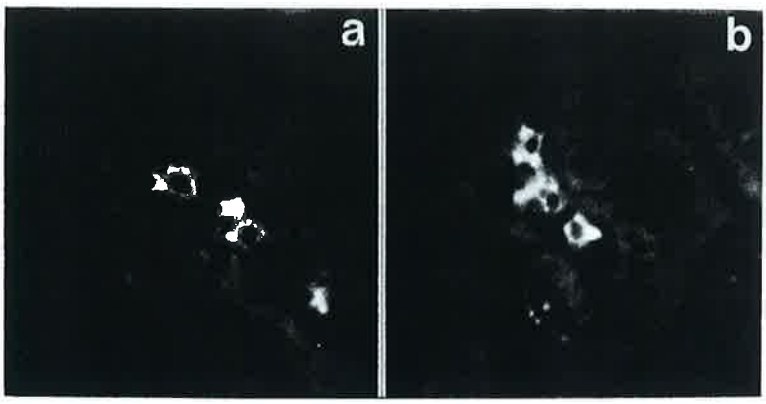

Figure 2. Immunofluorescence histology of neonatal intestines.
Neonatal intestines of euthymic Balb/c mice, age 2 days, were separately processed for immunofluorescence histology and incubated with biotynilated mAb's GL3 (anti-TcRδ chain) or H57-597 (anti-TcRβ chain), followed by incubation with streptavidin-PE. Panel A represents TcRβ chain staining, panel B shows TcRδ chain staining.

Changes in the intestinal CD3$^+$ lymphocyte population during the first weeks of life in athymic and euthymic Balb/c mice.

iIEL and LPL from euthymic Balb/c mice, isolated at 1, 28 and 70 days after birth, were analyzed by double fluorescence FCM analysis using mAb directed to CD3 and Thy-1. As one would expect from the histological studies (fig.2) virtually no CD3$^+$ cells can be detected in the intestinal epithelium of newborn mice (fig.3A). The lamina propria in such animals on the other hand contains significant numbers of LPL expressing both CD3 and Thy-1 in low membrane density (fig.3B).

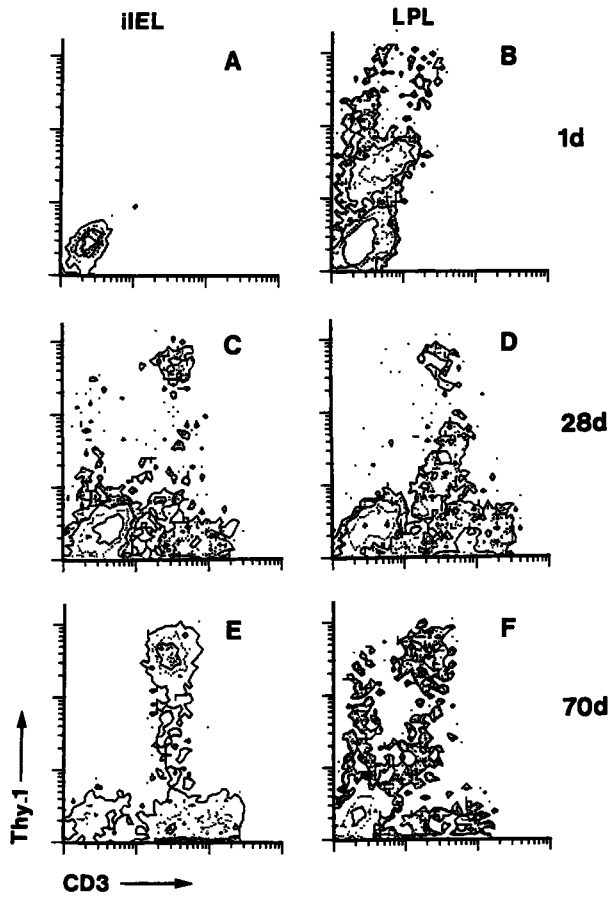

Figure 3. CD3/Thy-1 double fluorescence FCM analysis on iIEL (left panel) and LPL (right panel) isolated from euthymic Balb/c mice at 1 (A vs B), 28 (C vs D) and 70 (E vs F) days after birth.

iIEL and LPL were analyzed using mAb 145-2C11 (anti-CD3) and 59 AD 2.2 (anti-Thy-1). For LPL and iIEL isolation from of 1 day old animals the intestines of 3 mice were pooled and further processed simultaneously. Intestines of animals of 28 and 70 days of age were processed individually. The horizontal axis represents log green fluorescence, the vertical axis log red fluorescence. A representative staining pattern is shown. Negative control stainings, including streptavidin-PE and an irrelevant FITC conjugated mAb did not result in any significant background staining.

The number of $CD3^+$ cells increases with age in both the intestinal epithelium and lamina propria (fig.3CDEF). During neonatal development a variable expression of Thy-1 is seen on $CD3^+$ iIEL and LPL: Both Thy-1^{high} (fig.3CDEF), Thy-1^{low} (fig.3BCDEF) and Thy-1^- (fig.3CDEF) subsets can be detected.

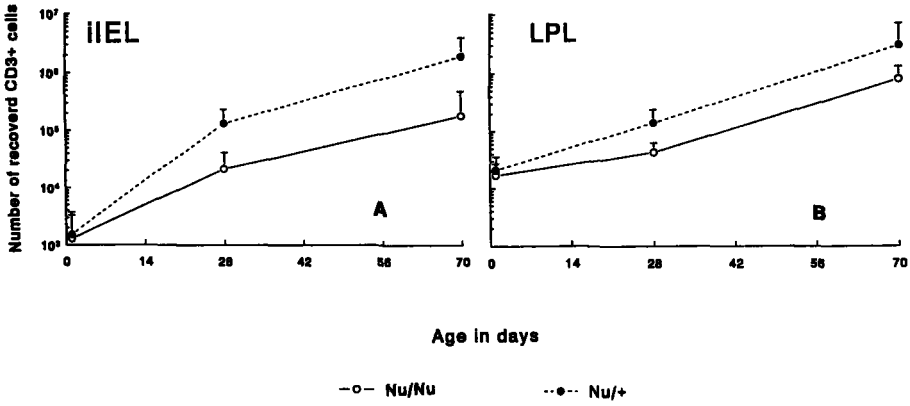

Figure 4. Analysis of the number of recovered $CD3^+$ iIEL and LPL from euthymic and athymic Balb/c mice at various time points after birth. The number of recovered $CD3^+$ LPL and iIEL were calculated by extrapolation of the percentage of $CD3^+$ cells in a FACS® life-gate setting on the total number of cells isolated. Panel A represents the number of recovered $CD3^+$ iIEL, panel B represents the number of recovered $CD3^+$ LPL. Open symbols represent the number of $CD3^+$ cells recovered from euthymic animals, closed symbols represent the number of recovered $CD3^+$ cells from athymic animals. Per time point the mean and standard deviation of data obtained in 3 separate experiments are shown. Per experiment 3 mice were used per time point. the intestines of the three 1-day old mice were pooled per experiment, the others were processed individually.

The development of iIEL and LPL populations in athymic, nude mice during neonatal life is comparable to the development of these populations in euthymic mice. The major difference between athymic and euthymic animals is the absence, in athymic mice, of $CD3^+$ iIEL and LPL expressing Thy-1 in high membrane density (results not shown). Also the developmental kinetics of the $CD3^+$ iIEL and LPL have similar

characteristics in athymic and euthymic mice (fig.4). As can be seen in figure 4A the $CD3^+$ iIEL population in euthymic animals expands rapidly during neonatal development. The expansion of this iIEL population in athymic mice is slightly slower and results in an approximately 10 fold lower number of $CD3^+$ iIEL than in euthymic animals at 70 days of age. Also the population of $CD3^+$ LPL increases in size with age (fig.4B), although at a somewhat slower rate than the $CD3^+$ iIEL population. Throughout the entire period of investigation the number of $CD3^+$ LPL from athymic mice is approximately five-fold lower when compared with euthymic mice.

TcR chain expression on LPL during early neonatal development in euthymic and athymic mice is associated with Thy-1 expression.

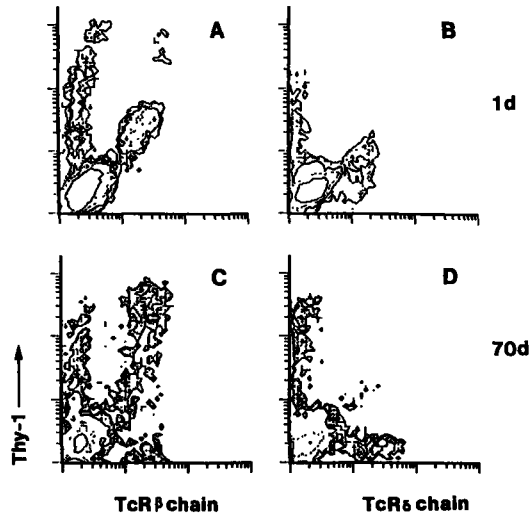

Figure 5. TcRβ chain/Thy-1 and TcRδ chain/Thy-1 double fluorescence FCM analysis of LPL isolated from euthymic Balb/c mice at 1 (top panel) and 70 (lower panel) days after birth.LPL were stained with FITC-conjugated mAb H57-597 (anti-TcRβ chain) in combination with biotinylated 59AD2.2 (anti-Thy-1) and streptavidin-PE (left panel, i.e. A and C) and with biotinylated mAb GL3 (anti-δ chain) in combination with FITC conjugated mAb 59AD2.2 (anti-Thy-1) and streptavidin-PE (right panel, i.e. B and D). Negative control stainings, including streptavidin-PE and a irrelevant FITC-conjugated mAb did not result in any significant background staining. Other control stainings are described at the end of the results section.

Double fluorescence FCM analysis of TcR and Thy-1 expression on LPL obtained from neonatal euthymic mice shows an identical staining pattern when compared to the expression of CD3 and Thy-1 on such cells (fig.5AB and fig.3B): TcR$\alpha\beta$ and TcR$\gamma\delta$ expression on LPL is completely associated with Thy-1^{low} expression. Similar results were obtained for TcR and Thy-1 expression on LPL of neonatal athymic, nude animals (data not shown).

In adult euthymic animals the majority of TcR$\alpha\beta^+$ LPL display Thy-1 in variable membrane density (fig. 5C). Most of the TcR$\gamma\delta^+$ LPL in adult euthymic mice do not express Thy-1 (fig. 5D). In adult athymic mice TcR$\alpha\beta$ and TcR$\gamma\delta$ positive LPL express predominantly Thy-1 in low membrane density (data not shown). Further flowcytometric analysis of TcR$^+$Thy-1^{low+} LPL in euthymic and athymic, nude animals reveals that such cells do not express CD8 or CD4 (data not shown). The overall phenotype of these cells is thus CD3$^+$TcR$^+$Thy-1^{low+}CD4$^-$CD8$^-$.

The percentage of CD3 expressing LPL in adult athymic Balb/c mice does not match with the total percentage of TcR expressing LPL in these animals.

Analysis of LPL from newborn athymic, nude and euthymic mice, as well as, of adult athymic mice reveals a marked difference when the percentage of CD3 and TcR expressing LPL are compared. In LPL of athymic mice these differences could be clearly shown at all life stages investigated. This is illustrated by the analysis of iIEL and LPL populations, isolated from individual adult athymic mice as shown in table 1. As shown in this table, the percentage of CD3+ iIEL in athymic nude mice matches well with the total percentage of TcR chain expressing cells. Suprizingly the same does not apply for the LPL population from athymic mice. The percentage of CD3$^+$ cells is significantly lower than the total percentage of TcR chain positive cells.

