International Immunology Advance Access published March 30, 2006

International Immunology 2006; 1 of 9
doi:10.1093/intimm/dxl018

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Reactivity of naive CD4$^+$CD25$^-$ T cells against gut microflora in healthy mice

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Keywords: inflammation, T lymphocytes, tolerance, Treg

Abstract

We have previously shown that conventional as well as germ-free CD4$^+$ T cells depleted of CD25$^+$ cells from the gut-associated lymphoid tissue and the periphery proliferate specifically in response to enterobacterial antigen exposure whereas unfractionated CD4$^+$ T cells are not reactive under these conditions. Here we show that the majority of the enteroantigen-specific CD4$^+$CD25$^-$ T cells are naive cells expressing a CD45RB^{high}, CD62L^{high} and CD44^{low} phenotype. These cells are also present in the thymus and data from adult thymectomized mice show that they represent late (>6 weeks) thymic emigrants. Upon enteroantigen activation, the CD4$^+$CD25$^-$ T cells secrete IL-4, IL-5, IL-10, granulocyte macrophage colony-stimulating factor, tumor necrosis factor-α and IFN-γ. Clonotype mapping of the TCRBV regions 1–18 of enteroantigen-reactive CD4$^+$CD25$^-$ T cells by TCR clonotype mapping revealed the polyclonal nature of this subset. In conclusion, we have for the first time demonstrated the presence of an evolutionary, functionally conserved subset of CD4$^+$ T cells, which are reactive against enterobacterial antigens. This subset resides both in the thymus and the periphery; it is not dependent on previous antigen experience and represents late thymic emigrants, which by enteroantigen-induced activation express a mixed T_h1–T_h2 phenotype. At homeostatic conditions, CD25$^+$ T cells maintain peripheral tolerance in this CD4$^+$ T cell subset.

Introduction

One of the major challenges in immunology is to understand the complex mechanisms that regulate immune responses. A key question is how the immune response mounts protective immunity to pathogens while preventing sustained inflammatory responses to self-antigens, food and commensal bacterial antigens. It is now evident that in addition to central tolerance, T regulatory (Treg) cells are crucial players in the fine-tuning of immune responses, and in the prevention of sustained inflammatory responses to the commensal microbial flora in the gut (1–4). Among the Treg cells, the so-called naturally occurring CD4$^+$CD25$^+$ Treg cells (5, 6) and the IL-10-producing Tr1 (7) cells are the best characterized.

Although the CD4$^+$CD25$^+$ Treg cells were first identified for their ability to prevent organ-specific autoimmunity in mice thymectomized on day 3 after birth (8, 9), it is now known that Treg cells play a more general role in immune regulation, and actively suppress the activation of effector T cells (10) and are involved in transplantation tolerance (11). Additionally, Treg cells have been shown to inhibit many autoimmune diseases (2, 12).

In our own studies, we have recently shown that CD4$^+$CD25$^+$ Treg cells can keep enterobacterial-reactive CD4$^+$CD25$^-$ T cells in a quiescent state (13). Unfractionated CD4$^+$ T cells from the gut-associated lymphoid tissue (GALT) and peripheral lymph nodes (PLNs) are unresponsive when exposed to enterobacterial antigens *in vitro*. However, when CD4$^+$ T cells are depleted *in vivo* or *in vitro* of CD4$^+$CD25$^+$ T cells, the CD4$^+$CD25$^-$ T cells proliferate extensively when exposed to enteroantigen. Additionally, we showed that CD4$^+$CD25$^-$ T cells obtained from the GALT of germ-free mice also proliferate when exposed to enterobacterial antigens, and adding back the conventional or germ-free CD4$^+$CD25$^+$ T cells

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Transmitting editor: W. Strober

Received 16 February 2005, *accepted* 27 February 2006

to the enteroantigen-stimulated $CD4^+CD25^-$ T cells abolished proliferation. Consistent with these data, $CD4^+CD45RB^{low}$ cells isolated from germ-free mice can inhibit colitis (14). Thus, apparently Treg cells in these model systems are neither antigen experienced nor specific for the same antigens as the effector cells.

Whereas our previous work for the first time demonstrated the presence of enteroreactive $CD4^+$ T cells in the periphery of normal mice (13), the present work deals with antigen experience, ontogenesis, clonality, life span and cytokine secretion of these cells.

Methods

Mice

Conventional 6- to 8-week-old female BALB/cA, C57BL/6 and SCID mice homozygous for the *scid/scid* mutation were purchased from M&B (Ry, Denmark). Female BALB/cA mice thymectomized or sham thymectomized at an age of 5–6 weeks were purchased from Taconic farms (Germantown, NY, USA). The mice were kept under controlled microbial conditions at the local animal facility of the Panum Institute.

$CD4^+$ T cell separation and negative and positive selection of $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells, respectively

All experiments were performed in complete media, RPMI 1640, supplied with Glutamax, $NaHCO_3$, 10% FCS, 50 μM 2-mercaptoethanol and antibiotics. In one separate experiment (data not shown), a batch of endotoxin-free media was kindly provided by Klaus Bendtzen, Copenhagen University Hospital, Denmark. Preparation of cells from lymphoid organs was performed as descried previously (15). $CD4^+$ T cells were positively selected from spleen and mesenteric lymph nodes (MLNs) using anti-CD4 mAb-coated Dynabeads and detach-a-bead from Dynal (Oslo, Norway). In separate experiments, $CD4^+$ T cells were purified from thymocytes and followed by depletion of $CD4^+CD8^+$ T cells by MACS using CD8a microbeads (Miltenyi Biotech, Belgisch Gladbach, Germany). The purity of $CD4^+$ T cells was >98% as assessed by flow cytometry. $CD4^+$ T cells were separated into $CD25^+$ and $CD25^-$ T cell populations by MACS (Miltenyi Biotech), using PE-labeled anti-CD25 mAb followed by addition of anti-PE microbeads, according to the manufacturer's description. The purity of the MACS-sorted cells was confirmed by FACS analysis using anti-CD4–FITC- and anti-CD25–PE-conjugated mAbs (PharMingen, San Diego, CA, USA) showed that >95% of the cells were either $CD25^+$ or >98% of the cells were $CD25^-$. The average cell yield was ~50% of the cell input. Flow cytometry was performed on a Becton Dickinson FACSCalibur and analyzed using CellQuest software.

