

Identification of a CD4⁺CD25⁺ T cell subset committed *in vivo* to suppress antigen-specific T cell responses without additional stimulation

Esther N. M. Nolte-'t Hoen^{1,5}, Josée P. A. Wagenaar-Hilbers¹, Elmieke P. J. Boot¹, Chia-Huey Lin², Ger J. A. Arkesteijn¹, Willem van Eden¹, Leonie S. Taams³ and Marca H. M. Wauben¹

¹ Department of Infectious Diseases and Immunology, Division of Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

² TeGenero AG, Science Park Wuerzburg, Wuerzburg, Germany

³ Infection and Immunity Research Group, Frankling-Wilkins Building, King's College London, London, UK

Naturally occurring CD4⁺ regulatory T cells can be identified on the basis of expression of CD25 and suppression of T cell responses *in vitro* after TCR triggering. Here, we demonstrate that a CD134⁺ subset of CD4⁺CD25⁺ T cells in naive rats suppresses antigen-specific T cell responses *in vitro* without additional TCR stimulation. In contrast, CD4⁺CD25⁺CD134[−] regulatory T cells and total CD4⁺CD25⁺ regulatory T cells have suppressive activity only during simultaneous activation of responder and regulatory T cells or after *in vitro* pre-activation. Furthermore CD4⁺CD25⁺CD134⁺ T cells have a more activated phenotype than CD4⁺CD25⁺CD134[−] T cells, as based on the expression of CD62L, CD45RC, and MHC class II. We propose that the CD134⁺ regulatory T cells contain an *in vivo* activated and highly suppressive regulatory T cell subset. CD4⁺CD25⁺CD134⁺ T cells can be found in several compartments of the immune system, including spleen, lymph nodes, and blood. Interestingly though, the relative amounts of these cells within the CD4⁺ population and their CD134 expression levels are highest in mucosa-draining lymph nodes and lowest in blood. This suggests that the presence of CD4⁺CD25⁺CD134⁺ T cells indicates sites of active immune suppression.

Key words: Regulatory T lymphocytes / Suppression / Cellular activation / CD134 / Lymphoid organs

Received	10/11/03
Revised	8/8/04
Accepted	16/8/04

1 Introduction

Regulatory T cells that suppress aberrant or surplus T cell responses play an important role in balancing the reactivity of the immune system and preventing autoimmunity [1, 2]. A minor subset of CD4⁺ T cells isolated from thymus and peripheral lymphoid organs of naive animals expresses the interleukin-2 receptor (IL-2R) α chain (CD25) and exerts suppressive effects on T cell responses *in vivo* [3]. In addition, such CD4⁺CD25⁺ regulatory T cells appeared to be hyporesponsive and suppressive *in vitro* [4].

In the assays most widely used to test *in vitro* suppression by regulatory T cells, the proliferation of CD4⁺CD25[−] responder T cells to a polyclonal stimulus, e.g. anti-CD3 mAb, is tested in the presence of CD4⁺CD25⁺ suppressor T cells. In these antigen-nonspecific suppression assays, both suppressor and responder T cells are activated simultaneously. Besides these antigen-nonspecific suppression assays, T cells of TCR-transgenic mice have been used to investigate the capacity of CD4⁺CD25⁺ T cells to suppress antigen-specific T cell responses [5–7]. The so-called two-antigen regulation assay, using CD4⁺CD25⁺ T cells and responder T cells of different TCR-transgenic backgrounds, allows independent titration of peptides recognized by responder and regulatory T cells. Based on these studies, it was shown that freshly isolated CD4⁺CD25⁺ T cells only exert suppressive activity after *in vitro* activation via TCR triggering. Once activated, CD4⁺CD25⁺ T cells were suppressive in an antigen-nonspecific manner without additional TCR stimulation [5, 6].

[DOI 10.1002/eji.200324725]

Abbreviations: BrdU: 5-Bromo-2'-deoxyuridine **CFSE:** Carboxy-fluorescein succinimidyl ester **MLN:** Mesenteric LN **NRS:** Normal rat serum **PLN:** Popliteal and brachial LN **PP:** Peyer's patches **SLN:** Submandibular LN

Several attempts have been made to identify a subset of CD4⁺CD25⁺ T cells to which the suppressive activity could be ascribed. However, in antigen-nonspecific suppression assays, subsets that have differential expression of CD62L, CD38, or CD45RB show a similar suppressive capacity compared to total CD4⁺CD25⁺ T cells [6, 8, 9]. Only α_E integrin-expressing CD4⁺CD25⁺ T cells stimulated by anti-CD3 mAb have a stronger suppressive capacity than the total regulatory T cell population [9].

Phenotypic analysis of CD4⁺CD25⁺ T cells has revealed differences between regulatory T cells derived from humans, mice and rats. Although the CD45 expression pattern of CD4⁺CD25⁺ T cells (CD45RB^{lo}CD45RO⁺ in humans, CD45RB^{lo} in mice, and CD45RC⁺ in rats) characterizes the “antigen-experienced” state of CD4⁺CD25⁺ T cells [4, 5, 10–13], CD25^{bright} human CD4⁺CD25⁺ T cells show high expression of CD62L [11, 14, 15], which is a marker for naive T cells, whereas mouse CD4⁺CD25⁺ T cells have a slightly increased amount of CD62L^{lo} cells [6]. Additionally, human CD4⁺CD25⁺ T cells show an increase in cells positive for MHC class II, a marker expressed by activated human and rat T cells, but not by mouse T cells [10, 11]. These differences may be partly due to the fact that most human studies are performed with peripheral blood lymphocytes, whereas for murine studies, spleen, thymus or lymph node (LN) suspensions are more frequently used.

Recently, we analyzed the expression of the T cell activation marker CD134 during the course of adjuvant arthritis, and we revealed that the number of CD4⁺CD134⁺ T cells in rats recovered from disease was increased compared to naive rats (unpublished results). This suggested that the CD4⁺CD134⁺ T cell subset contained regulatory T cells involved in disease down-regulation. Here, we therefore analyzed CD134 expression on CD4⁺CD25⁺ regulatory T cells, and we compared the suppressive capacity of CD134⁺ and CD134[−] regulatory T cells. Our data demonstrate that CD4⁺CD25⁺CD134⁺ T cells freshly isolated from naive rats, in contrast to CD4⁺CD25⁺CD134[−] T cells or total CD4⁺CD25⁺ T cells, do not require TCR triggering to mediate suppression of antigen-specific T cell responses *in vitro*. This direct suppressive effect is most likely incited by *in vivo* activation, as based on the overall activated phenotype of the CD4⁺CD25⁺CD134⁺ T cells. Finally, we provide evidence that the distribution of CD4⁺CD25⁺CD134⁺ T cells in the immune system appears to be related to sites of immunoregulation.