Table 1. CD3 and TcR chain expression in iIEL and LPL of adult athymic, nude mice[1].

	iIEL		LPL	
	Mouse 1	Mouse 2	Mouse 1	Mouse2
$CD3^+$	$23.1 \pm 2.2^{2,4}$	17.5 ± 3.6	15.6 ± 1.9	13.1 ± 2.7
$TcR\beta^+$	9.6 ± 0.2^2	10.6 ± 1.9	14.0 ± 0.7	11.0 ± 0.3
$TcR\delta^+$	15.6 ± 1.3^2	8.7 ± 0.9	6.2 ± 1.2	6.0 ± 1.2
$TcR\beta^+/\delta^+$	$25.3 \pm 1.1^{3,4}$	19.2 ± 1.8	20.3 ± 1.2	17.0 ± 1.0

[1] iIEL and LPL were isolated from 70 days old athymic, nude Balb/c mice.

[2] Mean and standard deviation of CD3 and individual TcR chain expression is calculated from the data obtained in three separate double stainings of CD3 and TcRβ and δ chains, respectively, in combination with Thy-1, CD4 and CD8α, per mouse.

[3] Mean and standard deviation of combined TcR$\beta+\delta$ expression is calculated over a total of 9 separate double staining combinations per individual mouse.

[4] Comparison of the values of CD3 and combined TcR $\beta+\delta$ chain expression was done by the Wilcoxon rank sum test, showing a significant difference ($P<0.05$ between the CD3 and combined TcR $\beta+\delta$ chain percentages for LPL and no significant difference for iIEL.

Unusual TcR expression by a subset of intestinal lamina propria lymphocytes.

The observed difference in percentage of TcR and CD3 expression by

TcRβ and TcRδ chains either as a CD3 associated TcR$\beta\delta$ heterodimer or through the simultaneous expression of both TcR$\alpha\beta$ ***and*** TcR$\gamma\delta$ on the cell surface.

In order to investigate this, we performed two colour FCM analysis on LPL and iIEL isolates, obtained from newborn and adult athymic and euthymic mice, using mAb specific for the TcRβ and TcRδ chains. In athymic mice cells double positive for the β ***and*** δ TcR chains can be detected in the LPL population of newborn and adult animals (fig.6AB).

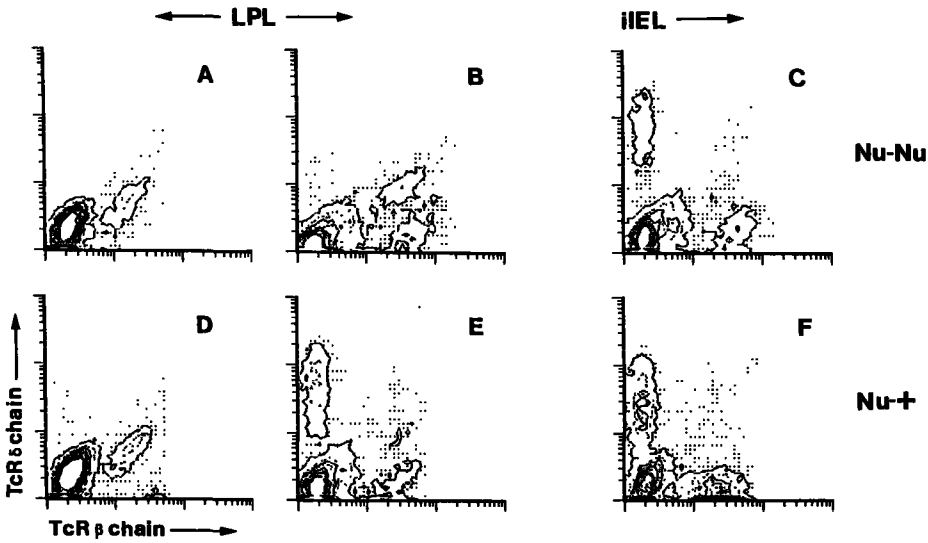

Figure 6. TcRβ/TcRδ chain double FCM analysis of iIEL and LPL isolated from newborn and adult euthymic and athymic Balb/c mice. LPL (AB vs DE) and iIEL (CF) were stained with biotinylated mAb GL3 (anti-TcRδ chain) in combination with streptavidin-PE and FITC conjugated mAb H57-597 (anti-TcRβ chain). LPL and iIEL were isolated from athymic (top panel) and euthymic (lower panel) mice at an age of 1 (A and D) and 70 (BC and EF) days, respectively. Negative control stainings, including streptavidin-PE and a irrelevant FITC-conjugated mAb did not result in any significant background staining. Other control stainings are described at the end of the results section.

Besides these TcRδ ***and*** TcRβ double positive LPL low numbers of TcRδ chain single positive cells are present and a larger population of TcRβ chain single positive cells is detectable in adult athymic animals (fig.6B). In

contrast to the overt presence of TcRβ *and* TcRδ chain positive cells in the LPL from athymic mice such cells are rare in the iIEL fraction of such animals. As can be seen in figure 6C the iIEL contain predominantly TcRδ *chain* single positive cells as well as TcRβ chain single positive cells. The presence of TcRβ chain expressing cells in the iIEL population of athymic mice varies between individual animals, as does the small population of TcRβ *and* δ chain double staining cells (results not shown).

The iIEL population, isolated from adult euthymic animals, displays a similar pattern of TcR chain expression as observed for the athymic animals (fig.6F). The same is true for LPL obtained from neonatal euthymic mice when compared with LPL from young athymic mice (fig.6AD). In contrast to the situation in adult athymic animals, however, the relative contribution of the TcRβ *and* TcRδ chain double positive LPL population in adult euthymic mice is rather small (fig.6BE). In the LPL population from adult euthymic animals both TcRδ or TcRβ chain, single positive cells can be detected (fig.6E).

To exclude the possibility that double staining of lymphocytes for the TcRβ *and* TcRδ chains is caused by aspecific staining or conjugate formation of cells we performed stainings, on LPL of athymic and euthymic neonatal mice, in the presence of normal hamster serum or 10mM EDTA. This latter addition prevents conjugate formation [21]. The staining patterns with the various mAb in these control experiments did not change nor did forward and side angle scatter patterns of the cells, indicating that the findings are not the result of aspecific staining or conjugate formation [results not shown].

Discussion

A significant proportion of the $CD3^+$ cell population in the perinatal period in mice is found in the gut (This report). These findings are supported by a recent report by Carding et al. [22] in which these authors show that in both euthymic and athymic mice mRNA encoding the TcRδ and γ chains can be detected in fetal gut and liver at 20 days of gestation [22]. Immune histology on neonatal intestinal tissue using TcR chain specific mAb furthermore shows that, in contrast to the situation in adult life in which the TcR^+ cells are distributed over both the intestinal epithelium and lamina propria, such cells are exclusively located in the

lamina propria during neonatal life, and probably also during late fetal life. Moreover, the composition of LPL is quite different in the perinatal and adult life stage: LPL in adult animals contain many TcR^+CD4^+ cells [4], which are mainly part of the efferent limb of the mucosal immune system. Besides these $CD4^+$ cells also $CD8^+$ cells can be detected in adult LPL. In the lamina propria of newborn mice CD4 and CD8 positive cells are virtually absent. The major difference, however, between perinatal LPL and adult LPL, as well as lymphocytes from other locations is the dominant presence of cells with a unique TcR expression in perinatal LPL. These cells co-express TcRβ and TcRδ chains on their surface (fig.6). This observation can be explained in two ways: [i] Such TcRβ and TcRδ chain positive cells express CD3 associated T cell receptors of heterodimeric associated TcRβ and δ chains, i.e a TcR$\beta\delta$, or [ii] these cells co-express simultaneously TcR$\alpha\beta$ and TcR$\gamma\delta$ on their cell surface. Although we cannot distinguish between these two options on basis of the experiments performed sofar, we favour the latter. The option of a TcR$\beta\delta$ heterodimeric structure is nevertheless possible, since such structures have been described on an human T cell leukaemia cell line [23]. One would expect, however, in that case an equimolar distribution of the two TcR chains on the surface of each individual cell, resulting in a diagonal staining pattern in a TcRβ vs TcRδ FCM analysis, which is not the case (fig.6). The option of cells co-expressing both TcR$\alpha\beta$ ***and*** TcR$\gamma\delta$ is strongly supported by recent observations of Rocha et al. who showed that cells both expressing the TcR$\alpha\beta$ ***and*** the TcR$\gamma\delta$ are present in the iIEL of TcR$\alpha\beta$ transgenic mice. Also in iIEL of normal mice low numbers of cells with this unusual TcR phenotype can be detected (Dr. B. Rocha, personal communication). Another explanation for the discrepancy between the CD3 and total TcR numbers in LPL (table 1), i.e. the assumption that part of the TcRβ and/or TcRδ chain positive cells may express T cell receptors which are not associated with CD3, seems rather unlikely since it has been shown that TcR expression is linked to the coordinated expression of CD3 genes [24,25]

We like to propose therefore a population of $TcR\alpha\beta^+$ ***and*** $TcR\gamma\delta^+$ cells which is found in high frequency in the intestinal lamina propria during early life, but also in low numbers in the intestinal epithelium later in life. Although the relative proportion of such TcR$\alpha\beta$ ***and*** TcR$\gamma\delta$ positive cells decreases with age in the LPL, which is mainly caused by the expansion of regular TcR expressing cells, it can be calculated that the actual number of

such cells remains at a rather stable level throughout life, at least for the time period investigated.

The mere existence, also under physiological conditions, of a significant population of TcR$\alpha\beta$ ***and*** TcR$\gamma\delta$ expressing cells raises obviously a series of questions with regard to the intracellular events involved in the regulation of TcR gene expression at the level of gene rearrangements, V gene usage and antigenic specificity of such receptors. In these TcR$\alpha\beta^+$ ***and*** TcR$\gamma\delta^+$ cells the traditional concept of allelic exclusion is obviously violated. Regular allelic- and isotype exclusion by TcR$\alpha\beta$ expressing cells has been described [26]. Also in TcR$\gamma\delta$ expressing T cells TcRγ-chain genes have been described to be subjected to allelic exclusion [27]. However, some TcR$\gamma\delta$ expressing T cell lines, derived from dendritic epidermal T cells, have been shown to express simultaneously two different TcRγ chains [28]. Thus isotype exclusion of TcRγ- chains does not occur in these cells. This indicates that in some TcR expressing cells the genetic regulation of the expression of TcR chains can be regulated in an alternative way.

Also the differentiational and functional implications of this intriguing population of TcR$\alpha\beta^+$TcR$\gamma\delta^+$CD4$^-$CD8$^-$Thy-1^{low+} cells needs to be clarified. In terms of differentiation these cells seem not to depend on the thymus for their generation since equal numbers of TcR$\alpha\beta$ ***and*** TcR$\gamma\delta$ positive cells are found in LPL from both euthymic and athymic, neonatal mice. Also in line with such an extrathymic origin of these cells, is the fact that even in euthymic mice the number of such CD3$^+$ cells in the intestine and liver exceeds greatly the total number of CD3$^+$ cells in the thymus during fetal life [This report]. In this respect it is also tempting to speculate that the 'N-region' diversity of the rearranged TcR genes in such TcR double positive cells must be low and non-randomized as is the case for the TcRδ chain in resident pulmonary lymphocytes, which are also believed to differentiate independently from the thymus [29,30]. Experiments to address this question are currently going on.

Although recently some progress has been made in our understanding of the selective processes which are involved in the extrathymic generation of iIEL [14,31], the precise nature and localization of the elements involved in these processes are still unknown. However, the murine intestinal epithelium expresses various MHC encoded proteins, which have been suggested to be involved in selective and antigen specific responses of T cells expressing a TcR$\gamma\delta$: Both MHC class II encoded

proteins [32], which are thought to be involved in the positive, extrathymic selection of TcR$\gamma\delta$ iIEL [14], as well as MHC class I encoded proteins, such as Tla-antigens [33,34] and CD1 [35], which have been reported to be involved in antigen specific responses of TcR$\gamma\delta$ expressing T cells [36,37] are expressed by the intestinal epithelium. However, a recent report by Correa et al. [38] describing the composition of the iIEL population in β2-microglobulin deficient mice, which do not express MHC Class I encoded proteins on their cell membrane [39], show that these mice express normal numbers of TcR$\gamma\delta$ iIEL, suggesting that MHC Class I encoded proteins are not involved in the development of these iIEL. Most of the studies quoted have focused on the epithelial cells as restricting elements. In view of our findings on the predominance of the CD3$^+$ LPL population during early life, however, an essential role for the lamina propria in the extrathymic generation of iIEL and perhaps other T cells should be considered.