Induction and assessment of IBD

IBD was induced in SCID mice by transplantation of $CD4^+$ T cells from normal BALB/c mice. The induction was performed as described previously (16). In brief, $CD4^+$ T cells were positively selected from spleen single-cell suspensions using anti-CD4 mAb-coated Dynabeads and detach-a-bead from Dynal. The $CD4^+$ T cells (>98% pure assessed by flow cytometry) were then stimulated *in vitro* for 3 days with 4 μg ml^{-1} ConA (Sigma, St Louis, MO, USA). SCID mice were injected intra-peritoneally with 3×10^5 of the activated $CD4^+$ T cells and monitored every week for weight loss, loose stools, bloody diarrhea and rectal prolapse.

Flow cytometry

Freshly prepared $CD4^+$ were stained for 30 min on ice for the expression of CD4 (anti-CD4–FITC; PharMingen) and CD25 (anti-CD25–PE; PharMingen). Subsequently, the cells were washed three times, re-suspended in PBS and analyzed using a FACSCalibur flow cytometer (Becton Dickinson). Data were analyzed using CellQuest software (Becton Dickinson). Specificity of the antibodies was confirmed by isotype-matched control mAbs.

FACS was performed on freshly prepared $CD4^+CD25^-$ T cells stained for the expression of CD45RB, CD62L or CD44. All antibodies were FITC labeled and purchased from PharMingen. A total of 5–15% of the cells within the brightest or most dim subsets were purified using a FACSVantage SE with DiVa option (BD Bioscience, Mountain View, CA, USA). The sorted cells were re-analyzed using a FACSCalibur flow cytometer (Becton Dickinson) and subsequently CellQuest software (Becton Dickinson) was used as described above. For intracellular Foxp3 staining, a FITC-conjugated anti-mouse kit was used according to the manufacturer's description (eBioscience, San Diego, CA, USA). In this case, CD4-purified T cells from MLN were first surfaced stained with anti-CD4–allophycocyanin and anti-$CD45RB^-$–, -$CD62L^-$–, -$CD44^-$– or $CD25^-$–PE mAbs followed by intracellular staining of Foxp3.

Proliferation assay

Unfractionated BALB/c splenocytes were used as antigen-presenting cells (APCs). They were pulsed overnight with fecal extract (200 μg protein ml^{-1}) or left unpulsed, washed and irradiated at 2000 rad. Preparation of the extract and pulsing of splenocytes were performed exactly as described previously (15). Antigen-pulsed or unpulsed APCs, 10^5, were added to each well of a 96-well flat-bottom microtiter plate. The added responder cells, 10^5, were either $CD4^+$ T cells from SCID mice with colitis (17) or freshly obtained $CD4^+CD25^-$ T cells from conventional (13), thymectomized or sham thymectomized mice. Responder cells were derived from either MLN or thymus. In separate experiments, freshly purified or pre-activated $CD4^+CD25^+$ T cells were titrated back to the $CD4^+CD25^-$ responder cells. The $CD4^+CD25^+$ T cells were pre-activated by plate-bound anti-CD3 (5 μg ml^{-1}) and IL-2 (100 U) for 3 days. The cells were cultured for 5 days and proliferation was measured by adding 0.5 μCi [^{3}H]thymidine (Amersham, UK) to each well for the last 18 h of the culture period. Then the cells were harvested to count the incorporated thymidine. All assay cultures were set up in four to six replicates.

TCR clonotype mapping by denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) analysis for clonotype mapping of the murine TCRBV regions 1–18 has been described (18, 19). Briefly, RNA was extracted using the Purescript Isolation Kit (Gentra Systems Inc., NC, USA) and synthesis of cDNA was carried out using 1–3 μg of total

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RNA, oligo-dT and SuperScript II reverse transcriptase (GIBCO-BRL, Gaithersburg, MD, USA). cDNA was amplified using primers specific for BV families 1–16 and a constant region primer. Amplified sequences were evaluated using the computer program MELT87, which predicts the melting of a double-stranded DNA molecule on the basis of its base composition (20). These calculations indicated that the DNA molecules amplified were suited for denaturing gradient gel analysis by the attachment of a 50-bp GC-rich sequence to the 5′ end of the constant region primer. Amplifications were performed in a GeneAmp PCR System 9700 (Applied Biosystems, Weiterstadt, Germany) using previously described conditions. DGGE analysis was done in 6% polyacrylamide gels containing a gradient of urea and formamide from 20 to 80%. Electrophoresis was performed at 160 V for 4.5 h in 1× TAE buffer at a constant temperature of 58°C.