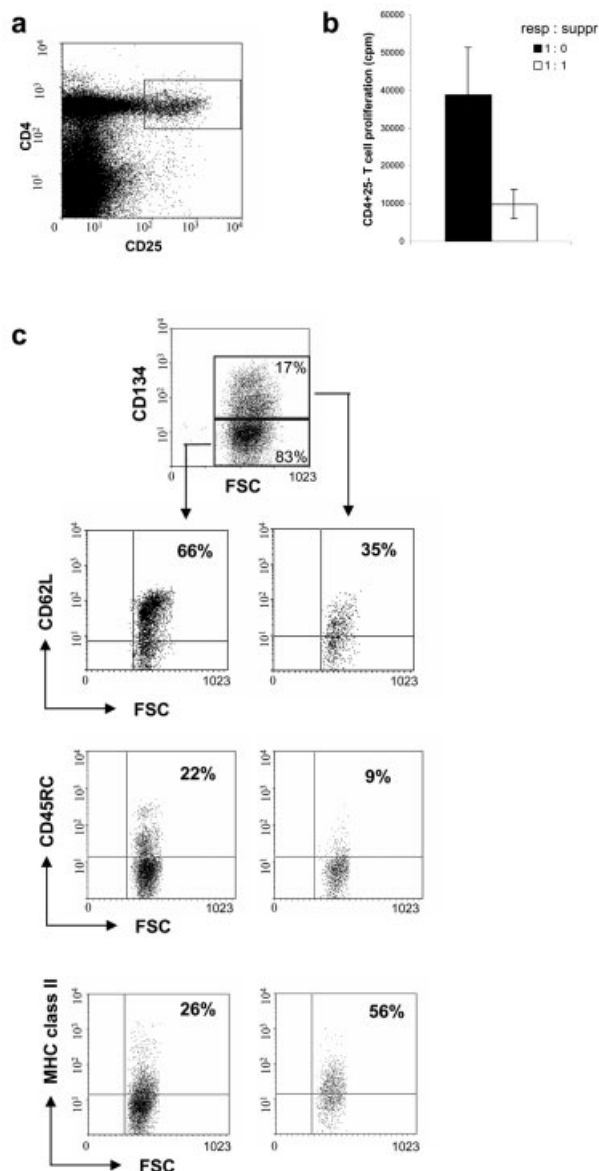
2 Results

2.1 Expression of T cell activation markers on naturally occurring CD4⁺CD25⁺ T cells

In spleen cell suspensions of naive rats, 7% of the CD4⁺ cells expressed CD25 (Fig. 1a). The suppressive capacity of sorted splenic CD4⁺CD25⁺ T cells was confirmed by the inhibition of proliferation of CD4⁺CD25[−] responder T cells in a co-culture stimulated by anti-TCR mAb and APC (Fig. 1b). The population of freshly isolated CD4⁺CD25⁺ T cells could be subdivided on the basis of expression of the T cell activation marker CD134 (Fig. 1c). To investigate whether the CD134⁺ subset of CD4⁺CD25⁺ T cells constitutes an activated population of regulatory T cells, we analyzed the expression of several T cell activation markers on both CD4⁺CD25⁺CD134⁺ and CD4⁺CD25⁺CD134[−] T cells. The reduced amount of cells positive for CD62L and CD45RC, combined with a higher expression of MHC class II molecules, marked the higher activation state of the CD134⁺ versus the CD134[−] subset of CD4⁺CD25⁺ T cells (Fig. 1c).

2.2 Suppression of polyclonal T cell responses

To compare the suppressive capacities of CD134⁺ and CD134[−] naturally occurring CD4⁺CD25⁺ T cells, we purified these populations from naive rat spleens by flow cytometric sorting. Both subsets expressed comparable amounts of CD25, but CD4⁺CD25⁺CD134⁺ T cells appeared to be larger (Fig. 2a). Both the CD134⁺ and CD134[−] CD4⁺CD25⁺ T cells were anergic, *i.e.* neither subset showed proliferation upon stimulation with anti-TCR mAb and irradiated APC (Fig. 2b). We compared the capacity of total CD4⁺CD25⁺ T cells, CD4⁺CD25⁺CD134⁺ and CD4⁺CD25⁺CD134[−] T cells to inhibit proliferation of CD4⁺CD25[−] responder T cells in an antigen-nonspecific *in vitro* proliferation assay, using polyclonal T cell activation by anti-TCR mAb and irradiated APC. Using [³H]thymidine incorporation, the suppressive capacity of all three regulatory T cells subsets was comparable (Fig. 2c). However, using the carboxy-fluorescein succinimidyl ester (CFSE) dye dilution method, we observed that co-cultured CD4⁺CD25⁺CD134⁺ T cells inhibited the proliferation of CFSE-labeled CD4⁺CD25[−] responder T cells to a higher extent than their CD134[−] counterparts (Fig. 2d).



◀ **Fig. 1.** Characterization of CD4⁺CD25⁺CD134⁺ and CD4⁺CD25⁺CD134⁻ subsets in naive rats. (a) Naive splenic lymphocytes expressing CD4 and CD25, as gated in (a), were purified by flow cytometric sorting. (b) Proliferation of naive CD4⁺CD25⁻ responder T cells co-cultured with CD4⁺CD25⁺ cells (1:1) or without added cells (1:0) was tested during stimulation with anti-TCR mAb and irradiated T cell-depleted spleen cells. Data are expressed as means $\pm$ SD of T cell [³H]thymidine incorporation (cpm) from triplicate wells. One out of three experiments is shown. (c) Naive rat splenic CD4⁺CD25⁺ cells can be subdivided into a CD134⁺ and a CD134⁻ population (upper dot plot). Cells gated as CD4⁺CD25⁺CD134⁺ or CD4⁺CD25⁺CD134⁻ were analyzed for expression of CD62L, CD45RC or MHC class II (RT1.B⁻). Shown are representative dot plots (from four experiments). The fluorescence intensity of cells stained with appropriate isotype control Ab was used to calculate the percentage of positive cells, as indicated in the upper right quadrant.

clones in the presence of CD4⁺CD25⁻ naive splenic T cells. Freshly isolated, total CD4⁺CD25⁺ T cells could not suppress the antigen-specific response of clonal T cells (Fig. 3a). However, the sorted CD4⁺CD25⁺CD134⁺ T cells suppressed the antigen-specific proliferation of both Z1a and A2b T cells to their stimulatory peptides (Fig. 3b, c). This effect was exclusive to CD4⁺CD25⁺CD134⁺ T cells, since no such effect was observed when CD4⁺CD25⁺CD134⁻ T cells were used. These data indicate that within the immune system of naive rats, a subset of activated CD4⁺CD25⁺ T cells exists that can be identified on the basis of CD134 and that suppresses antigen-specific T cell responses *in vitro* without additional stimulation.

2.3 Suppression of antigen-specific T cell responses

In the antigen-nonspecific suppression assay, due to the presence of anti-TCR mAb, both responder and suppressor T cells are stimulated. Therefore, this method cannot be used to investigate the requirement of *in vitro* activation of regulatory T cells for efficient suppression. Hence, we tested whether freshly isolated CD4⁺CD25⁺CD134⁺ and CD4⁺CD25⁺CD134⁻ T cells could suppress the proliferation of rat T cell clones A2b and Z1a reacting to their stimulatory peptides HSP65_{176–190} (for A2b) or MBP_{72–85} (for Z1a) presented by irradiated APC. As a control, we cultured the T cell

2.4 Suppression of antigen-specific T cell responses by *in vitro* activated CD4⁺CD25⁺ T cells

Next we tested whether both CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells activated *in vitro* to express CD134 could suppress antigen-specific T cell responses. *In vitro* stimulations were performed with the T cell-activating superagonistic anti-CD28 mAb JJ316. JJ316-activated CD4⁺CD25⁺ T cells display a more suppressive phenotype compared to freshly isolated CD4⁺CD25⁺ T cells [16]. After *in vitro* activation with JJ316, both CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells purified from naive rat spleens expressed CD134 and CD25, with the activated CD4⁺CD25⁺ T cells displaying higher levels of CD25 (Fig. 4a). However, only the activated CD4⁺CD25⁺ T cells were able to suppress antigen-specific proliferation of