Also the overall immature phenotype of the TcR double positive cell population points to a function as a differentiation intermediate, rather than an effector cell. When we restrict ourselves to the intestine, where the TcR$\alpha\beta$ ***and*** TcR$\gamma\delta$ positive cell is located almost exclusively, this cell type can well be an intermediate for all three major TcR$^+$ cell populations found in this organ: TcR$\alpha\beta^+$CD8$\alpha\beta^+$Thy-1$^+$ cells, TcR$\gamma\delta^+$CD8$\alpha\alpha^+$ cells and TcR$\alpha\beta^+$CD8$\alpha\alpha^+$ cells. However, numerous experiments have shown that TcR$\alpha\beta^+$CD8$\alpha\beta^+$Thy-1$^+$ cells are thymus dependent for their generation [40]. This leaves the TcR double positive cell as a candidate precursor cell type for both the TcR$\alpha\beta^+$CD8$\alpha\alpha^+$ and TcR$\gamma\delta^+$CD8$\alpha\alpha^+$ populations, which have been shown to be thymus independent [8,9,12,14,15,16]. However, recently reported experiments using mice treated with an anti-TcRβ chain mAb show that such a treatment depletes all the TcR$\alpha\beta^+$ lymphocytes from the intestinal epithelium and lamina propria but leaves the TcR$\gamma\delta^+$ population intact [41,42]. Although these results do not exclude a role for the TcR double positive cells in the differentiation pathway of TcR$\gamma\delta^+$CD8$\alpha\alpha^+$ cells, it can be concluded nevertheless that a major extrathymic differentiation route for this latter cell type must exist in which the TcR double positive cell is not involved. It seems thus, that, if the TcR double expressing cell is indeed a differentiation intermediate, it is part of the differentiation pathway of thymus independent TcR$\alpha\beta^+$CD8$\alpha\alpha^+$ cells.

In conclusion, we feel that the data described in this report fit well in the hypothesis, that the intestinal tissue, and especially the lamina propria, plays an important role in the generation of the T cell repertoire during early

(and adult) life. Furthermore, we propose the TcR double positive cell to be an intermediate in the extrathymic differentiation pathway of intestinal TcR$\alpha\beta^+$CD8$\alpha\alpha^+$ cells. To obtain more and detailed information on the 'double TcR' expressing lymphocytes it is important to culture and to immortalize such cells. These experiments are now in progress.

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APPENDIX PAPER 5

THE INTESTINAL INTRAEPITHELIAL LYMPHOCYTE POPULATION EXPRESSING THE TcR$\alpha\beta$ SHOWS VARIATION IN Vβ USAGE BETWEEN INDIVIDUAL MICE

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Published in: **From Gene to Man**, C.F.A. van Bezooijen et al. Eds. Stichting Gerontologie en Geriatrie. Leiden. 1990: 313.

Summary

We studied the composition of intestinal intraepithelial lymphocytes (iIEL) expressing the T cell receptor (TcR) $\alpha\beta$. The results indicate that the composition of this population, with respect to Vβ gene usage, displays individual characteristics.

Introduction

Relatively little is known about the cells involved in the local defense systems at the cell surfaces which are in direct contact with the environment (skin, lungs, gut, etc) and the effect of aging on the function and the composition of this population of cells. Experiments have been started therefore to investigate the response patterns of intestinal intraepithelial lymphocytes upon acute infection. These studies have focused on the IEL population in the intestine.

The iIEL population in mice is a heterogeneous population of cells with an as yet unknown function. The iIEL population in euthymic animals can be subdivided into two major populations, both expressing CD8 and a CD3 associated TcR. One of these expresses the TcR$\alpha\beta$. The other population expresses the TcR$\gamma\delta$. Both these cell types have been observed in the iIEL of all mouse strains investigated sofar [1]. However, from the data it appeared that the percentage of iIEL expressing the TcR$\alpha\beta$ differed between specific pathogen free (SPF) and conventional mice, suggesting a relationship between iIEL composition and antigenic status of the animals. Furthermore, a clear difference between individual mice can be shown with respect to TcR Vβ usage in the iIEL population expressing the TcR$\alpha\beta$. Such a difference is not detectable when TcR$\alpha\beta^+$ T cells from the mesenteric lymph nodes (MLN) are compared.

Methods and results

In order to test whether the gnotobiotic status of the animals influences the composition of iIEL, experiments were performed to determine the phenotypes of iIEL in SPF and germ free (GF) mice. The overall pattern of iIEL phenotypes was rather similar in both types of mice. However, a remarkable difference was found in Thy-1 expression by TcR$^+$ iIEL [2 and B de Geus, unpublished results]. The ratio of iIEL expressing a

TcR$\alpha\beta$ and a TcR$\gamma\delta$ differed between SPF and GF animals: In SPF mice the percentage of iIEL expressing the TcR$\alpha\beta$ is 50 - 70% of all $CD3^+$ cells, whereas in GF mice this is only 5 - 10% of the $CD3^+$ cells [3 and B de Geus, unpublished results]. It seems likely therefore to assume that the increase in TcR$\alpha\beta$ expressing iIEL in SPF mice is caused by the presence of a viable micro-flora in the gut of such mice. This assumption was further supported by data of experiments in which GF mice were contaminated with an SPF flora and the composition of the iIEL population was followed in time. This procedure resulted in an increase with time of TcR$\alpha\beta^+CD3^+$ iIEL [B de Geus, unpublished results].

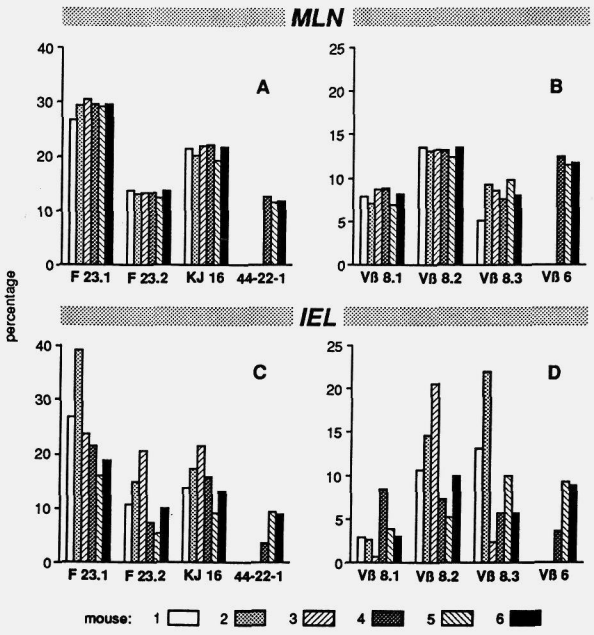

Figure 1. Expression of TcR Vβ8 and Vβ6 gene products on $CD8^+$ iIEL and MLN cells of the same individual BALB/c mice. Values are expressed as percentage of all $CD3^+CD8^+$ cells. Panels 1A and C represent staining percentages with mAb only. Panels 1B and D represent the actual percentages of expression of a Vβ family shown (mAb 44-22-1 is specific for the Vβ6 family, mAb KJ16 is specific for both the Vβ8.1 and Vβ8.2 families, mAb F23.1 is specific for Vβ8.1, Vβ8.2 and Vβ8.3 and finally F23.2 recognizes the Vβ8.2 family only).

Since there appears to be a relation between the presence of a viable micro-flora and the presence of TcR$\alpha\beta^+$ iIEL, we wondered whether there might be a preferential TcR Vβ gene usage of these cells. White et al. showed that bacterial enterotoxins can induce strong responses in T cells. Especially T cells expressing defined TcR Vβ gene families can be induced to proliferate in vitro after addition of bacterial enterotoxin [4]. Therefore we analyzed individually the TcR Vβ gene usage of CD8$^+$TcR$\alpha\beta^+$ expressing iIEL of 20-week-old SPF Balb/c female mice and compared this with the patterns of lymphocytes with a similar phenotype from mesenteric lymph nodes of the same mice. All animals used were littermates and were therefore supposed to be exposed to the same (food) antigens and micro-flora.

As shown in figure 1AB the individual mice did not differ in TcR Vβ gene usage for CD8$^+$ T cells of the MLN, with well defined values for the various TcR Vβ gene products. However, individual variations can be observed for the TcR Vβ gene usage of CD8$^+$ iIEL. Within all TcR Vβ gene families tested iIEL display, in contrast to the homogeneous expression of the MLN T cells, a clear individual heterogeneity for the TcR Vβ gene products, ranging from a 10-fold decrease (Vβ8.1 expression in iIEL from mouse 3) to a 2-fold increase (Vβ8.2 and Vβ8.3 expression in iIEL from respectively mouse 3 and mouse 2) as compared to MLN levels (fig.1CD). These results can not be explained by differences in antigenic history of the animals. However, one has to keep in mind that the composition of the intestinal bacterial flora may differ from animal to animal, even when housed together under the same conditions, i.e. although the same strains of intestinal bacteria are present in such animals the relative amount of each strain can differ significantly among individual mice.

Figure 2 shows that the observed differences display a similar pattern when analyzed for TcR$\alpha\beta^+$ iIEL only.

Conclusion

Viable micro-organisms appear to influence directly or indirectly the composition of the CD3$^+$TcR$\alpha\beta^+$ expressing iIEL population. They are probably the underlying cause for both the overall population shift towards cells expressing the TcR$\alpha\beta$ and the variations in individual levels of TcR Vβ gene family usage within this TcR$\alpha\beta^+$ iIEL population observed in

SPF (and conventional mice) as compared to GF mice.

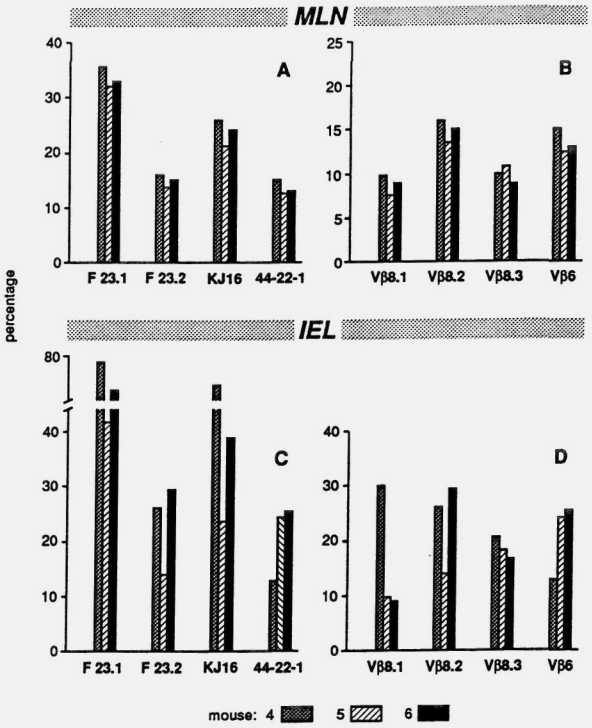

Figure 2. Expression of TcR Vβ8 and Vβ6 gene products on CD8$^+$ iIEL and MLN cells of the same individual BALB/c mice. Values are expressed as percentage of all TcR$\alpha\beta^+$CD8$^+$ cells. Panels 1A and C represent staining percentages with mAb only. Panels 1B and D represent the actual percentages of expression of a Vβ family shown. The legend from figure 1 explains the specificity of the mAb used.