Bio-Rad cytokine assay

Cell culture supernatants from enteroantigen-stimulated or polyclonally stimulated (anti-CD3: 2 μg ml^{-1} and anti-CD28: 100 ng ml^{-1}) CD4^{+} T cells were analyzed for cytokines [IL-4, IL-5, IL-10, granulocyte macrophage colony-stimulating factor (GM-CSF), IFN-γ, tumor necrosis factor-α (TNF-α)] using a multiplexed bead-based immunoassay kit (Bio-Plex Mouse Cytokine T_h1/T_h2 Panel # 171-F11081; Bio-Rad) in combination with Bio-Plex Cytokine Reagent Kit (# 171-304000; Bio-Rad). Blanks, standards and the samples were run in duplicates. After preparation, samples were processed using the Bio-Plex™ Suspension Array System and analyzed with Bio-Plex Manager™ Software Version 3.0. Generation of standard curves was performed using a five-parameter logistic regression model (%PL) with weighting including only the standards that were within 70–130% of the expected concentration. Cytokine concentrations are presented in picograms per milliliter. Samples with a CV > 15% were not used for final data analyses.

Results

CD4^{+}CD8^{-}CD25^{-} thymocytes are specific for enteroantigen

As is the case for CD4^{+}CD25^{-} T cells from GALT, spleen and peripheral blood (13), enteroantigen reactivity is also present in purified CD4^{+}CD8^{-}CD25^{-} thymocytes. As seen in Fig. 1(A), unfractionated thymocytes are unresponsive when exposed to enterobacterial antigens *in vitro*. However, purified CD4^{+}CD8^{-} thymocytes depleted for CD25^{+} cells proliferate extensively under these conditions. Additionally, as shown in Fig. 1(B), CD4^{+}CD8^{-}CD25^{+} thymocytes inhibit the enterobacterial-specific proliferation of MLN-derived CD4^{+}CD25^{-} T cells in a dose-dependent manner. Figure 1(A) and (C) include proliferative and inhibitory data obtained by CD4^{+} T cell subsets purified from the MLN and confirm our previous results (13). The figure also shows that prior activation of the CD4^{+}CD25^{+} T cell with plate-bound anti-CD3 and IL-2 enhances their inhibition of enterobacterial-specific proliferation in comparison with freshly derived CD4^{+}CD25^{+} T cells.

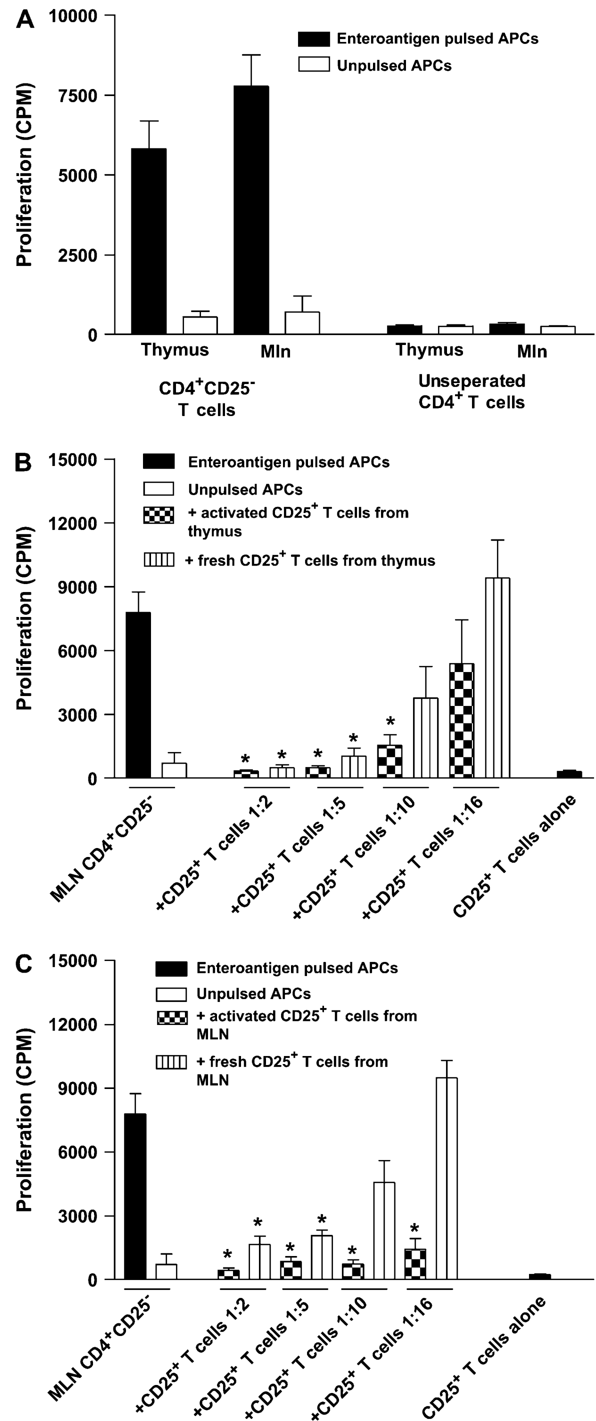

Fig. 1. Thymus-derived CD4^{+}CD25^{-} T cells are enteroantigen specific. (A) Unfractionated CD4^{+} T cells or fractionated CD4^{+}CD25^{-} derived from thymus and MLN were exposed to enteroantigen-pulsed (200 μg ml^{-1}) or unpulsed APCs. (B and C) MLN-derived CD4^{+}CD25^{-} T cells were exposed to enteroantigen-stimulated APCs in the presence of increasing numbers of CD4^{+}CD25^{+} T cells derived from MLN (B) or thymus (C). The CD4^{+}CD25^{+} T cells were either fresh or pre-activated with plate-bound anti-CD3 and IL-2. An asterisk denotes significant inhibition of CD4^{+}CD25^{-} T cell proliferation ($P < 0.05$). Each column represents the mean counts per minute value of four replicate microcultures containing 5 $\times$ 10^{4} CD4 T lymphocytes and 5 $\times$ 10^{4} antigen-pulsed or unpulsed APCs. Vertical bars represent the SD values. The data are from one of three typical experiments.