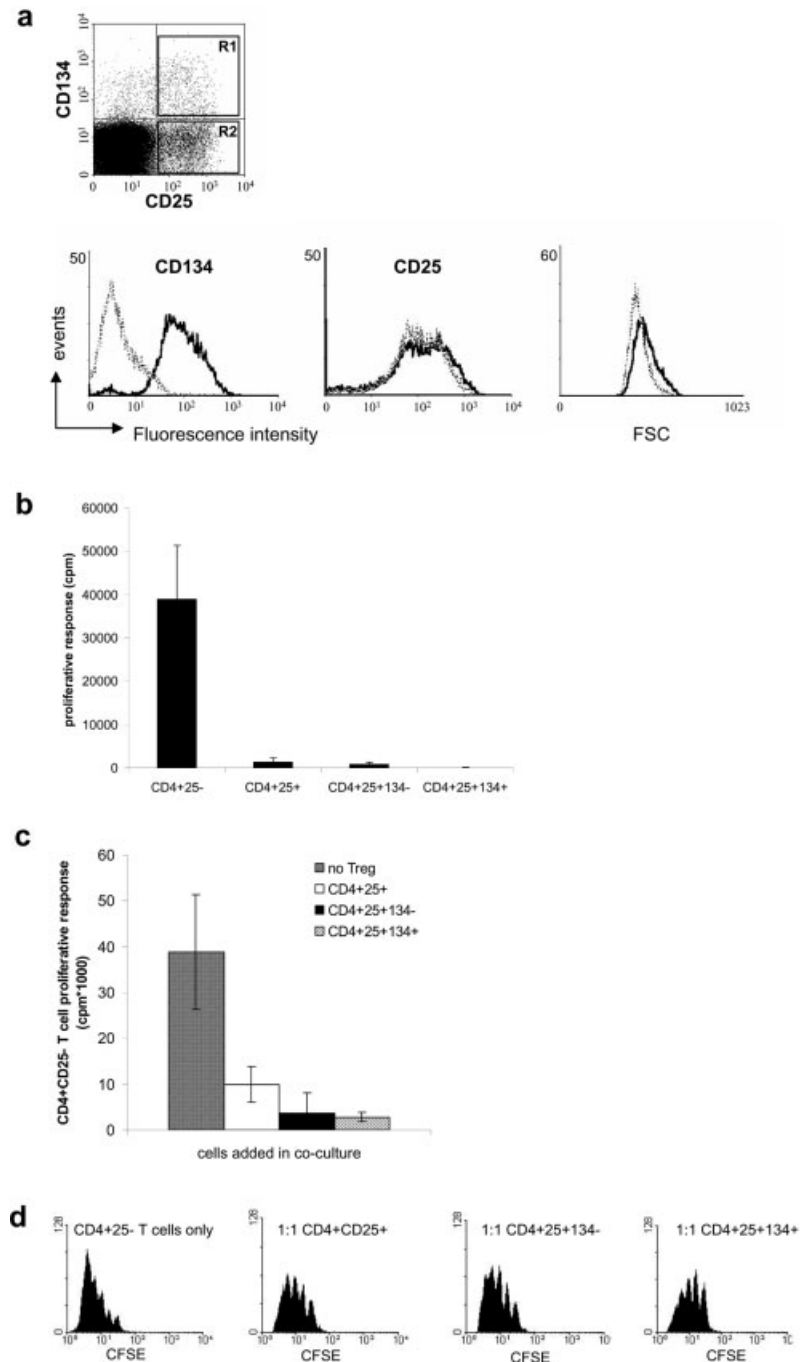
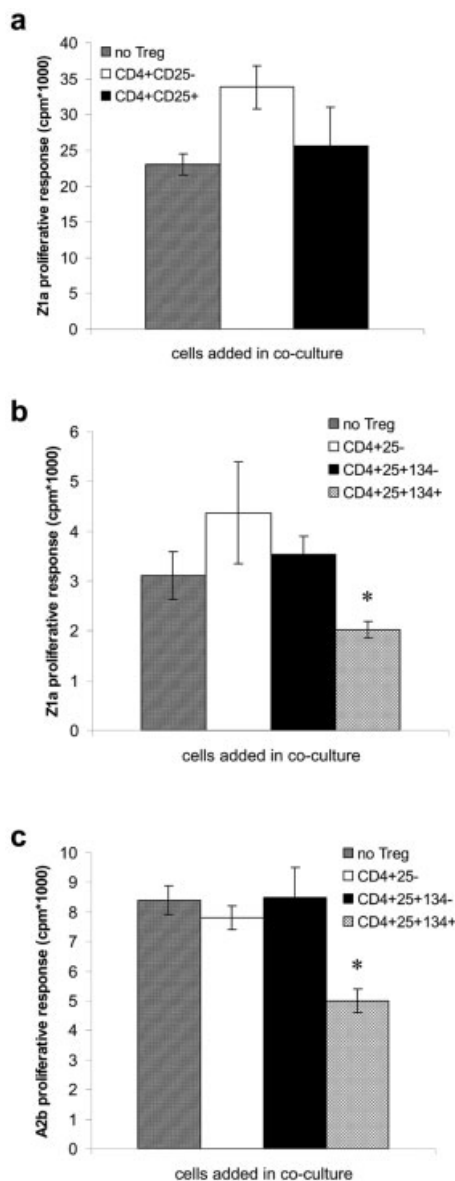


Fig. 2. CD4⁺CD25⁺CD134⁺ and CD4⁺CD25⁺CD134⁻ T cells suppress antigen-nonspecific T cell responses *in vitro*. (a) CD4⁺CD25⁺CD134⁺ and CD4⁺CD25⁺CD134⁻ T cells (gates R1 and R2 in the dot plot of CD4⁺ cells) were purified by flow cytometry (purity >95%). Expression of CD134, CD25 and the FSC of sorted CD4⁺CD25⁺CD134⁺ cells (black lines in histograms) and CD4⁺CD25⁺CD134⁻ cells (dotted lines) were compared. (b) CD4⁺ T cell fractions sorted on the basis of CD25 and CD134 expression were tested for their capacity to proliferate in the presence of anti-TCR mAb and irradiated T cell-depleted spleen cells. Data are expressed as means $\pm$ SD of T cell [³H]thymidine incorporation (cpm) from triplicate wells. The suppressive capacities of indicated CD4⁺ subsets were determined by stimulating naive CD4⁺CD25⁻ responder T cells, co-cultured in a 1:1 ratio with regulatory T cell subsets or without cells added, with anti-TCR mAb and irradiated T cell-depleted spleen cells. Responder CD4⁺CD25⁻ T cells were CD134⁻ (data not shown). Data of representative experiments (one out of three) are expressed as means $\pm$ SD of T cell [³H]thymidine incorporation (cpm) from triplicate wells (c), or visualized by the loss of CFSE content (d).



both A2b and Z1a T cells (Fig. 4b, c). In contrast, in co-cultures with activated CD4⁺CD25⁺ T cells, an increased proliferative response was observed. This could be the result of consumption of mutually produced IL-2. Freshly isolated CD4⁺CD25⁺CD134⁺ T cells and *in vitro* stimulated CD4⁺CD25⁺ T cells showed a similar suppressive capacity if tested next to each other in a suppression assay using Z1a responder T cells (Fig. 4d). These data show that the induction of CD134 expression does not *per se* lead to a suppressive phenotype in all T cell subsets. However, T cells expressing CD25 upon isolation from naive animals can acquire the capacity to inhibit antigen-specific T cell responses after *in vitro* stimulation.

◀ **Fig. 3.** CD4⁺CD25⁺CD134⁺ T cells suppress antigen-specific T cell responses *in vitro*. (a) Z1a responder T cells stimulated with 0.1 µg/ml MBP_{72–85} and irradiated thymus APC and co-cultured in a 1:1 ratio with naive splenic CD4⁺CD25⁺ T cells (black bar) or CD4⁺CD25⁺ T cells (white bar) were compared with stimulated Z1a T cells only (hatched bar). (b, c) CD4⁺CD25⁺CD134⁺ T cells (cross-hatched bars), CD4⁺CD25⁺CD134[−] T cells (black bars), and CD4⁺CD25[−] control T cells (white bars) were tested for their capacity to suppress the proliferation of the CD4⁺ rat T cell clones Z1a and A2b, stimulated with 0.1 µg/ml MBP_{72–85} and 0.001 µg/ml HSP65_{176–190}, respectively, and irradiated thymus APC. Proliferation without added cells (hatched bars) is compared to proliferation in co-cultures with the indicated regulatory or control T cell populations. Data in (a–c) are expressed as means ± SD of T cell [³H]thymidine incorporation (cpm) from triplicate wells. One out of three experiments is shown; * *p* < 0.05 compared to either no regulatory T cells or CD4⁺CD25⁺ T cells.