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APPENDIX PAPER 6

GPI-LINKED MEMBRANE PROTEIN EXPRESSION BY INTESTINAL INTRAEPITHELIAL LYMPHOCYTES

Bernard de Geus, Harm HogenEsch, Ralph. T Kubo and Jan Rozing

International Immunology 1992: In Press.

Summary

Murine intestinal intraepithelial lymphocytes (iIEL) represent a heterogeneous population of cells which contains both TcR$\alpha\beta^+$ and TcR$\gamma\delta^+$ cells. In contrast to the TcR$\alpha\beta^+$Thy-1$^+$ cell population in peripheral lymphoid organs, the TcR$\alpha\beta^+$ iIEL contain high numbers of Thy-1$^-$ cells. This negativity of iIEL for Thy-1, a glycosylphosphatidyl inositol (GPI) linked membrane protein, is not the result of conformational changes, since mAb to different epitopes of Thy-1 all give similar results. In order to determine whether the absence of Thy-1 on TcR$\gamma\delta^+$ and a subset of TcR$\alpha\beta^+$ iIEL is caused by a defect in the processing of GPI-linked proteins we examined the expression of the GPI-linked proteins Ly6A, Ly6C and Qa2 on these cells. TcR$\alpha\beta^+$ and TcR$\gamma\delta^+$ iIEL are virtually negative for Ly6A and Ly6C. They do express Qa2. This indicates that the lack of Thy-1 and Ly-6 expression on iIEL is not caused by downregulation of the synthesis of GPI-linked proteins. The expression pattern of GPI-linked proteins by iIEL might be related to the, yet unknown, function of this population of lymphocytes.

Introduction

The intestinal intraepithelial lymphocytes (iIEL) represent a heterogeneous population of cells with a variety of phenotypes. The majority of the iIEL expresses CD8 in combination with a CD3-associated T cell receptor (TcR) $\alpha\beta$ or $\gamma\delta$ [1-11]. Recent observations in athymic, nude mice [4,5] and in thymectomized, lethally irradiated, bone marrow reconstituted recipient mice indicate that TcR$\gamma\delta$ iIEL can mature in the absence of a thymus [8,12,13]. Also observations have been done that TcR$\alpha\beta^+$ iIEL may differentiate independently of the thymus [8,9,13]. The TcR$\alpha\beta^+$ iIEL can be subdivided, based on Thy-1 expression, in two populations of cells: One population expressing Thy-1 and another which does not express Thy-1 [7,8,9,11]. The TcR$\gamma\delta^+$ iIEL population is also for the most part negative for Thy-1 [4,7,8,11]. Only a low percentage of these cells express Thy-1 [4-7]. Thy-1 is a member of a group of proteins, linked to the cell membrane via an glycosylphosphatidyl inositol (GPI) anchoring structure [15]. Other members of the family of GPI linked proteins are Ly6A, Ly6C and Qa2 [16,17]. Mitogenic antibodies against these GPI linked proteins have been shown to mediate transmembrane signalling events [16-22]. These observations are supported by recent

findings that Thy-1 and Ly6A are associated with protein tyrosine kinases, suggesting a potential mechanism of signaltransduction via Thy-1 and Ly6A in those cells expressing these antigens [20]. Furthermore, after mitogen or antibody-induced activation expression of Ly6A becomes manifest on all activated splenic T cells [18]. The expression of the Ly6C antigen on murine T cells is restricted to cytolytic CTL [21,22]. In general it has been suggested that GPI linked membrane proteins play an important role in cell-cell interactions involved in the process of T cell activation and the maintenance of the cellular state of activation [16-22].

To investigate whether Thy-1$^-$ iIEL do not express the Thy-1 antigen due to a defect in the biosynthetic pathway of GPI linked membrane proteins in such cells we analyzed the iIEL population for expression of the GPI linked membrane proteins Qa2, Ly6A and Ly6C. The results show that Thy-1$^-$ iIEL are not defective in biosynthesis of GPI linked membrane proteins. Furthermore, the results suggets that GPI linked membrane protein expression by iIEL indicates that TcR$^+$ iIEL have an activational phenotype, different from activated peripheral T cells.

Materials and Methods

Animals

Male DBA/2 specified pathogen free mice of 8-10 weeks old were obtained from the Central Animal Facilities of the TNO Institute for Applied Radiobiology and Immunology, Rijswijk, The Netherlands.

Isolation of intestinal IEL

iIEL were isolated as previously reported from the small intestine of DBA/2 mice [3,4]. Briefly, the small intestine was removed and flushed with PBS. Peyers patches were excised and the iIEL released by incubation in HBSS, 1mM EDTA with shaking for 20 minutes at 37°C. The supernatant was filtered through cellulose acetate columns. The filtered cells were centrifuged over a discontinuous Percoll (Seromed, Berlin, FRG) density gradient ($\rho = 1.086$ g/ml, 1.054 g/ml, and 1.037 g/ml) at 600g and 4°C for 20 minutes. After centrifugation the most dense population of cells at the 1.086 g/ml / 1.054 g/ml interphase was harvested. Cells in this

interphase were further denoted as IEL.

FCM analysis and staining

Biotinylated or FITC conjugated mAb's 59AD2.2 [23] and 30H12 [23] (Becton Dickinson, Sunnyvale, CA) were used for Thy-1 staining. mAb H57-597, a *pan* TcR$\alpha\beta$ recognizing mAb [24], was directly conjugated to FITC or conjugated to biotin. TcR$\gamma\delta$ cells were detected with biotinylated mAb GL3 [7] (PharMingen, San Diego, CA). FITC-conjugated mAb Monts-1 [25] (PharMingen) was used for detection of Ly6C. mAb 5041-24.2 specific for Ly6A.2 [26] and mAb 141-15.8, specific for Qa2 [27] (Serotec, Oxford, UK) were used in combination with a FITC-conjugated goat anti-mouse polyclonal antibody reagent (Jackson, West Grove, PA). All biotin-conjugated mAb were used in combination with streptavidin-PE (Becton Dickinson). Hybridoma cells were grown in vitro culture systems. mAb were concentrated and purified using the Filtron minisette membrane system® (Filtron, Northborough, MA) For double fluorescence FCM analysis cells were washed once in PBS, 1% BSA and incubated with two mAb (FITC- and biotin conjugated) at the appropriate dilution for 30 minutes at 4°C. The first incubation was followed, after washing, by a second incubation with streptavidin PE. Staining for FCM triple fluorescence analysis was done as follows: Cells were first incubated with mAb 141-15.8. After washing the cells were incubated with PE-labelled goat anti- mouse $F(ab')_2$ polyclonal antibody reagent. Further incubation was done, after washing, with FITC-conjugated mAb H57-597 and biotinylated mAb 59AD2.2. After the last washing step the final incubation was done in presence of streptavidin-Duochrome (Becton Dickinson). Labelled cells were analyzed using a FACScan.

PI-PLC treatment of cells

Cells were washed once in HBSS, 2mM HEPES. PI-PLC (EC 3.1.4.10, Sigma, St. Louis, MO) incubation of 2.5×10^6 cells was done in 0.2 ml HBSS, 2mM HEPES in presence of 1 unit PI-PLC for 1 hour at 37°C.

Results

Thy-1 negativity of a subset of TcR$\alpha\beta$ iIEL is not the result of epitope modulation.

Thy-1 is not expressed on a subpopulation of TcR$\alpha\beta^+$ iIEL [6-9]. We confirm these observations using double fluorescence FCM analysis using mAb H57-597 (anti-TcR$\alpha\beta$) in combination with the anti-Thy-1 mAb's 59AD2.2 (fig.1A).

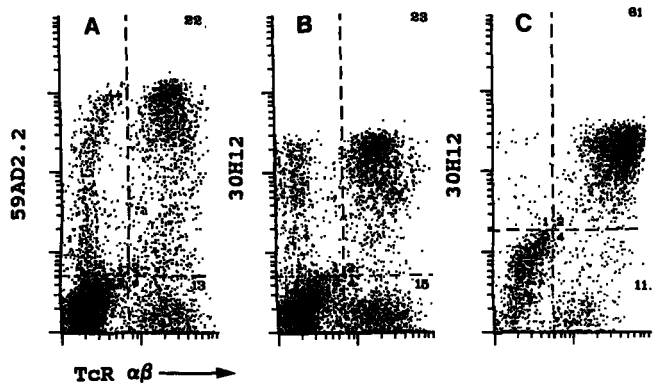

Figure 1. Thy-1 expression on TcR$\alpha\beta^+$ T cells, isolated from the intestinal epithelium (fig.1AB) and mesenterial lymph nodes (fig.1C). Antibodies used are FITC conjugated H57-597 for TcR$\alpha\beta$ staining, biotinylated 59AD2.2 for Thy-1 staining (fig.1AB) and biotinylated 30H12 for Thy1.2 staining (fig.1CB). Percentages of TcR$\alpha\beta$ positive cells are indicated in panels 1ABC.
No background staining was detected after staining with, FITC conjugated, irrelevant mAb and streptavidin PE.

The absence of Thy-1 on a subpopulation of murine TcR$\alpha\beta^+$ T cells might be due to conformational changes in the Thy-1 molecule itself, resulting in a different expression profile of antigenic determinants on the Thy-1 molecule. However, staining of iIEL with another mAb (30H12) directed against a different Thy-1 epitope [23] yielded an identical staining pattern (fig.1AB). These data suggest that modulation of Thy-1 epitopes as cause for the absence of Thy-1 reactivity on iIEL is unlikely. Furthermore, a small Thy-1$^-$TcR$\alpha\beta^+$ population is also present in the MLN TcR$\alpha\beta^+$ T cell population, but less abundant than in the TcR$\alpha\beta^+$ iIEL as shown in fig.1C.

GPI linked membrane protein expression is not deficient in TcR$^+$ iIEL.

The lack of Thy-1 expression by a subset of iIEL can be the result of a defective synthesis of GPI linked membrane proteins in these cells. Such defect has been described to be responsible for the lack of Thy-1 expression in certain mutant Thy-1$^-$ cell lines [28]. To investigate this option we analyzed the expression of the GPI linked membrane proteins Ly6A, Ly6C and Qa2 [16]. These antigens are expressed on peripheral TcR$\alpha\beta^+$ T cells [16-17].

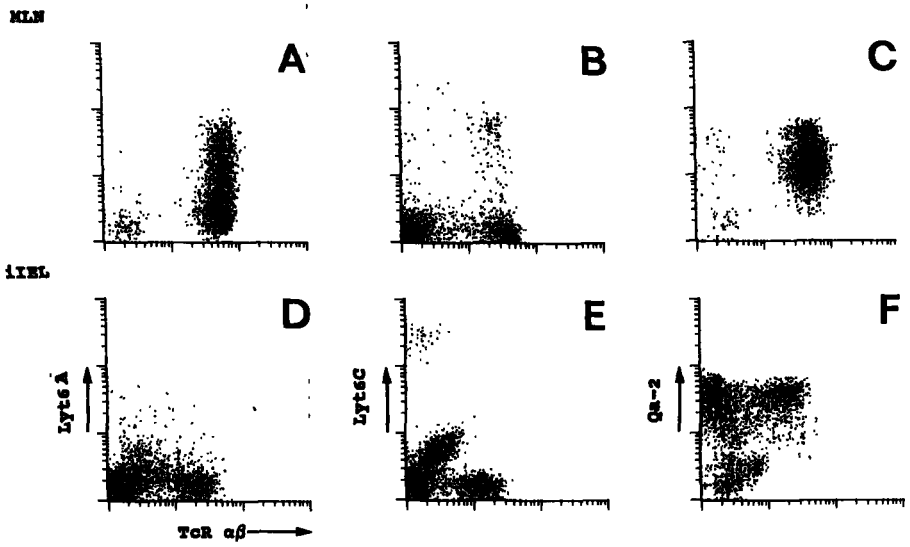

Figure 2. Analysis of GPI linked membrane protein expression by iIEL and MLN TcR$\alpha\beta$ expressing T cells. Fig.2ABC shows, respectively, Ly6A, Ly6C and Qa2 expression by MLN TcR$\alpha\beta^+$ T cells. Fig.2DEF show, respectively, Ly6A, Ly6C and Qa2 expression by TcR$\alpha\beta$ iIEL T cells. In figs. 2ACDF B cell depleted cell populations were analyzed, the populations analyzed in figs. 2BE were unfractionated. The horizontal axis represents log red fluorescence, the vertical axis represents log green fluorescence.