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Enteroantigen-specific CD4⁺CD25⁻ T cells are naive

Figure 2(A) shows the FACS profile for the expression of CD45RB, CD62L and CD44 in purified $CD4^+CD25^-$ cells obtained from the MLN. Five to ten percent of the cells within the most bright and most dim cell subsets were purified by cell sorting and purified cells as well as unfractionated $CD4^+CD25^-$ T cells were exposed to enteroantigen-pulsed APCs. Figure 2(B) shows that the majority of $CD4^+CD25^-$ T cells which proliferate against enteroantigen-pulsed APCs belong to the fraction of naive and non-activated cells. Thus, 60–70% of the enteroantigen-specific $CD4^+CD25^-$ T cells are $CD45RB^{high}$, $CD62L^{high}$ and $CD44^{low}$. As negative controls, the figure shows that $CD4^+CD25^-$ T cells incubated with unpulsed APCs do not proliferate.

Enteroantigen-stimulated CD4⁺CD25⁻ T cells are late thymic emigrants

$CD4^+CD25^-$ T cells were isolated and purified from the MLNs of adult mice thymectomized or sham operated 6–8 weeks previously. As post-thymic maturation of recent thymic emigrants probably occurs within 1–2 weeks, adult thymectomized mice can be considered to be devoid of these cells (21, 22). As shown in Fig. 3(A), $CD4^+CD25^-$ T cells from sham-operated as well as thymectomized mice proliferate strongly against enteroantigens. These results show that the enteroantigen-specific $CD4^+CD25^-$ T cells are not recent thymic emigrants but may circulate in the periphery for extended periods of time. As unfractionated $CD4^+$ or $CD4^+CD25^+$ T cells from MLN of conventional mice never respond when exposed to enteroantigen-pulsed APCs, the data indicate that, under homeostatic conditions, enteroantigen-specific $CD4^+CD25^-$ T cells are kept in a quiescent state by the presence of regulatory $CD4^+CD25^+$ T cells. Surprisingly, unfractionated $CD4^+$ T cells from adult thymectomized mice showed significant proliferation against enterobacterial-pulsed APCs, although to a lesser extent than the fractionated $CD4^+CD25^-$ T cells. This latter observation might reflect that Treg cells in adult thymectomized mice display a shorter life span than $CD4^+$ effector T cells and/or that the Treg cells are cell-by-cell less potent than Treg cells from control animals. However, the data in Fig. 3(B) and (C) argue against these ideas. In

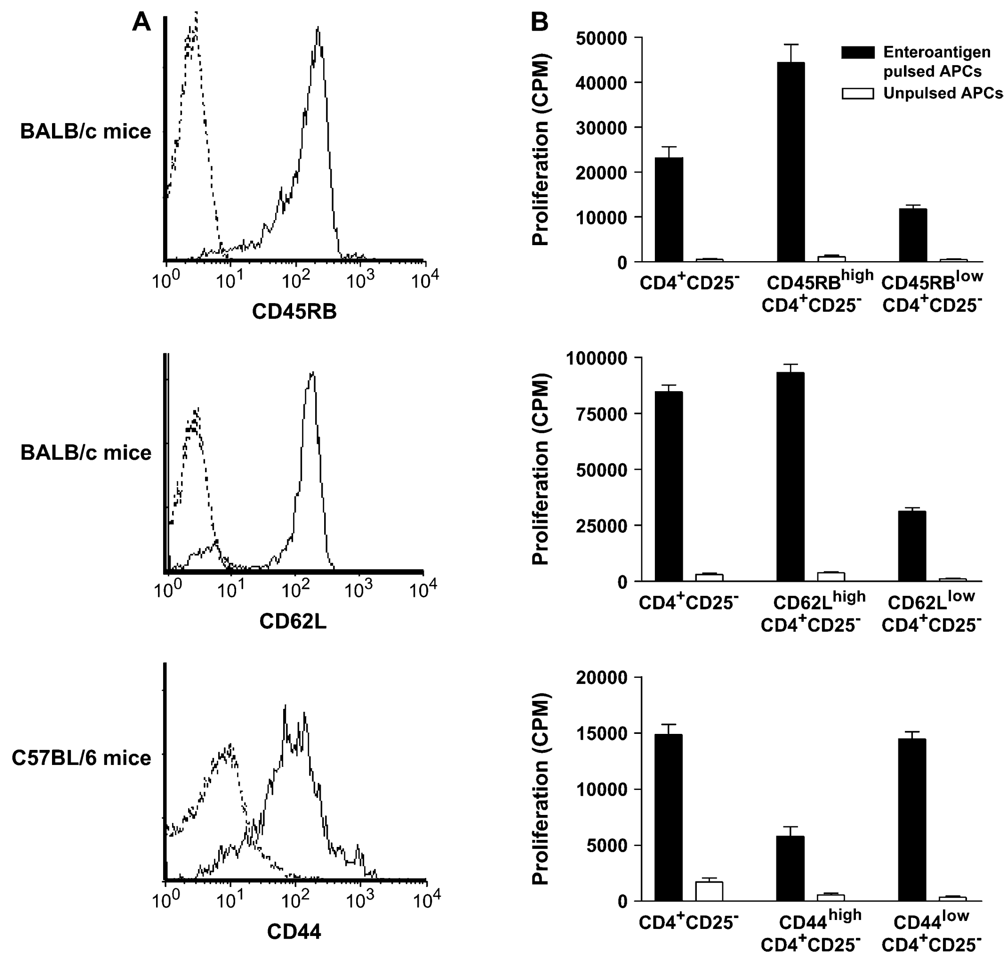

Fig. 2. Enteroantigen-specific $CD4^+CD25^-$ T cells are naive. (A) Distribution of CD45RB, CD62L and CD44 in FACS-purified $CD4^+CD25^-$ T cells obtained from MLN. Isotype controls are shown by dotted curves. (B) The majority of the enteroantigen-specific $CD4^+CD25^-$ T cells are naive cells expressing a $CD45RB^{high}$, $CD62L^{high}$ and $CD44^{low}$ phenotype. A total of 5–10% of the cells within the most bright and dim subsets were purified. Each bar represents the mean proliferation ($n = 4$) of 10^5 $CD4^+CD25^-$ T cells and 10^5 enteroantigen-pulsed (200 μg ml^{-1}) or unpulsed APCs. Error bars indicate SD values. The data show one representative experiment out of two.