2.5 Distribution and characterization of CD4⁺CD25⁺CD134⁺ T cells in different compartments of the immune system

We compared the percentage of CD25⁺CD134⁺ T cells within the CD4⁺ fraction of spleen, blood, popliteal and brachial LN (PLN), and mucosa-draining submandibular LN (SLN). Interestingly, the relative amount of CD25⁺CD134⁺ T cells varied per compartment, with blood containing the lowest and SLN containing the highest number of these cells (Fig. 5a). Similar to SLN, also mucosa-draining mesenteric LN (MLN) and Peyer's patches (PP) contained a higher percentage of CD25⁺CD134⁺ T cells compared to the non-mucosa-draining PLN (Fig. 5b). The percentage of total CD25⁺ T cells within the CD4⁺ T cell population was not significantly higher in mucosa-draining LN compared to non-mucosa-draining LN (data not shown). Not only the percentage of CD25⁺CD134⁺ T cells, but also the expression levels of CD134 on these T cells were highest in the mucosa-draining LN (Fig. 5c). To analyze whether the CD134[−] and CD134⁺ subsets of CD4⁺CD25⁺ T cells in spleen, blood, and LN had a similar surface expression of activation markers, we tested for the expression of CD62L, CD45RC and MHC class II on these cells. All analyzed CD4⁺CD25⁺CD134⁺ T cell populations showed reduced staining for CD62L and CD45RC compared to their CD134[−] counterparts (Fig. 6a, b). In blood, CD45RC expression on CD4⁺CD25⁺CD134⁺ T cells was almost absent. The MHC class II expression on CD134⁺ and CD134[−] subsets in spleen and LN was comparable, while regulatory T cell populations in blood were mostly negative for MHC class II (Fig. 6c).

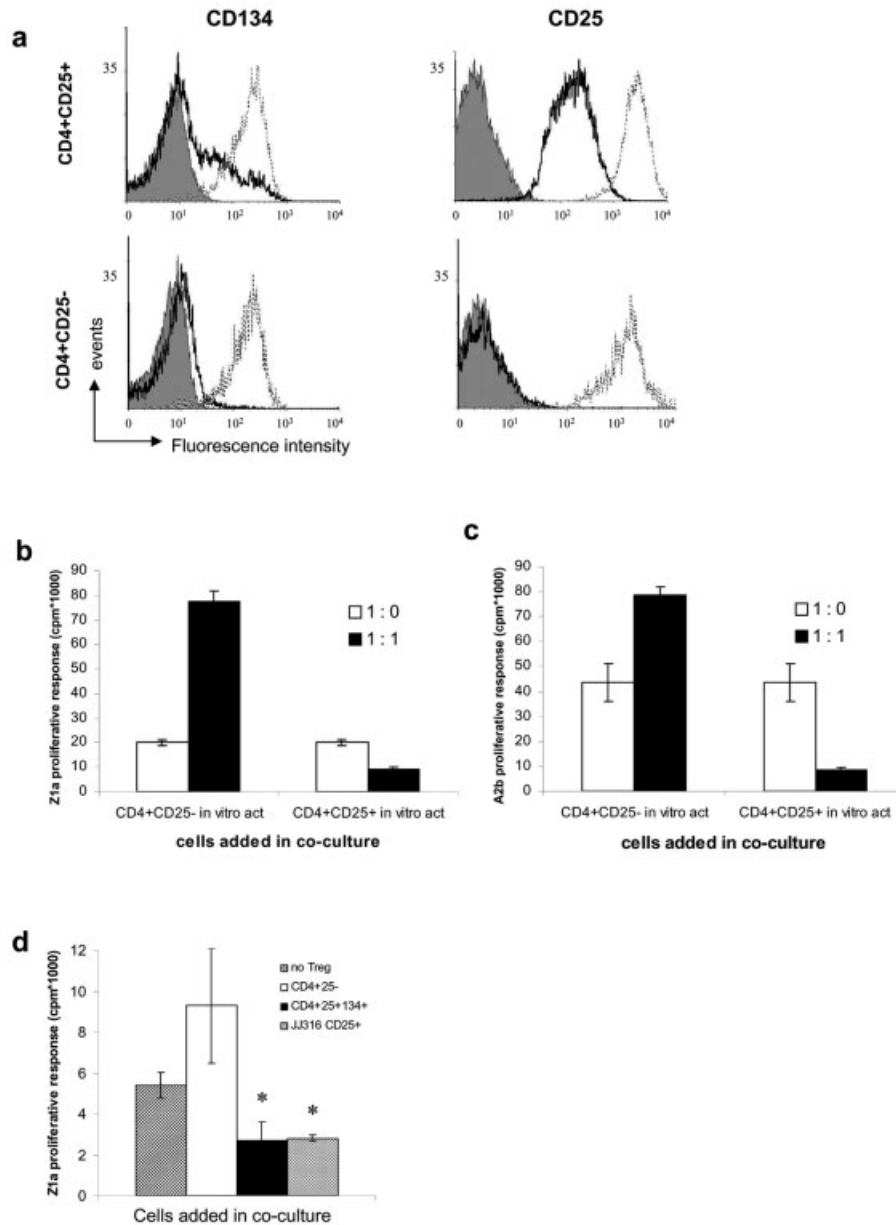


Fig. 4. CD4⁺CD25⁺ T cells, but not CD4⁺CD25⁻ T cells pre-activated *in vitro* to express CD134, suppress antigen-specific T cell responses. Sorted CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells were activated *in vitro* for 48 h with the superagonistic anti-CD28 mAb JJ316. (a) Flow cytometric analysis of CD4⁺CD25⁺ (top) and CD4⁺CD25⁻ T cells (bottom) stained for CD134 (left) and CD25 (right), before activation (black lines) and after activation (dotted lines). *In vitro* activated CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells were tested for their capacity to suppress the response of Z1a (b) and A2b (c) T cell clones to stimulatory peptide (1 µg/ml and 0.01 µg/ml, respectively) in the presence of irradiated thymus APC. Proliferation without added cells (1:0) is compared to proliferation in co-cultures (1:1) with the indicated regulatory T cell populations. Data are expressed as means ± SD of T cell [³H]thymidine incorporation (cpm) from triplicate wells. Proliferation of Z1a T cells to stimulatory peptide (0.1 µg/ml) presented by irradiated thymus cells was measured in the presence of *in vitro* activated CD4⁺CD25⁺ T cells or freshly isolated CD4⁺CD25⁺CD134⁺ T cells (d). Data are expressed as means ± SD of T cell [³H]thymidine incorporation (cpm) from triplicate wells; * *p* ≤ 0.001 compared to either no regulatory T cells or CD4⁺25⁻ T cells.

Besides the expression of specific activation markers, blast formation and down-regulation of the TCR are hallmarks of (recently) activated T cells. We tested these

two parameters for the CD134⁺ and CD134⁻ subsets of CD4⁺CD25⁺ T cells in the different organs. In all organs, CD4⁺CD25⁺CD134⁺ T cells were increased in size