Figure 2 shows the results of representative experiments using mAb against these GPI linked membrane proteins in combination with an anti-TcR$\alpha\beta$ mAb. As can be seen in this figure the Qa2 expression is similar for the TcR$\alpha\beta^+$ T cells both in the iIEL population and in the lymph node T cell population (fig.2CF). Comparison, however, of Ly6A and Ly6C expression between iIEL TcR$\alpha\beta^+$ T cells and MLN TcR$\alpha\beta^+$ T cells reveals striking differences: Only a low percentage (<5%) of TcR$\alpha\beta^+$ iIEL expresses the Ly6A antigen (fig.2D). In contrast Ly6A is expressed on 50% of the MLN TcR$\alpha\beta^+$ T cells (fig.2A). In further analysis 80% of these Ly6A$^+$ TcR$\alpha\beta^+$ MLN cells were found to belong to the CD4$^+$ subset and the remaining 20% to the CD8$^+$ subset (data not shown). A similar pattern is observed for Ly6C: this antigen is not expressed on TcR$\alpha\beta^+$ iIEL (fig.2E) while clearly expressed on a subpopulation of TcR$\alpha\beta^+$ cells in the MLN (fig.2B). These TcR$\alpha\beta^+$, Ly6C$^+$ MLN T cells comprise 50% of the CD8 expressing T cells (data not shown). The Ly6A and Ly6C data show that also expression of these GPI-linked proteins on iIEL is deficient when compared with the expression of these antigens on TcR$\alpha\beta$ T cells in MLN. However, the GPI-linked protein Qa2 is expressed on virtually all TcR$\alpha\beta^+$ iIEL as well as on TcR$\alpha\beta^+$ MLN T cells (fig.2CF).

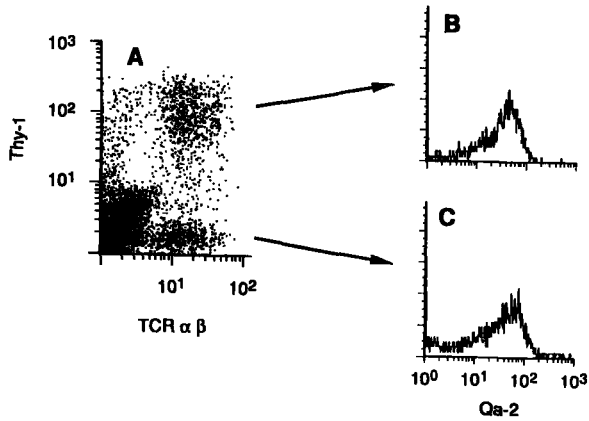

Figure 3. Triple staining of iIEL with FITC conjugated H57-597 (anti TcR$\alpha\beta$), 141-15.8 (anti-Qa2) in combination with an PE conjugated goat anti-mouse polyclonal antibody reagent and biotynilated 59AD2.2 in combination with streptavidin-Duochrome. Panel A represents the TcR$\alpha\beta$/Thy-1 staining on IEL, Panel B represents the Qa2 expression on the TcR$\alpha\beta$/Thy-1 double positive population and panel C represents the Qa2 expression on the TcR$\alpha\beta$ positive, Thy-1 negative,population.

These data indicate the existence of both a TcR$\alpha\beta^+$ Qa2$^+$ Thy-1$^+$ and a TcR$\alpha\beta^+$, Qa2$^+$, Thy-1$^-$ subset. This is confirmed by triple colour FCM analysis for TcR$\alpha\beta$, Qa2 and Thy-1 (fig.3). This figure shows that Thy-1$^-$ TcR$\alpha\beta^+$ iIEL display similar Qa2 staining characteristics as Thy-1$^+$ TcR$\alpha\beta^+$ iIEL do.

The observed pattern of expression of GPI linked membrane proteins by Thy-1$^-$TcR$\alpha\beta^+$ iIEL is not unique for this subset of iIEL since also TcR$\gamma\delta^+$ iIEL display similar characteristics. As shown in figure 4, the TcR$\gamma\delta^+$ iIEL T cell population is almost completely negative for Thy-1, Ly6C and Ly6A but strongly positive for Qa2. It is, however, difficult to compare GPI linked membrane protein expression on TcR$\gamma\delta^+$ iIEL with other populations because the frequency of TcR$\gamma\delta^+$ T cells in peripheral

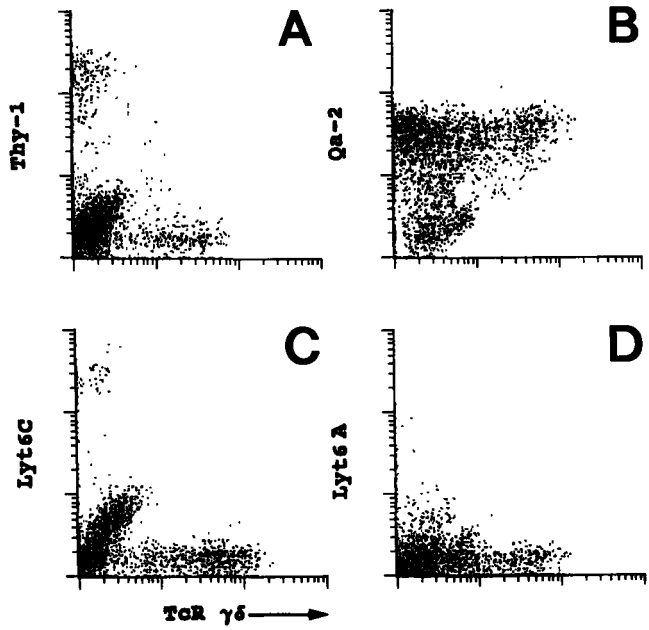

Figure 4. **Analysis of GPI linked membrane protein expression on TcR$\gamma\delta$ iIEL by double fluorescence FCM. TcR$\gamma\delta^+$ iIEL were analyzed for expression of Thy-1, Qa2, Ly6C and Ly6A, respectively shown in panels ABCD. For Thy-1 staining mAb 59AD2.2 was used. Other mAb used are indicated in materials and methods. The horizontal axis represents log red fluorescence, the vertical axis represents log green fluorescence.No background staining was detectable after incubation with FITC conjugated second stage mAb and streptavidin PE.**

lymphoid organs in the mouse is low. There is nevertheless one other large population of murine IEL expressing a TcR$\gamma\delta$: the skin IEL population or dendritic epithelial T cells (DETC). These DETC, however, display a different pattern of GPI-linked membrane expression when compared to other TcR expressing T cells. Freshly isolated DETC express Thy-1 [14] and Qa2, but express little Ly6A and no Ly6C (H.H. submitted for publication).

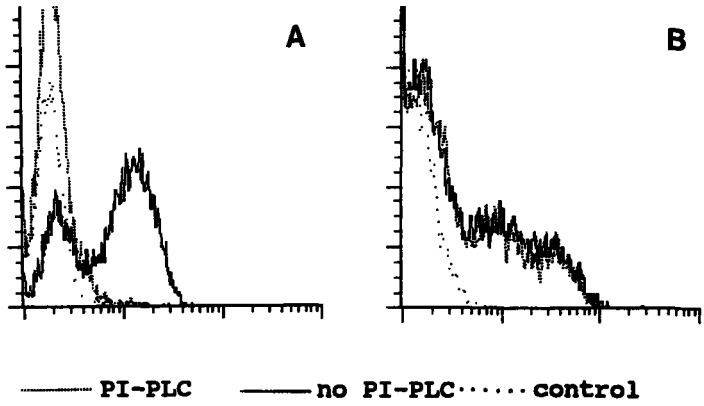

Figure 5. PI-PLC incubation of iIEL: Cells were treated as indicated in the legend of figure 5. Panel 5A represents Qa2 staining, Panel 5B represents TcR$\gamma\delta$ staining. The control represents second step staining only on PI-PLC treated cells: FITC conjugated rat-anti-mouse Ig, panel 5A and streptavidin-PE only, panel 5B.

Finally we investigated whether Qa2 on the various iIEL subsets is truly linked to the cell membrane via a Glycosyl Phosphatidyl Inositol anchoring structure. iIEL were incubated with Phosphatidyl Inositol specific Phospholipase C (PI-PLC) an enzyme which cleaves phosphatidylinositol between the glycerol backbone and the phosphate group [29]. This treatment resulted in the complete removal of Qa2 from TcR$^+$ iIEL as shown in figure 5A. Non-GPI linked proteins, like the TcR$\gamma\delta$, are not removed from the cell membrane during incubation with PI-PLC (fig. 5B). In a similar experiment Thy-1 was removed from Thy-1$^+$TcR$\alpha\beta^+$ iIEL (results not shown).

It can be concluded that deficiency in expression of the GPI linked membrane proteins Thy-1, Ly6A and Ly6C on TcR$\alpha\beta^+$ iIEL is not due to a defective biosynthesis of GPI linked membrane proteins in such cells.

Discussion

The precise function of GPI linked membrane proteins on T cells remains unclear, but current evidence suggests that these molecules are involved in mitogen and antigen specific responses involving TcR$\alpha\beta^+$ T cell-accessory cell interactions [16-20]. This is supported by recent findings that Thy-1 and Ly6A are associated with protein tyrosine kinases, key regulators of cell activation and signal transduction processes [20]. Furthermore, Ly6C has been shown to be expressed by CTL and is expressed by activated CTL regardless of the expression of CD8 or MHC restriction [21-22]. This is of interest in view of the possible activation state of TcR$^+$ iIEL. These cells have been claimed to be activated in vivo as Thy-1$^+$TcR$\alpha\beta$ show in vitro cytolytic activity in an anti-CD3 mAb redirected cytolysis assay using the mastocytoma cell line P815 as target cells [1,7,11,30]. In such an assay system Thy-1$^-$TcR$\alpha\beta^+$ and Thy-1$^-$TcR$\gamma\delta^+$ iIEL are inactive. However, such cells, show in vitro cytolytic activity when hybridoma cells secreting anti-CD3 or anti-TcR(β or δ chain) mAbs are used as target cells [31]. This strongly suggests that these cells are also in vivo activated. Because Thy-1$^+$TcR$\alpha\beta^+$ iIEL exhibit in vitro cytolytic activity and have a CTL phenotype common to peripheral CTL i.e. Thy-1$^+$TcR$\alpha\beta^+$CD8$\alpha\beta^+$ [6,8] one would expect that, if the same rule applies for these cells, Ly6C would be expressed. However, as shown above [fig.2E) Ly6C is not expressed at all on TcR$\alpha\beta^+$ iIEL. In general the same is true for Ly6A. This antigen is consistently expressed on activated peripheral TcR$\alpha\beta^+$Thy-1$^+$ T cells [16,28], but can only be detected on a small number of TcR$\alpha\beta^+$ iIEL (fig.2D). Similarly the absence of Ly6C and Ly6A expression on Thy-1$^-$TcR$\alpha\beta^+$ and on Thy-1$^-$TcR$\gamma\delta^+$ iIEL is not in agreement to the observed cytolytic activity of these cells.