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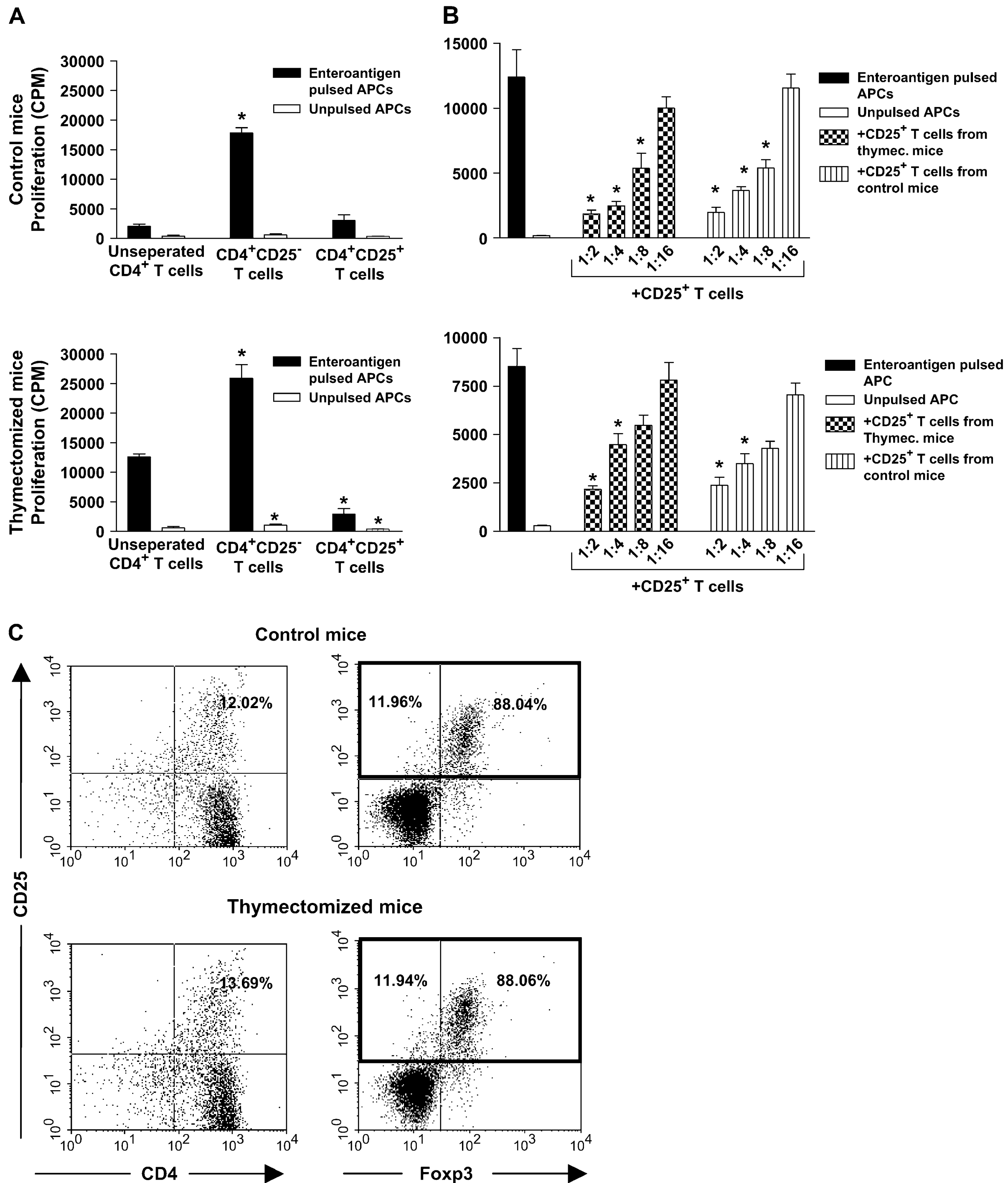

Fig. 3. Enteroantigen-stimulated CD4$^+$CD25$^-$ T cells are not recent thymic emigrants. (A) BALB/c-derived MLN CD4$^+$ T cells, obtained from sham thymectomized or 6–8 weeks post-adult thymectomized mice depleted of CD25$^+$ T cells, proliferate against enteroantigen. Unfractionated CD4$^+$ T cells from thymectomized mice also proliferate against enteroantigen although to a lesser extent than CD4$^+$CD25$^-$ T cells. In contrast, unfractionated CD4$^+$ T cells from sham thymectomized mice or fractionated CD25$^+$ T cells are unresponsive against enteroantigen. As control, the different T cell subsets were exposed to unpulsed APCs. An asterisk denotes significant difference between unfractionated and fractionated cells ($P < 0.05$). (B) MLN-derived CD4$^+$CD25$^-$ T cells from sham-operated or thymectomized mice were exposed to enteroantigen-stimulated APCs in the presence of increasing numbers of MLN-derived CD4$^+$CD25$^+$ T cells of either sham-operated or thymectomized mice. An asterisk denotes significant inhibition of CD4$^+$CD25$^-$ T cell proliferation ($P < 0.05$). Each column in (A) and (B) represents the mean counts per minute value of four replicates containing 10^5 CD4$^+$ T cells and 10^5 enteroantigen-pulsed (200 μg ml^{-1}) or unpulsed APCs. Vertical bars represent the SD values. (C) CD25 expression of CD4$^+$ T cells (double staining) and Foxp3/CD25 expression of CD4$^+$ T cells (triple staining) from normal and thymectomized mice is shown. A gate for all lymphocytes was set in the left-hand diagrams and a gate for CD4 cells was set in the right-hand diagrams. The bolded frame identifies the fraction of CD25$^+$ cells being either Foxp3 positive or negative.