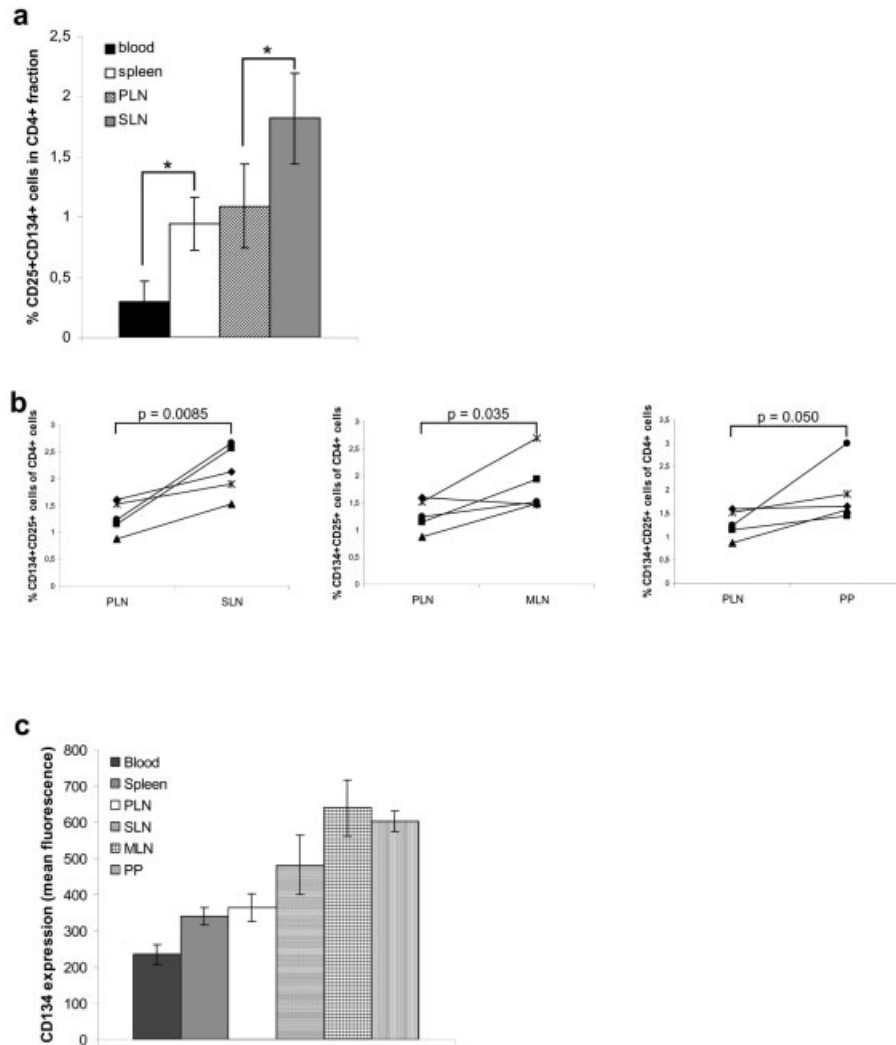


Fig. 5. CD4⁺CD25⁺CD134⁺ T cells reside in various compartments of the naive immune system. (a) Single-cell suspensions were prepared from spleen, PLN, SLN, and peripheral blood of naive rats. The percentage of CD25⁺CD134⁺ T cells within the CD4⁺ fraction was determined by flow cytometric analysis. Data are mean values $\pm$ SD of three independent experiments; * $p < 0.05$. (b) The percentage of CD25⁺CD134⁺ T cells was compared in mucosa-draining SLN, MLN, PP, and non-mucosa-draining PLN. Symbols indicate individual rats; p values were determined in a paired, one-tailed Student's t -test. (c) The CD134 expression on CD4⁺CD25⁺CD134⁺ T cells was compared for the six different lymphoid organs described in (a) and (b). Data are presented as the mean fluorescence intensity of CD134 staining within the CD25⁺CD134⁺ populations $\pm$ SD of five rats.

compared to the CD4⁺CD25⁺CD134⁻ T cells (Fig. 6d). In addition, CD4⁺CD25⁺CD134⁺ T cells in all lymphoid compartments except for blood showed a significantly decreased TCR expression compared to the CD134⁻ fraction (Fig. 6e). To investigate the *in vivo* proliferative capacity of the CD134⁺ and CD134⁻ subsets of CD4⁺CD25⁺ T cells, we assessed the incorporation of i.p.-injected 5-bromo-2'-deoxyuridine (BrdU) in these cells. In naive rats, generally 1–2% of the CD4⁺ cells were found to incorporate BrdU (Fig. 6f). Whereas the BrdU incorporation in the CD25⁺CD134⁻ fraction was low, cells in both the CD25⁺CD134⁻ and the CD25⁺CD134⁺ fraction

proliferated in each of the lymphoid compartments tested (Fig. 6g). Hence, the differences in expression of T cell activation markers on CD134⁺ versus CD134⁻ regulatory T cells are not reflected in their capacity to proliferate *in vivo*.

3 Discussion

We here demonstrate that CD4⁺CD25⁺ regulatory T cells from naive animals can be dissected, based on CD134 expression, into two populations that display different

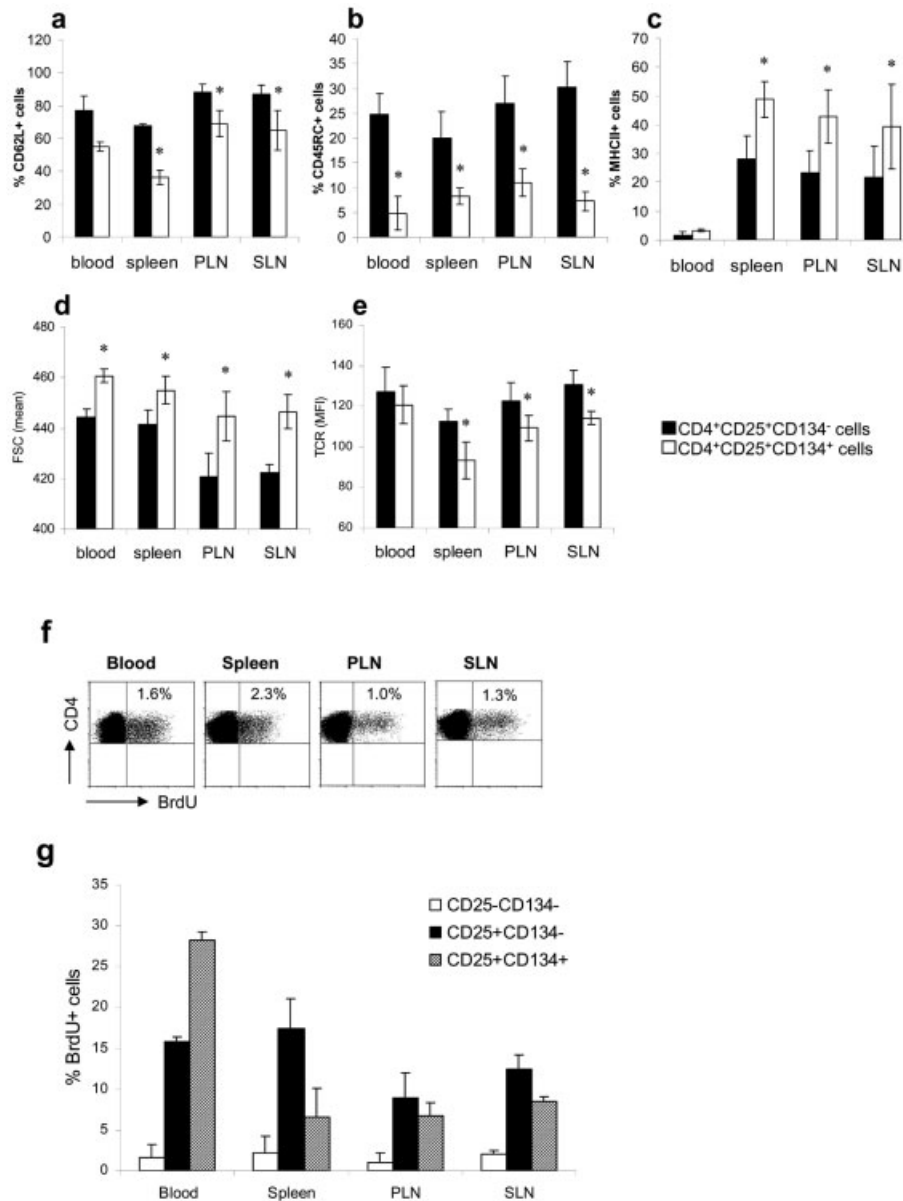


Fig. 6. CD4⁺CD25⁺CD134⁺ and CD4⁺CD25⁺CD134⁻ T cells in different compartments of the immune system have similar phenotypes. Expression of CD62L (a), CD45RC (b), and MHC class II (c) was determined on cells of various lymphoid compartments stained for CD4, CD25, and CD134. These cells were also analyzed for mean FSC height (d) and TCR expression (e). Data presented in (a–e) are the percentages of cells positive for the indicated markers within the CD4⁺CD25⁺CD134⁻ fractions (black bars) or the CD4⁺CD25⁺CD134⁺ fractions (white bars) $\pm$ SD of four to five rats. * $p \leq 0.05$, as determined in a paired, one-tailed Student's *t*-test, was considered significant. Cells of blood, spleen, popliteal LN, and SLN recovered from naive rats 24 h after injection with BrdU were stained for CD4, CD25, CD134 and incorporated BrdU. Shown are representative dot plots indicating the percentage of BrdU-positive cells within the CD4⁺ population (f). (g) The percentage of BrdU-positive cells within fractions gated as CD4⁺CD25⁻CD134⁻ (white bars), CD4⁺CD25⁺CD134⁻ (black bars), and CD4⁺CD25⁺CD134⁺ (hatched bars) cells of the different immune compartments was analyzed. Data are indicated as the mean percentage $\pm$ SD of BrdU-positive cells of three rats. Cells were gated as BrdU positive based on stainings with an isotype control Ab.

capacities to suppress antigen-specific T cell responses. CD4⁺CD25⁺CD134⁺ T cells have the capacity to inhibit antigen-specific proliferation of rat T cell clones without

additional stimulation. CD4⁺CD25⁺CD134⁻ T cells, on the contrary, only suppress T cell responses in anti-TCR Ab-stimulated co-cultures with responder T cells.