Taken together, these data suggest that either the majority of iIEL are resting cells or that iIEL are truly in vivo activated cells, but that this activation process is not associated with or dependent on an upregulation of certain GPI linked membrane proteins, such as Ly6A and Ly6C. In the first case the observed [1,7,11,30,31] in vitro cytolytic activity may just reflect the potential of a small population of in vivo activated iIEL. The second option suggests the possibility of different cellular activation and signal transduction pathways for TcR$^+$ iIEL when compared with T cells at other sites in the mouse.

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APPENDIX PAPER 7

THE COMPOSITION OF THE MURINE INTESTINAL INTRAEPITHELIAL LYMPHOCYTE POPULATION DURING AGING

Bernard de Geus, Margit van den Enden and Jan Rozing

Submitted for publication.

Summary

Many studies have addressed the changes that occur with age in T cells of the systemic immune system. Relatively few data have been reported on the T lymphocytes of the mucosal immune system during aging. This paper describes a phenotypical analysis of the composition of the intestinal epithelial lymphocyte (iIEL) compartment in C57Bl/Ka mice of various ages. From these experiments it can be concluded that the $CD3^+$ iIEL fraction remains quantitatively rather stabile throughout life. A striking shift, however, is noted with age within the $CD3^+$ iIEL T cell population from presumably thymus-dependent $TcR\alpha\beta^+Thy\text{-}1^+CD8\alpha\beta^+$ cells towards thymus-independent $TcR\gamma\delta^+CD8\alpha\alpha^+$ and $TcR\alpha\beta^+CD8\alpha\alpha^+$ cells. Furthermore, the number of $CD4^+$ and $CD4^+CD8^+$ iIEL increase significantly with age. These CD4CD8 double-positive cells may be involved in the local thymus independent generation of $CD3^+$ iIEL. In some mice of 6 months and older $TcR\alpha\beta^+CD4^+CD8^+$ cells form the majority of $CD3^+$ iIEL. Whether there is a relation between this phenomenon and the high frequency of monoclonal gammapathies found in this C57Bl/Ka strain of mice needs further investigation. Finally also the percentage of $CD3^-CD8^+$ iIEL, cells with phenotypical similarities with NK cells, rises with age.

Introduction

With aging a variety of changes have been described, which occur in the systemic immune system [1,2]. This system consists of the major secondary lymphoid organs, such as the spleen and lymphnodes, and is interconnected through the blood and lymph vessels. The various subpopulations of lymphocytes are structurally well organized in the lymphoid organs. Although several age-associated changes have been reported in the humoral part of the immune system, such as a decrease in the ability to mount a primary humoral immune response against neoantigens [3], a decreased heterogeneity of the antibody profiles, an increased occurrence of monoclonal gammapathies [4,5] and auto-antibodies [6], major changes during aging are observed in the cellular immune system. One of the most striking phenomena in this respect is the age-related involution of the thymus [7], the primary organ, essential for the generation of most of the lymphocytes of the cellular immune system.

Besides this reduction in the generative capacity also changes within the cellular immune system, the T lymphocyte population, occur with age. The major subset afflicted is the $CD4^+$ subset. Besides intrinsic changes, such as decreased receptor expression for the IL-2R [8,9] and the CD3/TcR complex [9] or the reduced potential for signal transduction [10], the most prominent change in the $CD4^+$ T cell subpopulation with age is the shift within this subset from naive to memory cells, determined both by phenotype [11-13] and lymphokine profiles [11,12,14].

In contrast to the detailed analysis of changes in the systemic immune system with age, just a few studies have addressed changes during aging in the mucosal immune system [15,16]. This system which is considered to be a rather separate entity from the systemic immune system [17], is associated with the epithelial cell layers, that are in direct contact with the environment in for instance the lungs, skin, urogenital tract and the gastro-intestinal tract. At these sites IgA is the predominant antibody secreted [18]. Most aging studies have therefore focused on changes in IgA production capacity with age [19,20]. Over the last decade, however, it became clear that also $CD3^+$ lymphocytes form an integral part of the mucosal immune system. The predominant localization site of such mucosal $CD3^+$ cells is the mucosa associated epithelium. They are therefore called intra epithelial lymphocytes (IEL). IEL can be detected readily in skin [21], the urogenital tract [22] and the gastro-intestinal tract [23-29]. IEL display a site-specific phenotype and characteristic T cell receptor (TcR) usage [21,30]. Intestinal IEL (iIEL) can be divided in several major subsets based on their phenotypes [23-29]. These are $CD3^+TcR\alpha\beta^+CD8\alpha\beta^+Thy\text{-}1^+$, $CD3^+TcR\gamma\delta^+CD8\alpha\alpha^+$ and finally $CD3^+TcR\alpha\beta^+CD8\alpha\alpha^+$. The first population is thymus dependent for its generation, the latter are not [25-28, 31-37]. *$CD4^+$ cells in the gut are in majority found in the lamina propria.* Furthermore an interesting population of $CD3^-CD8\alpha\alpha^+$ cells can be detected in the iIEL [38,39]. This population of cells displays several phenotypical similarities with NK cells in rat and man [39].

Sofar no experiments have been performed to study the iIEL *population during aging. This report provides the first, detailed set of data* on the composition of the iIEL in mice at higher age.

Materials and methods

Animals

Female C57Bl/Ka mice were bred and maintained under SPF conditions till the age of 10 weeks at the animal facilities of the Institute for Applied Radiobiology and Immunology TNO, Rijswijk, The Netherlands. Young mice (2 months old) were obtained from this stock. Older mice were derived from our aging colonies of mice. These animals were obtained from the SPF stock and housed under clean conventional conditions behind a physical barrier from the age of 3 months. Mice that showed any signs of pathology were excluded from this study.

Isolation of iIEL

iIEL were isolated as previously reported from the small intestine of BALB/c mice [26]. Briefly, the small intestine was removed and flushed with PBS. Peyers patches were excised and the iIEL released by incubation in HBSS, 1mM EDTA with shaking for 20 minutes at 37°C. The supernatant was filtered through cellulose acetate columns. The filtered cells were centrifuged over a discontinuous Percoll (Seromed, Berlin, FRG) density gradient ($\rho = 1.086$ g/ml, 1.054 g/ml, and 1.037 g/ml) at 600g and 4°C for 20 minutes. After centrifugation the cells at the 1.086-1.054 g/ml interphase were harvested. Cells in this interphase are further denoted as iIEL. Cell suspensions of mesenteric lymph nodes (MLN) were prepared as described previously [25].

FCM staining and analysis

Biotinylated or FITC conjugated mAb 59AD2.2 was used for Thy-1 staining [40]. mAb H57-597 (a kind gift of Dr. R.T. Kubo), a TcR β chain constant region recognizing mAb [41], was used directly conjugated to FITC or biotin. TcR δ chains were detected with biotinylated mAb GL3, recognizing the constant region of the δ chain [29] (PharMingen, San Diego, CA). Mouse CD3 was detected using FITC conjugated mAb 145-2C11 (kindly provided by Dr. J.A. Bluestone) [42]. CD4 was detected with biotin conjugated mAb GK1.5 [43] and CD8 was detected with biotin or FITC conjugated mAb 53-6.7 [40] (Becton Dickinson). All biotin-conjugated

mAb were used in combination with streptavidin-PE (Becton Dickinson). For double fluorescence FCM analysis cells were washed once in PBS, 1% BSA and incubated with two mAb (FITC- and biotin conjugated) for 30 minutes at 4°C. The first incubation was followed, after washing, by a second with streptavidin PE. Labelled cells were analyzed using a FACScan with a life gate setting.

Immunofluorescence histology

Small intestines were processed in so called "Swiss rolls" and snap frozen in liquid nitrogen: 6-μm-thick cryostat sections were cut and incubated for 30 minutes at room temperature with anti- CD3 mAb YCD3-1 [44]. After washing the sections twice with PBS and 1% BSA, a second incubation with a peroxidase conjugated mouse anti-rat polyclonal antibody (Jackson, West-Grove, PA) was performed.

Results

In order to investigate the influence of aging on the cellular composition of the iIEL population, tissue was isolated from mice of 2, 6, 12 and 24 months old. Cell suspensions of iIEL and mesenteric lymphocytes (MLN) were processed individually for each animal. The recovered cell number per animal did not change significantly for the various age points (data not shown).

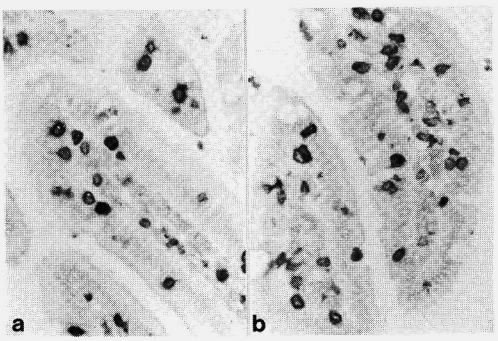

Figure 1. Immunohistology of the small intestine of 2- (A) and 24-months (B) old female C57Bl/Ka mice. Equal frequencies of $CD3^+$ iIEL, stained with mAb YCD3-1, can be observed in both age-groups. Representative patterns are displayed.

Also no major shifts in the overall balance between $CD3^+$ *iIEL and epithelial* cells were detected, as can be seen in figure 1. Immuno-histological comparison between $CD3^+$ cell frequencies in frozen sections of intestinal tissue from 2- (fig.1A) and 24-months (fig.1B) old mice did not result in dramatic differences.

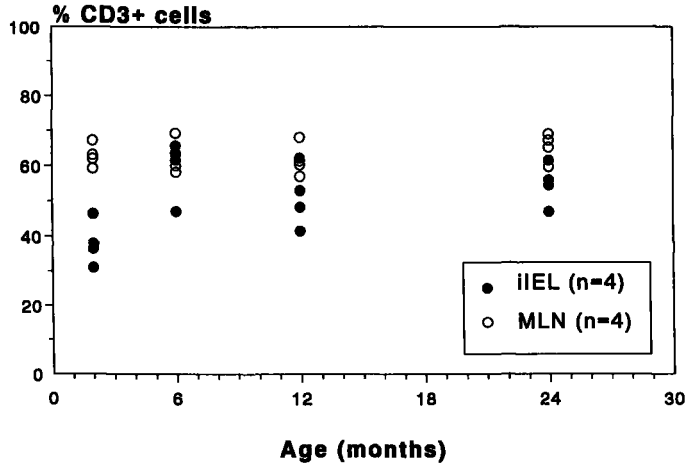

Figure 2. Percentages of $CD3^+$ cells in iIEL (closed symbols) and MLN (open symbols) of *C57Bl/Ka mice at various ages.* $CD3^+$ *cells were determined* by FCM and are individually expressed as percentage of gated, viable cells with a lymphoid appearance. Four individual mice were analyzed per age point.

However, quantitative measurement of the percentage of $CD3^+$, IEL using FCM analysis clearly showed an increase of $CD3^+$ cells after 2 months, with a stabilization at a higher level from 6 months on for the iIEL population, whereas no such changes were observed for MLN (Figure 2).

Detailed FCM analyses of the lymphocyte populations in the two tissues studied with a variety of cell surface markers revealed that $CD3^+$ MLN cells were composed of $CD8^+$ and $CD8^-$ (= $CD4^+$, data not shown) cells (fig.3A), whereas $CD3^+$ iIEL were virtually all $CD8^+$ (fig.3B). This characteristic tissue distribution remained essentially the same with some minor changes, throughout the age period investigated (data not shown).