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Fig. 3(B), the data show that $CD4^+CD25^+$ T cells from both groups of mice suppress equally well. In addition, Fig. 3(C) shows the same frequency (12–13%) of $CD4^+CD25^+$ T cells in both sham-operated and thymectomized mice. Moreover, MLN-derived $CD4^+CD25^+$ cells from both groups of mice show that ~90% of these cells express Foxp3. This latter observation excludes the possibility that a higher fraction of the $CD4^+CD25^+$ cells from the thymectomized mice are effector cells. However, the data in Fig. 3(B) suggest that the $CD4^+CD25^-$ T cells from thymectomized mice are more refractory to suppression than the same cell subset in control mice.

Enteroantigen-reactive $CD4^+CD25^-$ T cells are not clonally distributed

Figure 4 shows data from the clonotypic analysis of the TCRBV regions 1–18 of MLN-derived $CD4^+CD25^-$ T cells from healthy mice (Fig. 4A) and $CD4^+$ T cells from SCID mice (Fig. 4B) with T cell-induced colitis (see Methods), respectively. Prior to analysis, the $CD4^+$ T cells were exposed to enteroantigen-pulsed APCs for 4 days. The detection of clonally expanded T cells via this approach relies on the fact that clonotypic transcripts have no junctional diversity and therefore resolve at a fixed position in the denaturing gradient gel. Accordingly, the data show that the TCRs of $CD4^+$ T cells from healthy mice are not clonally distributed whereas TCRs from colitic $CD4^+$ T cells display an oligoclonal pattern of activation.

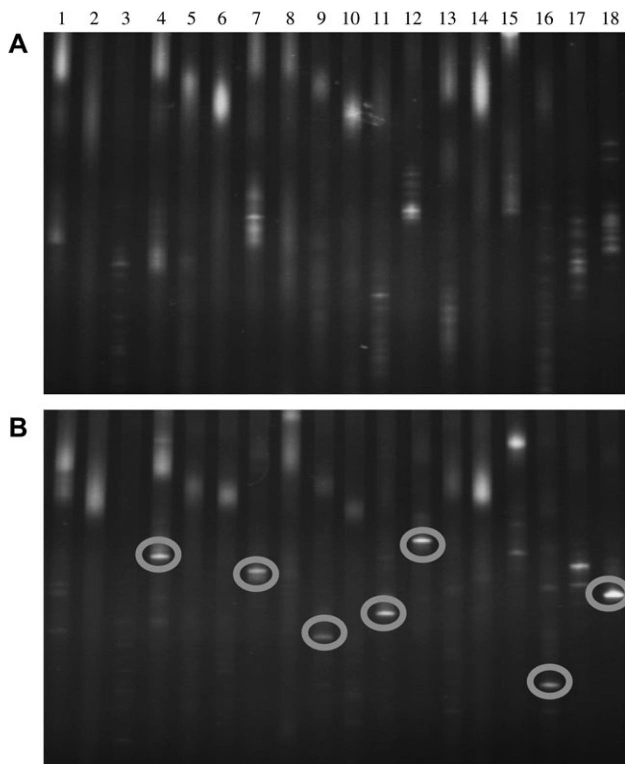

Fig. 4. The enteroantigen-reactive $CD4^+CD25^-$ T cell subset is polyclonal. TCR clonotype mapping of the TCRBV regions 1–18 of MLN-derived normal $CD4^+CD25^-$ T cells (A) and $CD4^+$ T cells from colitic mice (B) after exposure to enteroantigen-pulsed APCs for 4 days. Bands in circles indicate clonality. Notice that in contrast to $CD4^+$ T cells from colitic mice, normal enteroreactive T cells are polyclonal.

Proliferating $CD4^+CD25^-$ T cells display a T_h1–T_h2 cytokine secretion profile

The types of cytokines secreted into the culture supernatant of enteroantigen-exposed unfractionated $CD4^+$ T cells and fractionated $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells were studied. In addition to enteroantigen-pulsed APCs, the cells were also exposed to plate-bound anti-CD3 mAb and soluble anti-CD28 mAb. Figure 5 shows that after exposure to enteroantigen-pulsed APCs, the $CD4^+CD25^-$ T cells secrete IL-4, IL-5, IL-10, GM-CSF, TNF-α and IFN-γ reflecting a mixture of T_h1 and T_h2 cytokines. These data may support our finding above, that the TCRVβ repertoire of the enteroantigen-reactive $CD4^+CD25^-$ T cell subset is not clonally distributed. The data also show that $CD4^+CD25^-$ T cells secrete 2- to 25-fold higher amounts of cytokines after exposure to anti-CD3 mAb than after exposure to enteroantigen.