Many reports on naturally occurring CD4⁺CD25⁺ regulatory T cells are conclusive on the prerequisite of *in vitro* TCR triggering of these cells for efficient suppression of T cell responses (reviewed in [2, 17]). The CD134⁺ subset of freshly isolated CD4⁺CD25⁺ T cells has an activated phenotype, and as such, our data fit into the concept that only activated CD4⁺CD25⁺ T cells can efficiently suppress T cell responses. In rats, we observed a split staining pattern for CD134 expression on CD4⁺CD25⁺ T cells, and similar CD134 expression patterns have been described in humans and mice [13, 18]. The lower percentage of CD62L⁻ and CD45RC⁻ positive cells and the higher amount of MHC class II-positive cells within the CD134⁺ regulatory T cell population indicate a higher activation state compared to their CD134⁻ counterpart. Although we could not distinguish CD134⁺ and CD134⁻ regulatory T cells *in vivo* based on their proliferative response, our observation that CD4⁺CD25⁺CD134⁺ T cells are larger and have a lower TCR expression compared to CD4⁺CD25⁺CD134⁻ T cells suggests blast formation and TCR down-regulation as a result of recent activation. Our data are corroborated by a recent paper, in which phenotypic analysis of CD62L^{hi} and CD62L^{lo} regulatory T cells revealed the presence of an activated and a resting regulatory T cell population in naive mice [18]. Thus, two subsets of regulatory T cells are present in rodents: activated CD4⁺CD25⁺CD134⁺CD62L^{lo} regulatory T cells and resting/steady state CD4⁺CD25⁺CD134⁻CD62L^{hi} regulatory T cells. Supplementary, our data demonstrate that a functional difference exists between these two regulatory T cell populations, *i.e.* the activated CD4⁺CD25⁺CD134⁺CD62L^{lo} regulatory T cells, just like *in vitro* pre-activated CD4⁺CD25⁺ T cells, promptly suppress antigen-specific T cell responses, whereas the steady state regulatory T cells need to be activated first. Earlier attempts to dissect the suppressive capacity of CD4⁺CD25⁺ T cells on the basis of high and low CD62L expression have failed. In co-cultures with CD4⁺CD25⁻ responder T cells stimulated with anti-CD3 mAb, CD62L^{lo} and CD62L^{hi} CD4⁺CD25⁺ T cell populations showed similar degrees of suppression [6, 8]. However, these findings can be explained by the fact that culture with anti-CD3 mAb induced activation of both suppressor and responder cells. We found that CD134⁺ regulatory T cells suppressed polyclonally stimulated CD4⁺CD25⁻ primary responder T cells to a higher extent than antigen-specifically stimulated clonal T cells. This suggests that naive T cells are more susceptible to suppression by regulatory T cells than antigen-experienced T cells.

CD4⁺CD25⁺ T cells pre-activated *in vitro* with the superagonistic anti-CD28 mAb JJ316 acquired the capacity to suppress antigen-specific T cell responses. As described previously for the phenotype of CD4⁺CD25⁺ T cells

before and after *in vitro* activation [19], we observed that JJ316 stimulation leads to increased, homogeneous expression of CD134 on CD4⁺CD25⁺ T cells. Also the non-suppressive CD4⁺CD25⁻ T cells express CD134 after incubation with JJ316. CD134, like many other markers found on CD4⁺CD25⁺ T cells, is therefore not directly associated with the regulatory function of CD4⁺CD25⁺ T cells, but merely reflects the activation state of the cells. Furthermore, the expression of CD25 on activated CD4⁺CD25⁺ T cells was higher compared to CD25 on activated formerly CD25⁻CD4⁺ T cells. The latter finding corresponds to earlier observations in mice [8] and to the observation that in human peripheral blood, only CD25^{bright} T cells are suppressive [11]. Since both *in vitro* activated total CD4⁺CD25⁺ T cells and CD4⁺CD25⁺ T cells expressing CD134 upon isolation suppress antigen-specific T cell responses, we propose that CD134 expression on freshly isolated regulatory T cells is a result of recent stimulation *in vivo* and predicts their capacity to suppress antigen-specific T cell responses without additional stimulation.

The presence of regulatory T cells with an activated phenotype in naive animals could be the result of (repetitive) encounter with stimulatory ligands. Such stimulatory ligands could be tissue antigens, since the repertoire of CD4⁺CD25⁺ T cells was suggested to be skewed towards recognition of self-antigens [20, 21]. Alternatively, CD4⁺CD25⁺ T cell induction and activation could occur in the periphery in response to antigen presented in a "tolerogenic" form (*e.g.* in the presence of IL-10) or via a "tolerogenic" route [*e.g.* oral intake of (harmless) antigen] (reviewed in [22, 23]). Moreover, CD4⁺CD25⁺ T cells can acquire a more activated and suppressive phenotype as a result of MHC class II-driven homeostatic proliferation [24]. The origin of the isolated CD4⁺CD25⁺CD134⁺ T cells and the type of activation stimulus they received *in vivo* is currently under investigation.

Regarding the functional diversity of different compartments of the immune system, it is conceivable that (subsets of) regulatory T cells are distributed accordingly. Indeed, we found that mucosa-draining LN harbor a higher relative amount of CD4⁺CD25⁺CD134⁺ T cells, compared to spleen, blood and non-mucosa-draining LN. In addition, CD4⁺CD25⁺CD134⁺ T cells found in mucosa-draining LN showed the highest CD134 expression level. Mucosa-draining LN have an important role in induction of tolerance to innocuous antigens via the peripheral generation of regulatory T cells [25–29]. We propose that this strong immune regulatory function is reflected in the elevated number of activated CD134⁺ regulatory T cells found in these LN. Although a specific pattern of chemokine receptor expression has been

detected on CD4⁺CD25⁺ T cells [30], it is not known whether these cells continuously circulate via the bloodstream or whether antigen-experienced CD4⁺CD25⁺ T cells are retained within draining LN to exert their suppressive function at these locations [31, 32]. Although the number of CD4⁺CD25⁺CD134⁺ T cells found in blood was low (0.3% of CD4⁺ cells), the proliferative capacity of these cells was highest of all CD134⁺ or CD134[−] regulatory T cell subsets tested. This could imply that (some) regulatory T cells leave the lymphoid organs after being stimulated to proliferate and to express CD134. How proliferation and expression of activation markers relate to the migratory behavior of regulatory T cells and their effector site remains to be investigated.

In conclusion, we have shown that CD134 can be used to identify regulatory T cells committed to suppress T cell responses without additional stimuli. Since relatively high numbers of CD4⁺CD25⁺CD134⁺ T cells are present in LN draining mucosal sites, the local presence of these cells may discriminate sites of active immune regulation.

4 Materials and methods

4.1 Rats, T cell clones and peptides

Male inbred 7–10-week-old Lewis rats were obtained from Maastricht University (Maastricht, The Netherlands) and kept under conventional conditions. Isolation, maintenance and properties of the CD4⁺ T cell clones A2b [33] and Z1a [34] have been described previously. Briefly, T cell clone A2b was derived from LN of a Lewis rat immunized with *Mycobacterium tuberculosis* (Mt) in IFA and recognizes the mycobacterial 65-kDa heat shock protein (HSP65) peptide 176–190 (HSP_{176–190}) presented by the MHC class II molecule RT1.B^L. The T cell clone Z1a was derived from draining LN of a Lewis rat immunized with guinea pig myelin basic protein (MBP) in CFA and recognizes peptide 72–85 (MBP_{72–85}) presented by RT1.B^L. HSP_{176–190} and acetylated MBP_{72–85} [35] were obtained from Isogen Bioscience (Maarsse, The Netherlands).