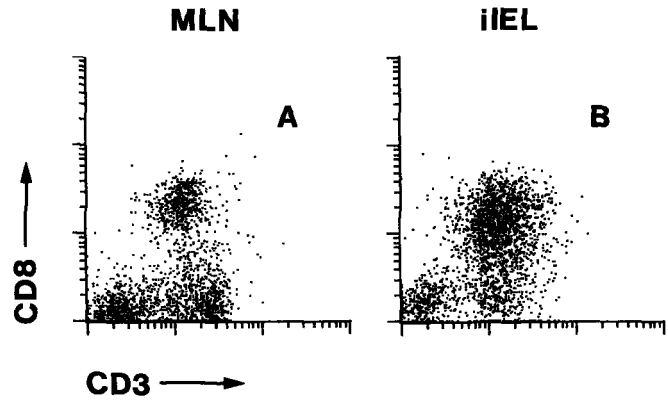

Figure 3. Dot plot FCM analysis after double-fluorescence staining of MLN (A) and iIEL (B) of a 12-months old C57Bl/Ka mouse with anti-CD3 (mAb 145-2C11), expressed on the horizontal axis, and anti-CD8 (mAb 53-6.7), expressed on the vertical axis. Representative fluorescence patterns of viable lymphoid cells, defined by forward and perpendicular light scatter, are shown.

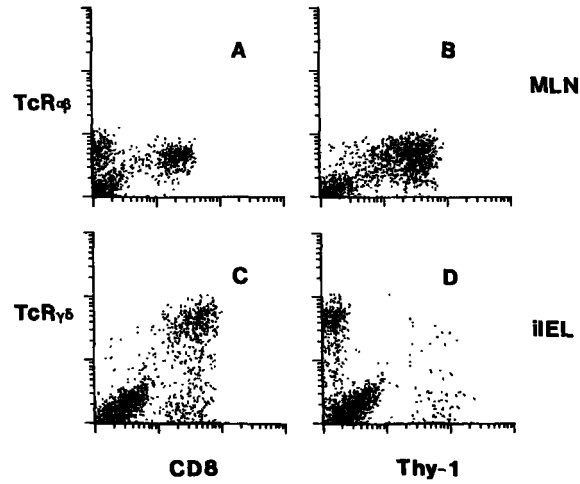

Figure 4. Dot plot FCM analysis after double-fluorescence staining of MLN (A&B) and iIEL (C&D) of a 24-months old C57Bl/Ka mouse with anti-CD8 (mAb 53-6.7) (A&C), or anti-Thy-1 (mAb 59AD2.2) (B&D) expressed on the horizontal axis, and anti-TcRβ (mAb H57-597) (A&C), or anti-TcRδ (mAb GL3) (C&D) expressed on the vertical axis.

Furthermore, CD3$^+$ MLN cells could be further characterized as TcR$\alpha\beta^+$Thy-1$^+$CD8$^+$ and TcR$\alpha\beta^+$Thy-1$^+$CD8$^-$(=CD4$^+$, data not shown) cells (fig.4AB), with virtually no TcR$\gamma\delta^+$ cells present at any time of life (data not shown). This balance was stable for all the time points investigated in the MLN (for CD4/CD8 shifts: see later).

The iIEL population on the other hand did contain significant numbers of TcR$\gamma\delta^+$ cells (fig.4CD). These cells turned out to be predominantly CD8$^+$ (fig.4C) and Thy-1$^-$ (fig.4D). The majority of TcR$\gamma\delta^-$ (=TcR$\alpha\beta^+$, data not shown) cells in iIEL were CD8$^+$Thy-1$^+$.

Although the specific phenotypes of these major iIEL populations did not change drastically during aging, the balance between the two major subsets, TcR$\alpha\beta^+$ and TcR$\gamma\delta^+$ cells, did. As can be seen from figure 5 the ratio between TcR$\alpha\beta^+$ cells and TcR$\gamma\delta^+$ cells in iIEL shifted from a clear dominance of the TcR$\alpha\beta^+$ population [2:1] in the young animals to an equal distribution between TcR$\alpha\beta^+$ and TcR$\gamma\delta^+$ cells [1:1] in 12-months old mice, whereafter it remained at that level.

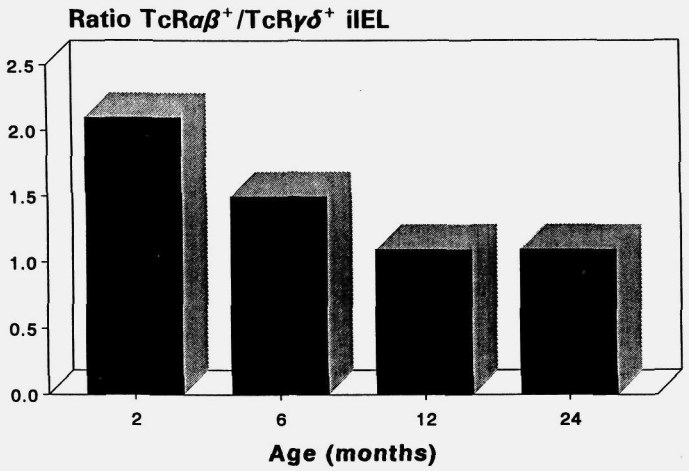

Figure 5. Ratio of TcR$\alpha\beta^+$ vs TcR$\gamma\delta^+$ iIEL at different ages. Percentages of TcR$\alpha\beta^+$ and TcR$\gamma\delta^+$ IIEL were determined by FCM and ratio's were calculated individually per mouse. Values of four (4) individual mice per age point were combined to calculate the overall ratio's as shown.

Also in the CD4$^+$ vs CD8$^+$ cell ratio shifts could be observed in the iIEL fraction during aging. As mentioned above MLN lymphocytes of young

mice could be divided into $CD4^+$ or $CD8^+$ cells, with virtually no $CD4^+CD8^+$ double positive cells (fig.6A). This picture remained identical also at higher ages (fig.6B), with only a slight shift in CD4/CD8 ratio from 2.0 in 2-months old mice to 1.6 in 24-months old animals.

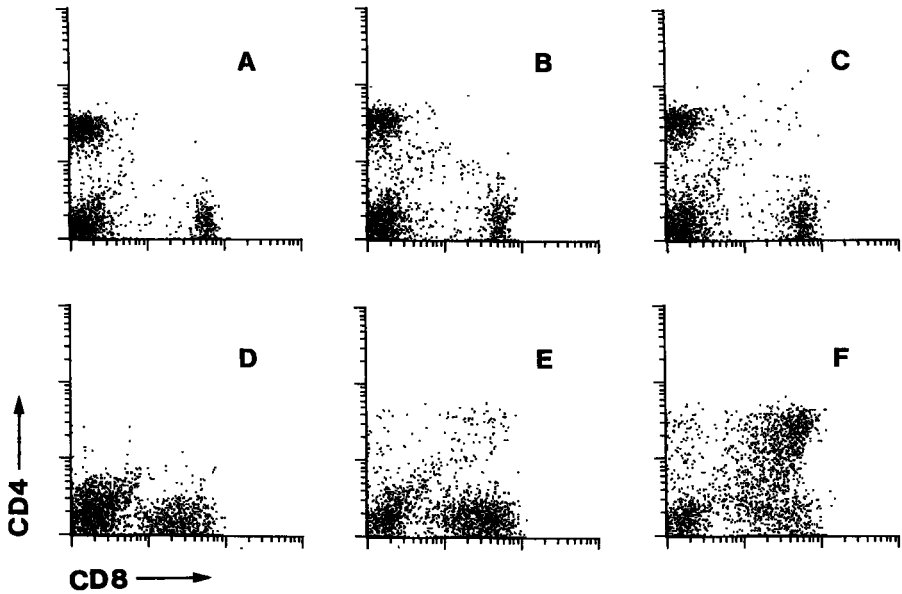

Figure 6. Dot plot FCM analysis after double-fluorescence staining of MLN (A,B&C) and iIEL (D,E&F) of a 2-(A&D) and two individual 6-months (B&E;C&F) old C57Bl/Ka mice with anti-CD8 (mAb 53-6.7), expressed on the horizontal axis, and anti-CD4 (mAb GK1.5), expressed on the vertical axis. Representative fluorescence patterns of viable lymphoid cells, defined by forward and perpendicular light scatter, are shown. The first 6-months old mouse (B&E) shows the characteristic increase of $CD4^+$ and $CD4^+CD8^+$ iIEL with age, the second 6-months old mouse (C&F) displays an overrepresentation of $CD4^+CD8^+$ iIEL as found in 1/5 C57Bl/Ka mice of 6 months and older.

Double fluorescence FCM analysis with anti-CD4 and anti-CD8 mAb of iIEL revealed that in young animals such cells were exclusively $CD8^+$, virtually

no $CD4^+$ or $CD4^+CD8^+$ iIEL could be detected (fig.6D). Although the $CD8^+$ population remained the dominant cell type in iIEL, both $CD4^+$ and $CD4^+CD8^+$ cells increased in number with age and were found in variable concentrations in the iIEL of all mice from 6 months on (fig.6E).
In a significant percentage of animals of 6 months and older (3/15) actually a hyperproliferation of $CD4^+CD8^+$ iIEL occurred, as illustrated in figure 6F. Some times also such $CD4^+CD8^+$ cells could be observed in the MLN of these animals (fig.6C). These mice were also excluded from the results.

Finally a tendency of higher numbers with age was noted for the population of $CD3^+CD8^+$ iIEL as shown in figure 7. The rather low number of such cells in FCM analysis is probably an underestimation of the actual number present in the gut, due to contra-selection of this cell type during the isolation procedure [38, B. de Geus, unpublished results]. No changes were observed in MLN (data not shown).

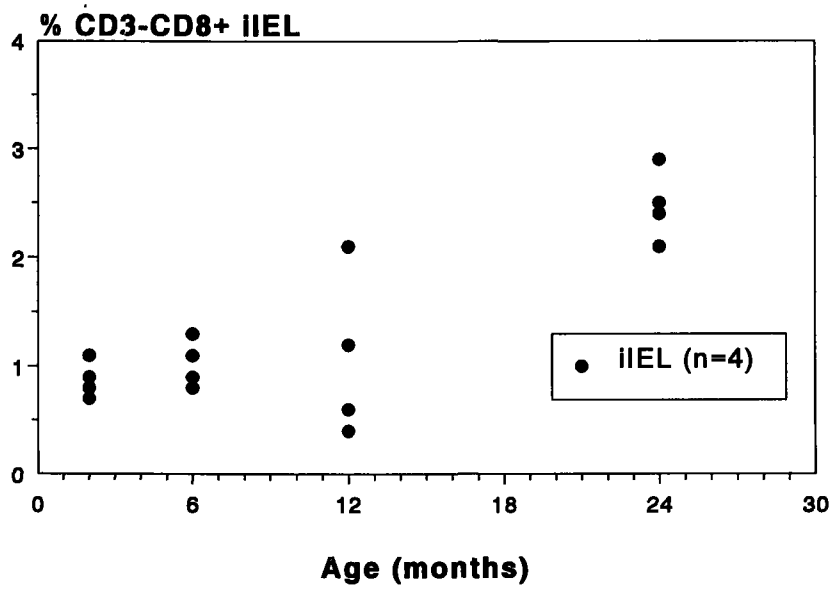

Figure 7. Percentage of $CD3^-CD8^+$ iIEL of C57Bl/Ka mice at various ages. $CD3^-CD8^+$ cells were determined by FCM after double fluorescence staining with anti-CD3 (mAb 145-2C11) and anti-CD8 (mAb 53-6.7), and are individually expressed as percentage of gated, viable cells with a lymphoid appearance. Four (4) individual mice were analyzed per age point. Note the expanded vertical axis.

Discussion

The vast majority of studies addressing the mucosal immune system in relation to aging have focused on the humoral and especially the IgA response of mucosal tissues [15,16,17,19]. Only a few investigations have been done on the influence of aging on T lymphocytes that are part of the mucosal immune system. Ernst et al. [45] reported that the high levels of IL-2 produced by Peyer's patch lymphocytes do not change with age, which is in striking contrast with the lymphocyte profiles of splenic T cells with age [8,46]. Green-Johnson and colleagues on the other hand showed a significant age-associated decline in the percentages of Peyer's patch cells containing IL-5 and IFN-γ [47]. They argue that such age-related changes in lymphokine production may affect the characteristic isotype of specific antibody responses at mucosal sites, and therefore may influence the host's defence against invading pathogens at mucosal surfaces. Also in the intestinal tissue itself significant numbers of IFN-γ and IL-5 secreting cells can be detected [48]. Whereas the majority of lamina propria lymphocytes (LPL) produces IL-5, equal numbers of IFN-γ and IL-5 secreting cells are found in the iIEL population following ConA activation [48]. These two cytokines are actually produced by the $CD8^+$ iIEL T lymphocytes. At present nothing is known about the age related changes in this population of intestinal T lymphocytes. the present report describes a first phenotypical analysis of this large population of mucosa associated intra-epithelial lymphocytes during aging.