Discussion

T cells specific for autoantigens are present in most individuals but are kept under control by multiple and diverse peripheral mechanisms. It is evident that in addition to T cells that mediate effector immune responses to combat infections, there are classes of Treg cells that control immunity. The present and previous data clearly illustrate that $CD4^+CD25^-$ T cells obtained from all investigated sources such as GALT, peripheral blood, PLN (13) and now also the thymus, proliferate vigorously when exposed to APC pulsed with entobacterial antigens. In contrast, the unfractionated $CD4^+$ T cells from all these sources are refractory. Thus, T cells directed against commensal bacteria exist everywhere but are silenced by regulatory $CD25^+$ T cells. Accordingly, it is evident that there exists a level of evolutionary adaptation in the selection of the TCR repertoire on one hand and of the microbial flora of the gut on the other hand. On the top of this, regulatory thymus-derived $CD4^+CD25^+$ T cells have evolved to keep enterobacterial-specific $CD4^+CD25^-$ T cells in a quiescent state. Thus, coexistence between effectors, regulators and microbial gut flora appears to have developed during evolution.

We find that activated $CD25^+$ Treg cells express an enhanced inhibitory activity compared with freshly isolated $CD25^+$ T cells in consistence with the current view on regulatory $CD25^+$ T cell physiology (23–26). Activation-induced Treg cell activity may reflect further up-regulation of CD25 (26) and a very efficient consumption of IL-2 secreted during the early phase of T cell activation. Although it is known that $CD4^+CD25^+$ Treg cells suppress the transcription of the IL-2 gene in co-cultures with $CD4^+CD25^-$ responder cells (27), it has been suggested (28) that $CD4^+CD25^+$ Treg cells must respond to IL-2 before they can suppress proliferation of naive responder cells.

Our results from previous work (13) showed that a fraction of $CD4^+CD25^-$ T cells obtained from germ-free mice are enteroantigen specific, suggesting that these effector cells are

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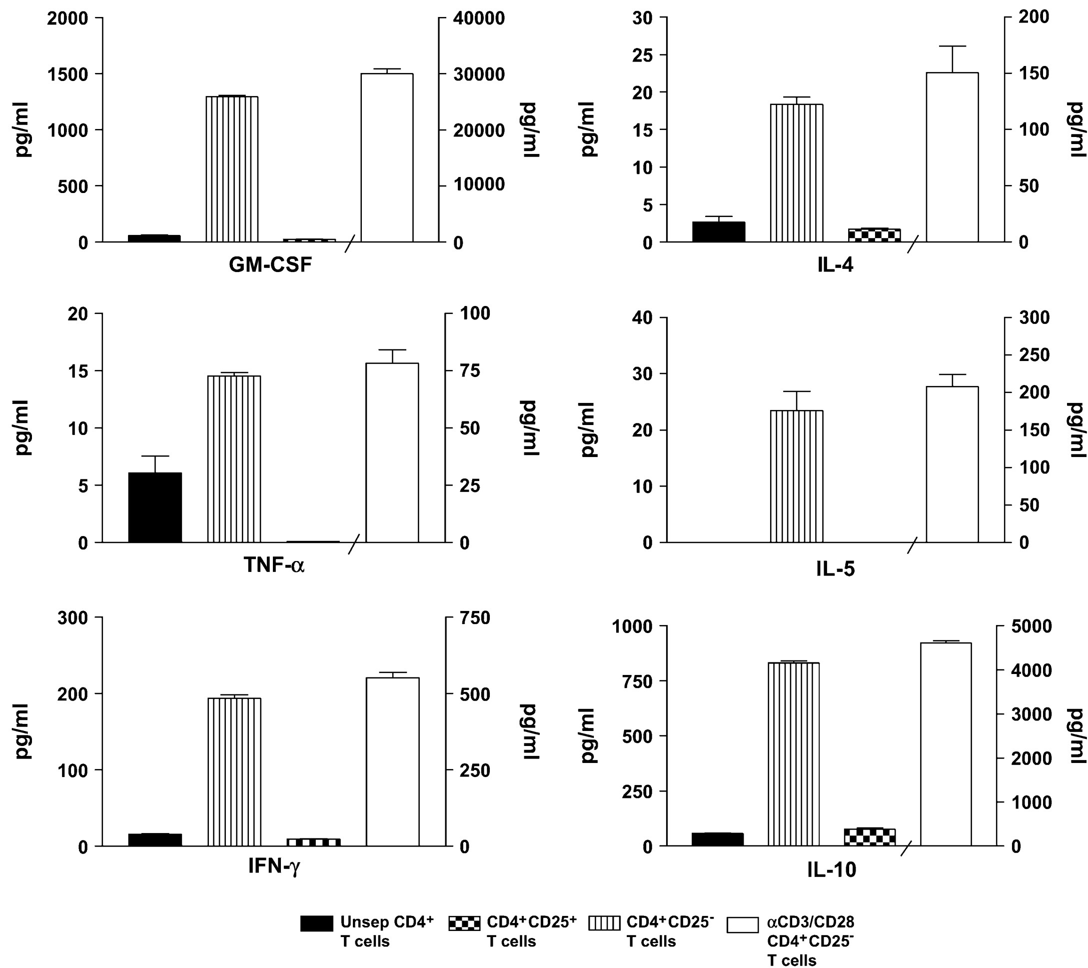

Fig. 5. Cytokine profile in the supernatant of MLN-derived unfractionated $CD4^+$ T cells, fractionated $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells exposed to enteroantigen-pulsed irradiated APCs. As a positive control, $CD4^+CD25^-$ T cells were stimulated with plate-bound anti-CD3 and soluble CD28. The cytokine production was measured at day 4 after stimulation by harvesting the supernatant and analyzing for a T_h1–T_h2 profile using a Bio-Rad cytokine assay (Bio-Rad Laboratories, CA, USA).

