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Cutting Edge

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Cutting Edge: CD4-Independent Development of Functional FoxP3$^+$ Regulatory T Cells[1]

Céline Blache,* Sahil Adriouch,* Sébastien Calbo,* Laurent Drouot,* Sophie Dulauroy,[2‡] Christophe Arnoult,* Stéphanie Le Corre,*† Adrien Six,[3‡] Michel Seman,* and Olivier Boyer[4*†]

The CD4 coreceptor is mandatory for the differentiation and function of conventional MHC class II-restricted T cells, but little is known about its contribution in regulatory T cells (Tregs). We thus investigated the Treg compartment in mice lacking CD4. CD3$^+$CD8$^-$ FoxP3$^+$ cells were readily detected in the periphery of CD4$^{-/-}$ mice, where their percentages were even increased as compared with wild-type animals. These cells had a classical CD25$^+$CD152$^+$GITR$^+$ Treg phenotype, were enriched in memory-type Tregs, and displayed a diversified TCR repertoire. Functionally, CD4$^{-/-}$ Tregs were equally as suppressive as CD4$^{+/+}$ Tregs in vitro as well as in vivo. Hence, the CD4 coreceptor is dispensable for the generation and function of FoxP3$^+$ Tregs. Furthermore, CD3$^+$CD8$^-$ FoxP3$^+$ Tregs were also found to develop in the absence of both CD4 and MHC-II molecules, demonstrating that the generation of Tregs can occur independently of MHC-II recognition. *The Journal of Immunology,* **2009, 183: 4182–4186.**

Among the different T cell subsets with regulatory activity, naturally occurring CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells (Tregs)[5] play an essential role in the control of immune responses (1, 2). Despite accumulating evidence illustrating the importance of the suppressive functions mediated by Tregs, the factors that influence their selection and development are still poorly known. Results obtained in double-TCR/Ag transgenic mice have suggested that high-affinity interactions with a single self-peptide may induce thymocytes bearing an autoreactive TCR to be selected to become CD4$^+$ CD25$^+$ Tregs (3). In line with this view, autoantigen expression on the thymic medulla was shown to favor Treg development (4, 5). This has led to the widely accepted paradigm that Tregs are autoreactive T cells whose selection is the result of high-avidity TCR/MHC class II interactions in the thymus (3–5). Yet, an alternative interpretation of the results of double-TCR/Ag transgenic experiments is that Tregs would be highly resistant to clonal deletion, which would favor their survival in conditions where conventional CD4$^+$ T cells (Tconv) are counterselected (6). Recently, it was also shown that *trans*-conditioning factors expressed by double-positive thymocytes may negatively influence Treg selection in the thymus (7). By linking the size of the Treg compartment to that of Tconv precursors, such a mechanism may explain the apparent Treg enrichment observed in models with reduced thymic cellularity such as pre-TCRα-deficient mice (7).

The CD4 coreceptor plays essential roles in the development and function of CD4$^+$ Tconv cells. Its extracellular domain interacts with MHC class II molecules and thus increases the avidity of the TCR/MHC class II interaction. Also, its cytoplasmic domain interacts with the p56^{lck} kinase, which is a key event in the initiation of TCR signaling (8). Therefore, CD4-deficient (CD4$^{-/-}$) mice have a profound defect in the development of the Tconv cell population that normally expresses CD4 and displays strongly reduced functional helper activity (9, 10). In contrast, little is known about the CD4 requirements for Treg development and function. Hence, we investigated how the Treg compartment would be affected by the absence of CD4.

Materials and Methods

Mice

CD4$^{-/-}$, CD3$^{-/-}$, I-A$\beta^{-/-}$, and MHC-II$^{\Delta/\Delta}$ mice (all of the B6 background) were described previously (10–12).

Flow cytometry

Fluorescently conjugated mAbs directed against CD3 (145-2C11), CD4 (L3T4), CD8 (Ly2 53-6.7), CD25 (PC61), CD44 (IM7), I-A/I-E (AF6–120.1), and Ki-67 (B56) from BD Biosciences or to CD62L (MEL-14), CD103 (M290), CD152 (UC10-4B9), GITR (DTA-1), TCRδ (UC7–13D5),

*INSERM, Unité 905, Rouen, France; University of Rouen, Institut Fédératif de Recherches Multidisciplinaires sur les Peptides, Institute for Biomedical Research, Rouen, France; †Rouen University Hospital, Department of Immunology, Rouen, France; ‡Université Pierre et Marie Curie-Paris 6, Centre National de la Recherche Scientifique Unité de Recherche Associée 1961, Immunophysiopathologie Infectieuse, Institut Pasteur, Paris, France

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[2] Current address: Développement des Tissus Lymphoïdes, Institut Pasteur, Paris, France.