4.2 Flow cytometric analysis

Single-cell suspensions of naive rat spleens and various LN were obtained by forcing the organs through a 70-μm mesh. Lymphocytes from peripheral blood and spleen cell suspensions were isolated by Ficoll-Hypaque gradient centrifugation. Cells (7 × 10⁵ cells/sample) were incubated for 30 min on ice with mAb in PBS containing 1% BSA (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands), 2% normal rat serum (NRS) and 0.1% NaN₃. To characterize CD4⁺CD25⁺ T cells, lymphocytes were labeled with Cychrome-labeled

OX35 (anti-CD4 Ab), PE-labeled OX39 (anti-CD25 Ab) and biotinylated anti-CD134 Ab/streptavidin-APC (all from BD-PharMingen, San Diego, CA). In addition, cells were stained with FITC-labeled mouse IgG1 or hamster Ig isotype control, HRL1 (anti-CD62L Ab), OX22 (anti-CD45RC Ab), OX6 (anti-MHC class II Ab), or R73 (anti-TCR Ab) (BD PharMingen). Samples were measured using a FACSCalibur and CellQuest software (Becton Dickinson, San Jose, CA). Percentages of CD25⁺CD134⁺ cells within the CD4⁺ fractions of the different lymphoid organs were compared using a paired Student's *t*-test; *p* < 0.05 was considered significant.

4.3 Isolation of CD4⁺ T cell subsets and T cell-depleted spleen cells

Lymphocytes isolated from single-cell suspensions of naive rat spleens by Ficoll-Hypaque gradient centrifugation were incubated in PBS containing 5% FCS and 4% NRS and stained with FITC-labeled OX8 (anti-CD8 Ab) and OX33 (anti-CD45RA Ab), PE-labeled OX39 (anti-CD25 Ab), biotinylated OX40 (anti-CD134 Ab) and streptavidin-APC (BD PharMingen). FITC-negative CD4⁺ T cells were sorted into CD25[−], CD25⁺, CD25⁺CD134[−] and CD25⁺CD134⁺ fractions using a FACS Vantage SE (Becton Dickinson) and CellQuest software. T cell-depleted spleen cells were obtained by magnetic separation with MACS (Miltenyi Biotec, Auburn, CA) according to the manufacturer's procedure, using biotinylated R73 (anti-TCR Ab) and streptavidin MACS beads.

4.4 Analysis of suppression in *in vitro* lymphocyte proliferation assays

Proliferation of responder T cells was measured in 96-well plates (Costar, Corning, NY) in triplicate cultures. Proliferation assays were performed using culture medium [Isocove's Modified Dulbecco's Medium (IMDM) supplemented with L-glutamine (2 mM), 2-ME (50 μM), penicillin (50 U/ml), streptomycin (50 U/ml)] containing 2% heat-inactivated NRS.

For antigen-nonspecific suppression assays, wells were coated with goat anti-mouse Ig (100 μg/ml, Sigma-Aldrich, St. Louis, MO) for 2 h at room temperature (RT), washed, incubated with 2 μg/ml purified R73 (anti-TCR Ab) for 1 h at RT and washed again. CD4⁺CD25[−] responder T cells (2 × 10⁴) were cultured in 200 μl culture medium, in the presence of 4 × 10⁴ irradiated (30 Gy) T cell-depleted spleen cells and 2 × 10⁴ sorted CD4⁺CD25⁺ suppressor T cells for 72 h. For antigen-specific suppression assays, T cell clones A2b or Z1a were used as responder T cells. A2b or Z1a cells (2 × 10⁴/per well) were cultured in 200 μl culture medium in the presence of irradiated (30 Gy) thymus cells as the source of APC (1 × 10⁶), the specific peptide, and different amounts of sorted CD4⁺CD25⁺ suppressor T cells or CD4⁺CD25[−] control T cells. Suppression was analyzed in the linear part of the

antigen dose-response curve of the T cell clones. After 3 days of culture, proliferation was determined by overnight incorporation of [^{3} H]thymidine (0.4 μ Ci per well; Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands). Results are expressed as the mean counts per minute (cpm) of triplicate cultures $\pm$ SD. For the analysis of differences in the suppressive capacity of the different T cell populations, an unpaired Student's *t*-test was used; $p < 0.05$ was considered statistically significant. Alternatively, CD4⁺CD25⁻ responder T cells were labeled before co-culture with CFSE (Molecular Probes, Inc, Eugene, OR) at a final concentration of 0.5 μ M in PBS for 10 min at RT. CFSE cell division patterns were measured after 3 days of culture, using a FACSCalibur and CellQuest software (Becton Dickinson).

4.5 *In vitro* activation of CD4⁺CD25⁺ T cells

Sorted CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells were stimulated with the superagonistic anti-CD28 mAb JJ316, as described by Tacke et al. [36]. Briefly, 24-well plates (Greiner, Bio-one b.v., Alphen a/d Rijn, The Netherlands) were coated with sheep anti-mouse Ig (0.5 mg/ml, Roche Biochemicals) for 1 h at 37°C. After extensive washing of the wells with PBS/1%BSA, CD4⁺CD25⁺ or CD4⁺CD25⁻ cells (2.5×10^5 /ml) were added to the wells together with JJ316 (5 μ g/ml) and cultured for 72 h in culture medium supplemented with 10% FCS (Bodinco B.V., The Netherlands).

4.6 BrdU labeling and detection by flow cytometry

Rats were injected i.p. with BrdU (33 mg/kg body weight; Becton Dickinson). At 24 h after injection, cell suspensions of blood, spleen, popliteal LN and SLN were stained for CD4, CD25 and CD134, and thereafter stained for incorporated BrdU according to the manufacturer's procedure (FITC BrdU Flow Kit, Becton Dickinson). BrdU incorporation in CD4⁺ cells gated according to CD25 and/or CD134 expression was measured by flow cytometry. FITC-labeled mouse IgG1 (Becton Dickinson) was used as isotype control for the FITC-labeled anti-BrdU Ab.