As is obvious from the results a dramatic increase of the proportion of $CD3^+$ cells is observed between 2 and 6 months of age, followed by a stabilization. It is questionable, however, whether this change actually has to do with the aging process. A first argument against an age-association is the fact that this phenomenon is seen at a rather young age and that this trend does not continues at higher ages. Secondly, one has to keep in mind that in this particular time period the mice undergo a dramatic change in environment: they are moved from the SPF facilities into the aging cohorts, where they encounter a clean conventional environment. Various studies have shown that such changes in microbiological status have a major impact on the composition of the T cell compartment both in the periphery [49,50] and in the iIEL population of the gut [50,51]. It seems thus that in terms of aging the size of the overall $CD3^+$ T cell compartment in the iiEL, after adopting itself to the microbiological environment, remains stabile.

The composition of the intestinal iIEL population, however, does change with age. One of the most obvious alterations is the shift from TcR$\alpha\beta^+$ cells to TcR$\gamma\delta^+$ cells with age. The likely explanation for this phenomenon seems to be a decrease of thymus dependent TcR$\alpha\beta^+$ cells, due to the age-related involution of the thymus, which is compensated by an increase of thymus-independent TcR$\gamma\delta^+$ cells. One would assume that such a process would also be reflected in an increase of the number of CD8$\alpha\alpha^+$ cells in the iIEL, since T cells with a CD8$\alpha\alpha^+$ phenotype are considered to be thymus-independent for their generation [31,33]. Preliminary data of experiments looking at the Lyt3 (CD8β) expression of iIEL of mice at various ages suggest that this is indeed the case (B. de Geus, unpublished results).

At the age of one year also a balance between the TcR$\alpha\beta^+$ and the TcR$\gamma\delta^+$ IIEL subset is observed, i.e. no further reduction of the TcR$\alpha\beta^+$ subpopulation is observed, indicating an autonomous thymus independent regulation of the maintenance of the thymus derived TcR$\alpha\beta^+$ population or an age related shift in the iIEL from thymus dependent TcR$\alpha\beta^+$CD8$\alpha\beta^+$ cells towards thymus independent TcR$\alpha\beta^+$CD8$\alpha\alpha^+$ cells. With respect to this latter option recently data have been obtained in our laboratory suggesting an important role for the lamina propria environment for such a thymus-independent generation of TcR$\alpha\beta^+$CD8$\alpha\alpha^+$ iIEL [52]. Strong indications for local factors involved in the regulation of the intestinal TcR$\alpha\beta^+$ cells can also be derived from experiments in which the TcR Vβ usage of individual , 2 months old SPF, mice was measured, both in mesenteric lymph nodes and in iIEL [53]. Whereas the Vβ profiles were identical between the individual mice for MLN, a striking heterogeneity was observed in the iIEL of the same mice. The obvious conclusion of an autonomous regulation of the intestinal TcR$\alpha\beta^+$ population is further supported by a recent report of Rocha et al [32], in which they showed that in DBA/2 mice the TcR Vβ6 family is not deleted in the TcR$\alpha\beta^+$CD8$\alpha\alpha^+$ iIEL, whereas this Vβ family is deleted in the thymus from all thymus dependent subsets. Finally strong arguments for a role of gut associated microbiological components, also in the expansion of thymus derived T cells have come from studies in which the peripheral T cell compartment has been analyzed phenotypically and functionally in mice of various ages with different microbiological status, such as germ free, SPF and conventional mice [50,51]. From these experiments it can be concluded that factors, derived from the intestinal flora, play a key role in the expansion of the peripheral naive T cell compartment, also at higher ages.

Another remarkable change in the phenotypical composition of the iIEL population with age is the appearance of $CD4^+$ T cells. Such cells are normally in younger animals only detectable in the lamina propria. Perhaps the increased number $CD4^+$ iIEL in older mice reflects an increased migration of $CD4^+$ LPL into the intestinal epithelium. Moreover, most of the $CD4^+$ iIEL in older mice co-express CD8 on their surface, resulting in a double positive $CD4^+CD8^+$ phenotype. Such an immature phenotype is predominantly found on T cells in the thymus and is recently described to be expressed by a subset of iIEL [28,54]. The presence of such cells seems to be in line with an increase in the local generation of TcR^+ iIEL, as suggested above. Actually, in 20% of mice of 6 months and older (3/15) an overrepresentation of this $CD4^+CD8^+$ iIEL population was observed. An increase of $CD4^+CD8^+$ iIEL in aging rats has recently also been reported [55]. In two out of three cases the high numbers of double positive iIEL in aging mice were associated with supra-normal numbers of $TcR\alpha\beta^+$ iIEL (data not shown), suggesting again a possible role for such cells in the local generation of $TcR\alpha\beta^+$ cells. However, it must be pointed out that the exact phenotype of these CD8 and CD4 'double positive' iIEL is different from that of CD8 and CD4 expressing thymocytes [28]. With respect to the high frequency of mice of 6 months and older, displaying a hyperproliferation of $CD4^+CD8^+$ iIEL, it is worthwhile to mention that the strain of mice, used for these experiments, the C57Bl/Ka strain, is well-known for its high frequency of monoclonal gammapathies in older mice [4]. Whether there exist a causal relationship between these two age-associated phenomena is presently not known and needs further investigation.

Finally, also an increase in the frequency of $CD3^-CD8^+$ iIEL can be observed during aging in the present experiments. This cell type, which shows several phenotypical similarities with NK cells from rat and man [39] is probably present in the epithelial layers of the intestine in larger numbers than one would deduce from the FCM analysis, since the isolation procedure seems to counter-select for these cells [39]. An increase with age of this cell type in the intestine would be in line with the observed increase of NK cells in the peripheral blood of healthy aged individuals [56,57], suggesting perhaps a more important role for NK-cell mediated responses in the host defence at mucosal sites with age.

In conclusion, it seems from these studies that the population of intestinal iIEL remains rather intact in quantitative terms during aging. This is probably caused by and associated with a gradual shift from

predominantly thymus dependent cells towards more extrathymic derived cells within the iIEL population, whereas also a local expansion of thymus derived lymphocytes cannot be excluded. Double positive $CD4^+CD8^+$ cells have increased significantly in the iIEL of older animals and in 1/5 mice of 6 months and older such cells represent the majority of $CD3^+$ iIEL. The function of these cells and their possible relationship with the high frequency of age-associated monoclonal gammapathies in the C57Bl/Ka substrain of mice, needs to be established further.

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ABBREVIATIONS

BSA	Bovine serum albumin
C	Constant
CD	Cluster of differentiation
D	Diversity
DNA	Deoxyribonucleic acid
FACS	Fluorescence activated cell sorter
FCM	Flowcytometry
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GALT	Gut-associated lymphoid tissue
GF	Germ free
HBSS	Hank's balanced salt solution
IEL	Intraepithelial lymphocytes
Ig	Immunoglobulin
Ig-SC	Immunoglobulin-secreting cell
IL	Interleukin
J	Joining
kb	Kilo bases
LPL	Lamina propria lymphocytes
mAb	Monoclonal antibody
MALT	mucosa-associated lymphoid tissue
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
NK cell	Natural killer cell
PBS	Phosphate buffered saline
PE	Phycoerythrin
PP	Peyer's patches
RNA	Ribonucleic acid
SPF	Specific pathogen free
TcR	T cell receptor
TRITC	Tetramethyl rhodamine isothyocyanate

NAWOORD

Dit proefschrift kon tot stand komen dankzij de medewerking van velen, van wie een aantal hier ongenoemd zal blijven. Hiervoor bij voorbaat mijn excuses!

Professor Hoedemaeker wil ik danken voor zijn enthousiaste begeleiding gedurende de laatste maanden van het schrijfwerk. Vooral het gegeven dat hij altijd even tijd had heeft mij bijzonder aangesproken. Ook de suggesties van professor Knook zijn waardevol geweest.

De experimentele fase die ten grondslag ligt aan dit manuscript heeft tot stand kunnen komen onder de stimulerende begeleiding van Dr. Jan Rozing. De enorme mate van vrijheid die hij mij gegeven heeft, heb ik als bijzonder prettig en waardevol ervaren.

Een groot deel van de experimenten had nooit gedaan kunnen worden zonder de prettige samenwerking met Margit van den Enden. Margit vormde samen met Co Coolen het rustpunt in het, vaak hectische, laboratorium. De tijd die Co steeds weer wist vrij te maken om zijn toetsenbord voor de immunohistologie te ruilen waardeer ik bijzonder.

Veel werk is ondersteund door de inspanningen van stagiaires. Van hen, wil ik met name Renate Sonneveld en Boudewijn Hinlopen bedanken voor hun goede bijdrage.

Zonder proefdieren was het hele onderzoek helaas op niets uitgelopen daarom: Gerrit Schijf, Jan Pater en Anneriek Winkelaar bedankt voor de goede zorgen.

Hoewel vele discussies met Lex Nagelkerken, Harm HogenEsch, Emile de Heer, Frans Nooy en Jan Jore vaak over wetenschap(?) gingen bewaar ik vooral goede herinneringen aan het niet-wetenschappelijke deel.

Verder moeten - met een meer of minder belangrijke bijdrage aan wat dan ook - vermeld worden: Trees, familie, vrienden en collega's: Ruud Dobber, Frans Tielen (borrels), Paula van den Bergh, Erik Nijhuis, Frank Hopstaken (tekeningen), Sjeng Horbach, Tom Glaudemans (foto's!), Margret Tielemans, Mo Weijtens, Marlène van Velzen, Erik Braakman, Eline Slagboom (la la la), Arno Kleijweg, Anja Roos, Alita van der Sluis, Jiri Radl, Pleuni van den Berg, Anneke van Vliet , Lia Hoogeveen, Sjef van Asten en Nico Sakkee.

CURRICULUM VITAE

Bernard de Geus werd geboren op 30 maart 1960 te Vlaardingen. In 1978 haalde hij het HAVO diploma na een prettig verblijf op het Erasmus college te Zoetermeer. Vervolgens startte hij met de HBO A opleiding chemie aan het Van 't Hoff instituut te Rotterdam alwaar het diploma behaald werd in 1981. Tijdens deze opleiding heeft hij stage gelopen in diergaarde Blijdorp te Rotterdam. Dit leidde er uiteindelijk toe dat hij, na een kortstondig verblijf in militaire dienst gevolgd door werkzaamheden bij een assuradeur, van augustus 1982 tot november 1987 biologie studeerde aan de rijksuniversiteit te Utrecht. Tijdens deze studie deed hij een hoofdvak moleculaire celbiologie (Prof. Dr. H. O. Voorma) en een hoofdvak moleculaire genetica (Prof. Dr. G. van Arkel). Dit laatse vak werd uitgevoerd op het medisch biologisch laboratorium TNO te Rijswijk onder leiding van drs. Jan Jore. Vanaf juni 1987 tot juni 1991 was hij als promovendus, onder leiding van Dr. J. Rozing, werkzaam binnen de afdeling Immunologie van het TNO Instituut voor Experimentele Gerontologie te Rijswijk. Vanaf juni 1991 werkt hij binnen de afdeling Immunologie en Pathologie van het TNO Instuut voor Verouderings en Vaatziekten Onderzoek te Leiden.

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