[3] Current address: Unité Mixte de Recherche 7211 Centre National de la Recherche Scientifique and Université Pierre et Marie Curie, I2D3 Team "Integrative Immunology," Bâtiment CERVI, 83 Boulevard de l'Hôpital, F-75013 Paris, France.

[4] Address correspondence and reprint requests to Dr. Olivier Boyer, INSERM, Unité 905, 22 Boulevard Gambetta, F-76000 Rouen, France. E-mail address: olivier.boyer@chu-rouen.fr

[5] Abbreviations used in this paper: Treg, regulatory T cell; IBD, inflammatory bowel disease; LN, lymph node; Tconv, conventional T cell.

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TCRβ (H57-597), and MHC-II (ME/114.15.2) from eBioscience were used. V_β usage was determined using biotinylated anti-Vβ2 (B20.6), anti-Vβ3 (KJ25), anti-Vβ4 (KT4), anti-Vβ6 (RR4-7), anti-Vβ8 (KJ16), anti-Vβ10 (KT10b), and anti-Vβ11 (KT11) staining followed by PE-conjugated streptavidin (eBioscience). Intracellular FoxP3 (FJK-16s) staining was performed following the manufacturer's protocol (eBioscience). Analyses were performed on a FACSCalibur or FACSCanto flow cytometer (BD Biosciences) using the FlowJo software.

Immunoscope analysis

Lymph node (LN) cells were enriched using a CD4 negative isolation kit (Invitrogen) and stained with anti-CD8, anti-CD25, and anti-CD3 Abs. $CD3^+CD8^-CD25^+$ or $CD3^+CD8^-CD25^-$ cells were sorted (purity $\geq$ 97%) using a FACSAria flow cytometer (BD Biosciences). Total RNA was extracted from 2–10 $\times$ 10^6 sorted cells using TRIzol (Sigma-Aldrich). Protocols for Vβ-Cβ RT-PCR and gel analysis were described elsewhere (13). Immunoscope software was used to obtain CDR3 length profiles.

Functional Treg assays in vitro

LN cells were enriched using a CD4 negative isolation kit, yielding a $CD3^+CD8^-$ population (purity $\geq$ 98%). Then, $CD25^-$ (purity $\geq$90%) and $CD25^+$ (purity $\geq$89%) cells were isolated using PE-labeled anti-CD25 staining followed by immunomagnetic sorting with anti-PE beads (Miltenyi Biotec).

$CD3^+CD8^-CD25^+$ cells were added at different ratios to $CD4^+CD25^-$ responder cells (5 $\times$ 10^4) from B6 mice. Cells were activated for 72 h by 2.5 μg/well Con A in the presence of 5 $\times$ 10^4 splenocytes from syngeneic $CD3^{-/-}$ mice and pulsed with 1 μCi/well [^{3}H]TdR for the last 24 h of culture.

Alternatively, $CD3^+CD8^-CD25^+$ or $CD3^+CD8^-CD25^-$ cells (1 $\times$ 10^5) were cultured separately for 4 days with anti-CD28/CD3 Dynal beads (Invitrogen) before IL-2 was assayed in supernatants on a Bio-Plex/Luminex apparatus (Bio-Rad).

Functional Treg assay in vivo

FACS-purified $CD3^+CD8^-CD25^-$ T cells (1 $\times$ 10^6) from $CD4^{+/+}$ mice supplemented or not with 0.2 $\times$ 10^6 FACS-purified $CD3^+CD8^-CD25^+$ Tregs (purity $\geq$ 97%) from $CD4^{+/+}$ or $CD4^{-/-}$ mice were injected i.v. into B6 $CD3^{-/-}$ recipients. The weight curve was monitored for 7 wk after cell transfer. Then, colons were removed and 5-μm paraffin-embedded sections stained with hematoxylin/eosin/saffron.