References

- 1 Sakaguchi, S., Regulatory T cells: key controllers of immunologic self-tolerance. *Cell* 2000. **101**: 455–458.
- 2 Shevach, E. M., CD4⁺ CD25⁺ suppressor T cells: more questions than answers. *Nat. Rev. Immunol.* 2002. **2**: 389–400.
- 3 Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. and Toda, M., Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α -chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* 1995. **155**: 1151–1164.
- 4 Thornton, A. M. and Shevach, E. M., CD4⁺CD25⁺ immunoregulatory T cells suppress polyclonal T cell activation *in vitro* by inhibiting interleukin 2 production. *J. Exp. Med.* 1998. **188**: 287–296.
- 5 Takahashi, T., Kuniyasu, Y., Toda, M., Sakaguchi, N., Itoh, M., Iwata, M., Shimizu, J. and Sakaguchi, S., Immunologic self-tolerance maintained by CD25⁺CD4⁺ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int. Immunol.* 1998. **10**: 1969–1980.
- 6 Thornton, A. M. and Shevach, E. M., Suppressor effector function of CD4⁺CD25⁺ immunoregulatory T cells is antigen nonspecific. *J. Immunol.* 2000. **164**: 183–190.
- 7 George, T. C., Bilsborough, J., Viney, J. L. and Norment, A. M., High antigen dose and activated dendritic cells enable Th cells to escape regulatory T cell-mediated suppression *in vitro*. *Eur. J. Immunol.* 2003. **33**: 502–511.
- 8 Kuniyasu, Y., Takahashi, T., Itoh, M., Shimizu, J., Toda, G. and Sakaguchi, S., Naturally anergic and suppressive CD25(+)CD4(+) T cells as a functionally and phenotypically distinct immunoregulatory T cell subpopulation. *Int. Immunol.* 2000. **12**: 1145–1155.
- 9 Lehmann, J., Huehn, J., de la Rosa, M., Maszyra, F., Kretschmer, U., Krenn, V., Brunner, M., Scheffold, A. and Hamann, A., Expression of the integrin α E β 7 identifies unique subsets of CD25⁺ as well as CD25⁻ regulatory T cells. *Proc. Natl. Acad. Sci. USA* 2002. **99**: 13031–13036.
- 10 Jonuleit, H., Schmitt, E., Stassen, M., Tuettenberg, A., Knop, J. and Enk, A. H., Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. *J. Exp. Med.* 2001. **193**: 1285–1294.
- 11 Baecher-Allan, C., Brown, J. A., Freeman, G. J. and Hafler, D. A., CD4⁺CD25^{high} regulatory cells in human peripheral blood. *J. Immunol.* 2001. **167**: 1245–1253.
- 12 Fowell, D. and Mason, D., Evidence that the T cell repertoire of normal rats contains cells with the potential to cause diabetes. Characterization of the CD4⁺ T cell subset that inhibits this autoimmune potential. *J. Exp. Med.* 1993. **177**: 627–636.
- 13 Taams, L. S., Smith, J., Rustin, M. H., Salmon, M., Poulter, L. W. and Akbar, A. N., Human anergic/suppressive CD4(+)CD25(+) T cells: a highly differentiated and apoptosis-prone population. *Eur. J. Immunol.* 2001. **31**: 1122–1131.
- 14 Ng, W. F., Duggan, P. J., Ponchel, F., Matarese, G., Lombardi, G., Edwards, A. D., Isaacs, J. D. and Lechler, R. I., Human CD4(+)CD25(+) cells: a naturally occurring population of regulatory T cells. *Blood* 2001. **98**: 2736–2744.
- 15 Wing, K., Ekmark, A., Karlsson, H., Rudin, A. and Suri-Payer, E., Characterization of human CD25⁺ CD4⁺ T cells in thymus, cord and adult blood. *Immunology* 2002. **106**: 190–199.
- 16 Lin, C. H. and Hunig, T., Efficient expansion of regulatory T cells *in vitro* and *in vivo* with a CD28 superagonist. *Eur. J. Immunol.* 2003. **33**: 626–638.
- 17 Sakaguchi, S., Sakaguchi, N., Shimizu, J., Yamazaki, S., Sakihama, T., Itoh, M., Kuniyasu, Y., Nomura, T., Toda, M. and Takahashi, T., Immunologic tolerance maintained by CD25⁺ CD4⁺ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol. Rev.* 2001. **182**: 18–32.
- 18 Fisson, S., Darrasse-Jeze, G., Litvinova, E., Septier, F., Klatzmann, D., Liblau, R. and Salomon, B. L., Continuous activation of autoreactive CD4⁺ CD25⁺ regulatory T cells in the steady state. *J. Exp. Med.* 2003. **198**: 737–746.
- 19 McHugh, R. S., Whitters, M. J., Piccirillo, C. A., Young, D. A., Shevach, E. M., Collins, M. and Byrne, M. C., CD4(+)CD25(+) immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity* 2002. **16**: 311–323.
- 20 Jordan, M. S., Boesteanu, A., Reed, A. J., Petrone, A. L., Hohenbeck, A. E., Lerman, M. A., Naji, A. and Caton, A. J.,

- Thymic selection of CD4⁺CD25⁺ regulatory T cells induced by an agonist self-peptide. *Nat. Immunol.* 2001. **2**: 301–306.
- 21 Seddon, B. and Mason, D., Peripheral autoantigen induces regulatory T cells that prevent autoimmunity. *J. Exp. Med.* 1999. **189**: 877–882.
 - 22 von Herrath, M. G. and Harrison, L. C., Antigen-induced regulatory T cells in autoimmunity. *Nat. Rev. Immunol.* 2003. **3**: 223–232.
 - 23 Akbar, A. N., Taams, L. S., Salmon, M. and Vukmanovic-Stejic, M., The peripheral generation of CD4⁺ CD25⁺ regulatory T cells. *Immunology* 2003. **109**: 319–325.
 - 24 Gavin, M. A., Clarke, S. R., Negrou, E., Gallegos, A. and Rudensky, A., Homeostasis and anergy of CD4(+)CD25(+) suppressor T cells *in vivo*. *Nat. Immunol.* 2002. **3**: 33–41.
 - 25 Faria, A. M. and Weiner, H. L., Oral tolerance: mechanisms and therapeutic applications. *Adv. Immunol.* 1999. **73**: 153–264.
 - 26 Thorstenson, K. M. and Khoruts, A., Generation of anergic and potentially immunoregulatory CD25⁺CD4⁺ T cells *in vivo* after induction of peripheral tolerance with intravenous or oral antigen. *J. Immunol.* 2001. **167**: 188–195.
 - 27 Akbari, O., DeKruyff, R. H. and Umetsu, D. T., Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat. Immunol.* 2001. **2**: 725–731.
 - 28 Unger, W. W., Jansen, W., Wolvers, D. A., van Halteren, A. G., Kraal, G. and Samsom, J. N., Nasal tolerance induces antigen-specific CD4⁺CD25⁺ regulatory T cells that can transfer their regulatory capacity to naive CD4⁺ T cells. *Int. Immunol.* 2003. **15**: 731–739.
 - 29 Prakken, B. J., Roord, S., van Kooten, P. J., Wagenaar, J. P., van Eden, W., Albani, S. and Wauben, M. H., Inhibition of adjuvant-induced arthritis by interleukin-10-driven regulatory cells induced via nasal administration of a peptide analog of an arthritis-related heat-shock protein 60 T cell epitope. *Arthritis Rheum.* 2002. **46**: 1937–1946.
 - 30 Iellem, A., Mariani, M., Lang, R., Recalde, H., Panina-Bordignon, P., Sinigaglia, F. and D'Ambrosio, D., Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4(+)CD25(+) regulatory T cells. *J. Exp. Med.* 2001. **194**: 847–853.
 - 31 Belkaid, Y., Piccirillo, C. A., Mendez, S., Shevach, E. M. and Sacks, D. L., CD4⁺CD25⁺ regulatory T cells control *Leishmania* major persistence and immunity. *Nature* 2002. **420**: 502–507.
 - 32 Walker, L. S., Chodos, A., Eggens, M., Dooms, H. and Abbas, A. K., Antigen-dependent proliferation of CD4⁺ CD25⁺ regulatory T cells *in vivo*. *J. Exp. Med.* 2003. **198**: 249–258.
 - 33 Holoshitz, J., Matitiaou, A. and Cohen, I. R., Arthritis induced in rats by cloned T lymphocytes responsive to mycobacteria but not to collagen type II. *J. Clin. Invest.* 1984. **73**: 211–215.
 - 34 Ben-Nun, A., Wekerle, H. and Cohen, I. R., The rapid isolation of clonable antigen-specific T lymphocyte lines capable of mediating autoimmune encephalomyelitis. *Eur. J. Immunol.* 1981. **11**: 195–199.
 - 35 de Haan, E. C., Wauben, M. H., Wagenaar-Hilbers, J. P., Grosfeld-Stulemeyer, M. C., Rijkers, D. T., Moret, E. E. and Liskamp, R. M., Stabilization of peptide guinea pig myelin basic protein 72–85 by N-terminal acetylation – implications for immunological studies. *Mol. Immunol.* 2004. **40**: 943–948.
 - 36 Tacke, M., Hanke, G., Hanke, T. and Hunig, T., CD28-mediated induction of proliferation in resting T cells *in vitro* and *in vivo* without engagement of the T cell receptor: evidence for functionally distinct forms of CD28. *Eur. J. Immunol.* 1997. **27**: 239–247.

Correspondence: Marca H. M. Wauben, Department of Immunohematology and Blood Transfusion, E3-Q, Leiden University Medical Center, Albinusdreef 2, P.O. Box 9600, 2300 RC Leiden, The Netherlands
 Fax: +31-71-5216751
 e-mail: M.H.M.Wauben@lumc.nl

Present addresses: E. N. M. Nolte-‘t Hoen, Faculty of Biological Sciences, Imperial College London, South Kensington Campus, London SW7 2AZ, UK; E. P. J. Boot, Genmab, Utrecht, The Netherlands