naive non-activated cells—a view supported by the presence of intrathymic enteroantigen-specific cells. This assumption was further confirmed by our present data showing that enteroantigen-specific $CD4^+CD25^-$ T cells from MLN are $CD45RB^{high}$, $CD62L^{high}$ and $CD44^{low}$. However, some of the memory $CD4^+CD25^-$ T cells, that is, $CD45RB^{low}$, $CD62L^{low}$ or $CD44^{high}$ also proliferate in response to enteroantigen exposure, although to a lesser extent. Thus, the data presented here suggest that $CD25^+$ Treg cells act to prevent the priming of naive potentially pathogenic T cells, but also actively control cells being part of the enteroantigen-experienced memory T cell pool. To exclude the presence of suppressive memory cells in the $CD4^+CD25^-$ $CD45RB^{low}$, $CD4^+CD25^-$ $CD62L^{low}$ and $CD4^+CD25^-$ $CD44^{high}$ fractions, a Foxp3 staining was performed for these subsets: $<2\%$ of these cell fractions expressed Foxp3 (data not shown). Additionally, we showed that the enteroantigen-specific $CD4^+CD25^-$ T cells are not recent thymic emigrants but rather long-lived cells present in the periphery. In adult thymectomized mice, we also found that unfractionated $CD4^+$ T cells proliferate against enterobacterial-pulsed APCs although the size of the pool of Foxp3-expressing $CD4^+CD25^+$ T cells in the MLN is comparable in the thymectomized and sham-operated mice. Additionally, we observed that the regulatory subset of the $CD25^+$ T cells derived from the thymectomized mice is just as potent as the regulatory subset of the $CD25^+$ T cells derived from control mice. However, our data also strongly suggest that the effector subset of enterobacterial-specific $CD4^+CD25^-$ T cells from thymectomized mice are more resistant to regulation than similar cells from intact animals. Recent data demonstrate that the neonatal thymus exports long-lived fully committed T cell precursors which colonize the gut (29). Thus, enteroantigen-specific effectors and regulators, demonstrated in the present work, might in part be replenished in the GALT by such T cell-committed thymic precursors and not being late mature emigrants from the thymus.

Finally, the aim of the paper was to address the cytokine profile of the enteroantigen-specific $CD4^+CD25^-$ T cells. After 4 days of stimulation, a secretion of IL-4, IL-5, IL-10, GM-CSF, TNF-α and IFN-γ was found reflecting a mixed T_h1–T_h2 cytokine profile. These data support our finding,

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based on TCRVβ repertoire analysis, showing that the enteroantigen-reactive TCR repertoire is non-clonally distributed. Thus, our data suggest that a variety of antigenic epitopes are responsible for the proliferative activity, an issue that we are currently working on. The antigen specificity and activation status of the $CD25^+$ Tregs are also topics important to address in order to understand the control of autoimmunity and colitis. *In vitro* studies of $CD4^+CD25^+$ T cells have demonstrated that these cells require stimulation through their TCR in order to be suppressive (30, 31). However, once activated their suppressor effector function is antigen non-specific and does not require re-engagement of the TCR (30, 32). In addition, suppressive $CD4^+CD25^+$ T cells do not necessarily share specificity with the targets of their suppression (31). Thus, from the general view on $CD4^+CD25^+$ Treg cell function, the $CD25^+$ T cells become activated through their TCR in order to obtain their suppressive function, and there is no evidence for a limited antigen repertoire of Treg cells (30, 32). However, what type of antigenic epitopes, self or non-self, which is recognized, is still unsolved. We have demonstrated that $CD4^+CD25^+$ T cells derived from germ-free mice have the ability to suppress the *in vitro* proliferation of $CD4^+CD25^-$ T cells stimulated with enteric bacteria (13). Whether these $CD25^+$ T cells from germ-free mice recognize food-derived antigens or whether they see enteric bacteria antigen like the $CD4^+CD25^-$ effector T cells remains to be elucidated. Previous experiments (28, 32) showed that freshly isolated wild-type $CD25^+$ Treg cells co-cultured with TCR transgenic $CD4^+$ T cells stimulated with APCs and the transgene-relevant peptide did not lead to suppression. Thus, these experiments indicate that $CD25^+$ Treg cells are not activated by self-peptides presented by the APCs. Based on these data, we assume that the $CD25^+$ Treg cells in our *in vitro* culture are being activated by the supplied enteroantigens. We finally exclude endotoxins to be of relevance in our *in vitro* suppressor assay since the effect of Tregs was observed also in culture media completely free of endotoxins (data not shown).

Taken together, the data presented here together with our previous work demonstrate the presence of an evolutionary conserved subset of $CD4^+$ T cells, which possess reactivity against enterobacterial antigens. This subset shows a generalized distribution, is not dependent on previous antigen experience and represents a polyclonal population of late thymic emigrants, which by activation express a naive mixed T_h1–T_h2 phenotype. At homeostatic conditions, $CD25^+$ T cells maintain peripheral tolerance in this $CD4^+$ T cell subset. These and previous data, have shed new light on the pathogenesis of chronic T cell-induced colitis by demonstrating the dynamic equilibrium of the gut immune homeostasis where local inflammatory changes at the same time may reflect too strong stimulation and/or insufficient counter regulation triggered by the commensal microbial flora recognized by a pre-existing TCR repertoire.

Acknowledgements

This work was supported by the Colitis Crohn Association, Physician SCE Friis and Wife's Fund, Novo Nordic Fund, Kong Christian den tiendes Fund, Gerda and Aage Haensch's fund, Else and Mogens Wedellsborgs Fund and by The Danish Medical Research Agency.

Abbreviations

APC	antigen-presenting cell
GALT	gut-associated lymphoid tissue
GM-CSF	granulocyte macrophage colony-stimulating factor
MLN	mesenteric lymph node
PLN	peripheral lymph node
TNF	tumor necrosis factor
Treg	T regulatory

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