RNA preparation and RT-PCR

Total cellular RNA was extracted from sorted $CD3^+CD8^-CD25^+$ or $CD3^+CD8^-CD25^-$ cells using TRIzol (Invitrogen). The Moloney murine leukemia virus enzyme was used to synthesize cDNA from 500 μg of RNA. Primers for PCR were murine cKrox-forward (5′-ACATGAGGACCCA CACTGGTG-3′) and murine cKrox-reverse (5′-CTTCCTCTTCCTCC TCCTCAG-3′).

Results and Discussion

$FoxP3^+$ T cells develop despite the absence of CD4

In mice that lack expression of the CD4 glycoprotein, T cells of the CD4 lineage are identified according to their $CD3^+CD8^-$ "CD4-like" phenotype (14). As expected from previous reports (9, 10), the frequency of CD4-like T cells recovered from peripheral lymphoid organs was dramatically reduced in $CD4^{-/-}$ mice; $CD3^+CD8^-$ T cells represented <10% of LN mononuclear cells as compared with >30% in $CD4^{+/+}$ mice (Fig. 1*A*), corresponding to a ~70% reduction in absolute numbers (Fig. 1*B*). To further study the $CD3^+CD8^-$ T cell subset found in $CD4^{-/-}$ mice, we investigated the expression of the CD4-specific transcription factor cKrox (also called ThPOK) (15). Similarly as MHC class-II restricted $CD4^+$ OT-II but not MHC class-I restricted $CD8^+$ OT-I TCR-transgenic T cells, cKrox was expressed by $CD3^+CD8^-$ T cells from $CD4^{+/+}$ as well as $CD4^{-/-}$ mice (Fig. 1*C*).

We next investigated whether the Treg compartment would be similarly affected by the lack of CD4. In agreement with the healthy status of $CD4^{-/-}$ mice that do not exhibit obvious autoimmune manifestation, $FoxP3^+$ cells were readily found within the thymi and LNs of mice lacking CD4 and mainly corresponded to the cells expressing CD25 (Fig. 1*A*). Analysis of thymocytes from animals of different ages shows that the percentages of $FoxP3^+$ within the $CD3^+CD8^-$ subset represent ~3–4% throughout the 40-wk observation period, whereas their total numbers tend to decline in aged mice (supplemental Fig. 1).[6] In 4- to 5-wk-old mice, the numbers of $CD3^+CD8^-$ $FoxP3^+$ thymocytes were reduced 4-fold in $CD4^{-/-}$ mice as compared with $CD4^{+/+}$ mice, whereas the conventional $FoxP3^-$ thymic compartment was 3-fold diminished (Fig. 1*B*). Thus, the genetic deletion of the CD4 coreceptor affects the development of both conventional and regulatory CD4-like T

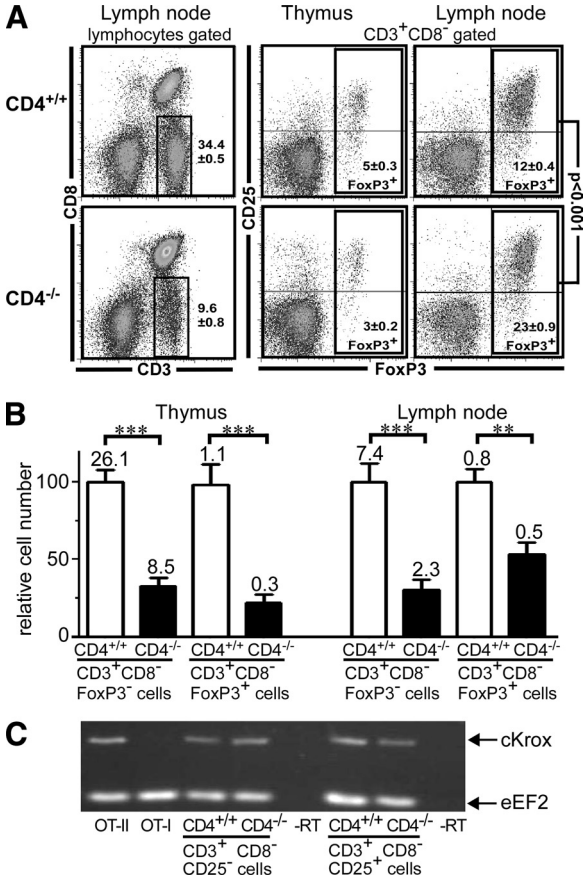

FIGURE 1. Increased frequency of $FoxP3^+$ cells within the $CD3^+CD8^-$ population in the LNs of $CD4^{-/-}$ mice. *A*, Thymocytes and LN cells from 4- to 5-wk-old $CD4^{-/-}$ (n = 14) or $CD4^{+/+}$ (n = 12) mice were stained with FITC anti-CD8, PE anti-FoxP3, PerCP anti-CD25, and allophycocyanin anti-CD3. Dot plots display the results of flow cytometry analysis after gating on lymphocytes based on forward and side scatter (*left*) or on $CD3^+CD8^-$ cells (*right*). Numbers indicate the frequency (mean ± SEM) of $CD3^+CD8^-$ cells within the lymphocyte gate (*left*) or the frequency of $FoxP3^+$ cells within the $CD3^+CD8^-$ gate (*right*). *B*, Histograms indicate normalized mean cell numbers in $CD4^{-/-}$ mice relative to $CD4^{+/+}$ mice taken as 100% (error bars represent SEM). The values indicated above the histograms are mean absolute cell numbers ($\times 10^6$) of $CD3^+CD8^-FoxP3^+$ or $CD3^+CD8^-FoxP3^-$ cells within the thymus (*left*) or 12 pooled LNs (*right*). **, $p < 0.01$; ***, $p < 0.001$ (Mann-Whitney U test). *C*, RT-PCR analysis of cKrox mRNA expression in immunomagnetic bead-purified LN $CD4^+$ cells from OT-II or $CD8^+$ cells from OT-I mice and in FACS-sorted LN $CD3^+CD8^-CD25^+$ or $CD3^+CD8^-CD25^-$ cells from $CD4^{-/-}$ or $CD4^{+/+}$ mice.

[6] The online version of this article contains supplemental material.

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cells within the thymus, indicating that the proposed higher affinity of Treg TCRs for self-MHC/peptide complexes may not be able to compensate for the lack of CD4 contribution to TCR avidity. Alternatively, this result suggests that Tregs have a degree of autoreactivity to self similar to that of their Tconv counterparts when CD4 is absent. Such an interpretation would be in agreement with the significant overlap found between the TCR repertoires of $CD4^+$ Tconv cells and Tregs, which are more closely related than the TCR repertoires of $CD4^+$ and $CD8^+$ cells (16).

Surprisingly, despite lower absolute numbers of $FoxP3^+$ cells in the thymi of $CD4^{-/-}$ mice, the percentages of $CD3^+CD8^-FoxP3^+$ T cells within the peripheral compartments appeared to be dramatically increased in $CD4^{-/-}$ mice (23% ± 0.9) as compared with their wild-type counterpart (12% ± 0.4; $p < 0.001$) (Fig. 1*A*). Hence, whereas $CD4^{-/-}$ mice had reduced total numbers of $CD3^+CD8^-FoxP3^-$ Tconv peripheral cells (3-fold reduction), the total numbers of cells in the LN $CD3^+CD8^-FoxP3^+$ compartment were less affected (2-fold reduction) (Fig. 1*B*). These cell numbers appeared stable over the lifetimes of the mice, because 8- to 10-wk, 15- to 20-wk, and 20- to 40-wk animals had $0.5 \pm 0.14 \times 10^6$ ($n = 6$), $0.4 \pm 0.04 \times 10^6$ ($n = 8$), and $0.7 \pm 0.15 \times 10^6$ ($n = 6$) LN $CD3^+CD8^-FoxP3^+$ cells, respectively.

$CD3^+CD8^-FoxP3^+$ T cells from $CD4^{-/-}$ mice are enriched in memory-type Tregs

We further determined whether the CD4-like $FoxP3^+$ cells present in $CD4^{-/-}$ mice display the classical phenotypic features of Tregs. $CD3^+CD8^-FoxP3^+$ cells were almost exclusively $TCR\alpha\beta^+$ and virtually no $TCR\gamma\delta^+$ cells were detected in this subset (supplemental Fig. 2*A*). They did not express the NK and invariant NK T cell marker CD49b (supplemental Fig. 2*A*). As in normal mice, the vast majority (77% ± 3) of $CD3^+CD8^-FoxP3^+$ cells from $CD4^{-/-}$ animals were $CD25^+$ (Fig. 1*A*). Also, expression of other classical Treg markers such as surface GITR (glucocorticoid-induced TNF receptor family-related protein) and intracellular CD152 was comparable in $CD4^{-/-}$ and $CD4^{+/+}$ mice (supplemental Fig. 2*B*).

One hypothesis for explaining the high frequency of $FoxP3^+$ cells among the peripheral CD4-like compartment could be a preferential survival of $FoxP3^+$ cells and/or a higher level of homeostatic proliferation as compared with Tconv cells. Although we did not find any difference in the frequencies of proliferating Ki-67^+ cells in adult mice (~30% of $CD3^+CD8^-FoxP3^+$ in both $CD4^{-/-}$ and $CD4^{+/+}$ mice; data not shown), the proportion of memory-type $CD44^+CD62L^{low}$ cells within the $CD3^+CD8^-FoxP3^+$ subset appeared increased in $CD4^{-/-}$ mice, as was the frequency of $CD103^+$ cells (supplemental Fig. 3*A*). The proportion of these $CD44^+CD62^{low}$ and $CD103^+$ cells increased in aged animals (supplemental Fig. 3*B*), presumably because either activated/memory Tregs gradually expand and/or adaptive Tregs are generated to compensate for the lower number of Tregs. These results suggest that homeostatic mechanisms contribute to the maintenance of a significant Treg pool in the periphery of $CD4^{-/-}$ mice.

CD4-like Tregs from $CD4^{-/-}$ mice display a diversified TCR repertoire

We next evaluated whether the absence of CD4 would impair the development of a normally diversified repertoire. TCR-Vβ usage of $CD3^+CD8^-FoxP3^+$ cells appeared undistinguishable in $CD4^{-/-}$ and $CD4^{+/+}$ mice (supplemental Fig. 4*A*), as was also the case for the $CD3^+CD8^+FoxP3^-$ subset (data not shown). To further assay the level of TCR diversity, we also analyzed the TCR-Vβ CDR3 length distribution using the Immunoscope method. This confirmed that CD4-like Tregs from $CD4^{-/-}$ mice had a fully diversified TCR repertoire (supplemental Fig. 4*B*). This was also the case when comparing the Immunoscope profiles of $CD3^+CD8^+CD25^-$ cells in both groups (not shown). Hence, generation of the Treg compartment in $CD4^{-/-}$ mice is not due to the peripheral expansion of a pauciclonal cell population. Rather, our data indicate that the lack of CD4 does not impair the production of a highly diversified Treg repertoire.

$FoxP3^+$ T cells can develop in CD4/MHC class II double-deficient mice

The observation that $FoxP3^+$ Tregs can develop in the absence of CD4 raises the possibility that their generation may be, at least partly, independent of interactions with MHC class II molecules. To investigate this point, we bred $CD4^{-/-}$ mice under an MHC-II-deficient I-A$\beta^{-/-}$ background (11). Surprisingly, the frequencies and absolute numbers of peripheral $CD3^+CD8^-FoxP3^+$ cells in $CD4^{-/-}$I-A$\beta^{-/-}$ mice were not further reduced by the absence of MHC-II molecules as compared with that of $CD4^{-/-}$ mice (supplemental Table I). Because I-A$\beta^{-/-}$ mice could still harbor some cross-paired I-Aα I-Eβ MHC-II chains, we additionally used another model of mice that lack all conventional MHC-II chains due to a large deletion of the entire class II region, i.e., MHC-$II^{\Delta/\Delta}$ mice (12). Even in this more stringent system, $CD3^+CD8^-FoxP3^+$ cells from $CD4^{-/-}$MHC-$II^{\Delta/\Delta}$ were still found at similar frequencies and absolute numbers than in $CD4^{-/-}$ mice (supplemental Table I). Hence, CD4-like Tregs can develop in the absence of both CD4 and MHC-II molecules, demonstrating that the generation of Tregs can be, at least partly, independent of MHC class II recognition. This conclusion is consistent with the observation that not only $CD8^+$ Tregs (17) but also CD4-like Tregs may be found in MHC class II-deficient mice (18), and even in double-MHC class I- and class II-deficient mice (19).

$CD3^+CD8^-CD25^+$ T cells of $CD4^{-/-}$ mice are functionally suppressive in vitro and in vivo

To evaluate the functional characteristics of CD4-like Tregs, we investigated their proliferative response to Con A activation and their capacity to suppress the proliferation of responder T cells. As expected for Tregs, $CD3^+CD8^-CD25^+$ cells were profoundly hyporeactive in vitro in response to mitogenic activation (Fig. 2*A*). This in vitro hyporesponsiveness was further attested to by the fact that upon stimulation by anti-CD3/anti-CD28 beads for 4 days, $CD3^+CD8^-CD25^+$ cells from both $CD4^{-/-}$ and $CD4^{+/+}$ mice similarly produced ~100-fold less IL-2 than $CD3^+CD8^-CD25^-$ cells (Fig. 2*B*).

To evaluate the suppressive function of CD4-like Tregs, different ratios of $CD3^+CD8^-CD25^+$ cells were assayed for their capacity to inhibit the proliferative response of syngeneic $CD4^+CD25^-$ responder cells. $CD3^+CD8^-CD25^+$ cells from $CD4^{-/-}$ mice were found as efficient as those from $CD4^{+/+}$ animals in suppressing the proliferation of effector cells in a dose-dependent manner (Fig. 2*C*).

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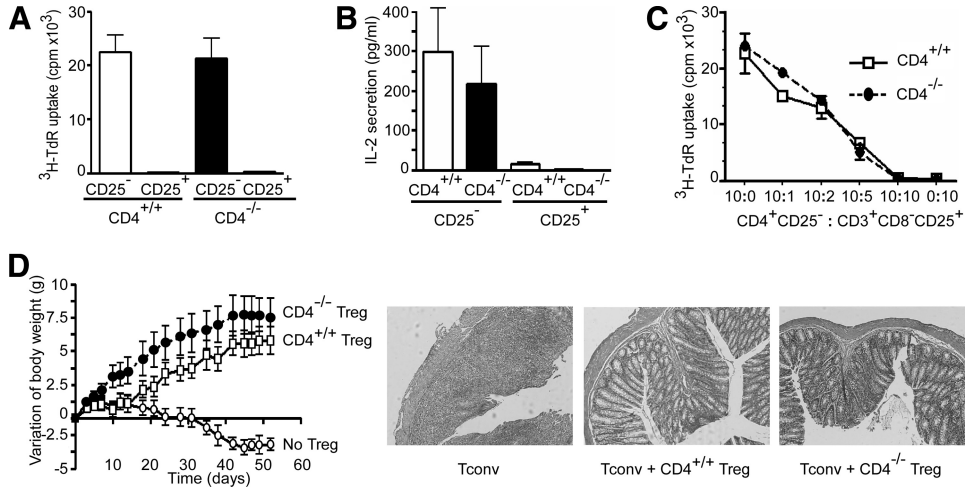

FIGURE 2. Suppressive function of $CD3^+CD8^-CD25^+$ Tregs from $CD4^{-/-}$ mice in vitro and in vivo. *A*, Proliferative response of Tregs from $CD4^{-/-}$ or $CD4^{+/+}$ mice upon activation with Con A. One representative result of three independent experiments is shown. *B*, IL-2 secretion by Tregs from $CD4^{-/-}$ or $CD4^{+/+}$ mice upon activation by anti-CD3/anti-CD28 beads. One representative result of three independent experiments is shown. *C*, Inhibition of Con A-activated effector T cell proliferation by $CD3^+CD8^-CD25^+$ Tregs from $CD4^{+/+}$ or $CD4^{-/-}$ mice. One representative result of four independent experiments is shown. *D*, Inhibition of $CD3^+CD8^-CD25^-$ (Tconv)-induced IBD by $CD3^+CD8^-CD25^+$ Tregs from $CD4^{+/+}$ or $CD4^{-/-}$ mice. The graph in the left displays variations of body weight. The pictures in the right correspond to colon histological analysis of H&E/saffron-stained paraffin-embedded sections (7 wk after transfer). One representative result of two independent experiments ($n = 6$ for each group) is shown.

To further evaluate the suppressive capacity of Tregs in the absence of CD4, we also analyzed their potency to prevent inflammatory bowel disease (IBD) in vivo. For this, purified Tconv cells were transferred with or without purified Tregs from $CD4^{+/+}$ or $CD4^{-/-}$ mice to T cell-deficient $CD3^{-/-}$ animals. Mice injected with Tconv cells alone lost 14% of their maximal weight 7 wk after transfer (Fig. 2*D*). This weight loss was associated with diarrhea, rectal prolapse, and local inflammation as indicated by histological signs of microvilli destruction and extensive mononuclear cell infiltrates. The coinjection of Tregs from $CD4^{+/+}$ or $CD4^{-/-}$ mice completely prevented the wasting syndrome and also histological signs of inflammation (Fig. 2*D*). Together, these data demonstrate that CD4-like Tregs from $CD4^{-/-}$ mice are functionally suppressive in vitro and, remarkably, also in vivo.

It follows from the present findings that CD4-like Tregs can develop in the absence of both CD4 and MHC-II molecules, indicating that the generation of Tregs can be, at least partly, independent of TCR/MHC class II recognition. This is in marked contrast with the current view that Treg development is highly dependent on high-avidity interactions with MHC class II molecules (3–5). This prevailing paradigm relies on experiments performed in TCR/Ag transgenic mice (i.e., with a repertoire biased toward the recognition of given specificities). In fact, it was shown that the total numbers of developing Tregs do not increase upon an encounter with the cognate Ag on thymic epithelial cells even if their proportion appears enriched relative to conventional $CD4^+$ thymocytes (6). This has suggested that selective survival, rather than induced differentiation, is the reason that explains the apparent Treg enrichment observed in previous studies. This hypothesis is compatible with the view raised by the present data that high affinity for self-Ags may not constitute the only mechanism promoting differentiation toward the Treg pathway. Indeed, if high affinity to self was to play a major role, we would expect that deletion of the CD4 coreceptor, by limiting the possibility to recognize MHC with high overall avidity, should more severely impact the differentiation of Tregs than their conventional counterparts. Our results rather show that the absence of CD4 similarly impairs the thymic differentiation toward the Treg lineage and toward the Tconv pathway (Fig. 1).

Yet, this interpretation relies on the underlying assumption that CD4-like lymphocytes generated in $CD4^{-/-}$ mice are restricted to MHC class II molecules. Our finding that $FoxP3^+$ CD4-like lymphocytes are also found in CD4/MHC class II double-deficient mice raises the possibility that Treg differentiation may not strictly depend on MHC-class II. In line with this view, it has recently been suggested that lymphocytes that differentiate in mice deficient for all coreceptors (CD4/CD8) are autoreactive and, surprisingly, are not restricted to MHC molecules (20). Indeed, by linking $p56^{lck}$ recruitment to MHC recognition, coreceptors restrict the specificity of nascent thymocytes to MHC molecules while inhibiting MHC-independent signaling by sequestering $p56^{lck}$. Because the CD4 coreceptor binds more abundantly to $p56^{lck}$ than CD8, it is plausible that the thymocytes developing in $CD4^{-/-}$ mice are partly independent of MHC molecules. This is consistent with our findings, because such thymocytes would be independent of the presence of both CD4 and MHC class II for their development and could be reactive to yet unidentified autoantigens. In such a situation, affinity for MHC-independent Ags would not be dependent on the presence of CD4 and could equally affect the selection of both Tconv cells and Tregs, as found in this study.

In conclusion, the present findings demonstrate that functional $FoxP3^+$ CD4-like cells displaying a fully diversified TCR repertoire can develop in the absence of a CD4 coreceptor. Although their differentiation in the thymus is impaired, as it is for Tconv cells, the situation is different in the periphery where Tregs are found in higher proportions than conventional CD4-like cells. Consistently, their total numbers are less affected by lack of CD4 than conventional T cells when compared with wild-type mice. Conceivably, this differential behavior may

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be linked to peripheral homeostatic mechanisms—as suggested by the augmented representation of memory-type $CD44^+CD62^{low}$ and $CD103^+$ Tregs—that contribute to the prevention of autoimmune manifestations in $CD4^{-/-}$ animals. Consistent with this notion, $FoxP3^+$ CD4-like cells from these animals are fully capable of preventing IBD mediated by the transfer of $CD4^+$ T cells in $CD3^{-/-}$ hosts. Thus, caution should be taken in studies that use $CD4^{-/-}$ or even MHC class II-deficient animals as models of Treg-free mice. Finally, it would be interesting in future studies to define the fine antigenic specificities of Tregs that can develop in $CD4^{-/-}$ as well as in CD4/MHC class II double-deficient mice. Their characterization would provide a proof of principle that non-MHC-restricted Tregs may function independently of individual MHC backgrounds, paving the way to a potential therapeutic use upon activation by non-MHC-dependent ligands.

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Disclosures

The authors have no financial conflict of interest.

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