

Inducible costimulator (ICOS) is a marker for highly suppressive antigen-specific T cells sharing features of T_H17/T_H1 and regulatory T cells

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Background: $CD4^+CD25^+$ regulatory T (Treg) cells are involved in the downmodulation of numerous immune responses to pathogens, tumors, or allergens.

Objective: In this study, we further characterized the nature of Treg cells that control skin inflammatory reactions to haptens.

Methods: In a model of contact hypersensitivity to 2,4-dinitrofluorobenzene, we have investigated the phenotype, the specificity, and the origin of Treg cells that modulate the priming of effector $CD8^+$ T cells responsible for the development of the pathology.

Results: 2,4-Dinitrofluorobenzene immunization induced a population of $CD4^+CD25^+$ Treg cells that controlled $CD8^+$ T-cell effector responses in a hapten-specific manner *in vivo*. High levels of inducible costimulator (ICOS) expression defined a population of $CD4^+CD25^+FoxP3^+$ (forkhead box protein 3) Treg cells that presented superior suppressive activity.

Importantly, $ICOS^+$ Treg cells were distinguishable from all other $FoxP3^+$ Treg cells by the expression of IL-10, IL-17, and IFN- γ . Hapten-specific Treg cells proliferating in response to their cognate antigen *in vivo* predominantly displayed a $CD25^+FoxP3^+ICOS^+$ phenotype. By using reporter mice, we showed that $ICOS^+$ Treg cells derived from the expansion of natural $CD4^+FoxP3^+$ Treg cells rather than generation of adaptive Treg cells. Furthermore, the generation of $ICOS^+$ Treg cells depended on innate cells rather than the effector $CD8^+$ T-cell population.

Conclusion: Taken together, our data show that a population of $CD4^+CD25^+FoxP3^+$ T cells upregulates ICOS on *in vivo* sensitization and specifically suppresses hapten-reactive $CD8^+$ T cells both *in vivo* and *in vitro*. (J Allergy Clin Immunol 2010;■■■■:■■■-■■■.)

Key words: Regulatory T cells, ICOS, antigen-specificity, T_H17/T_H1 features, skin inflammation, allergic contact dermatitis, haptens

Abbreviations used

CHS: Contact hypersensitivity
DC: Dendritic cell
dLN: Draining lymph node
DNBS: 2,4-Dinitrobenzene-sulfonic acid
DNFB: 2,4-Dinitrofluorobenzene
FACS: Fluorescence-activated cell sorting
FoxP3: Forkhead box protein 3
ICOS: Inducible costimulator
LN: Lymph node
MFI: Mean fluorescence intensity
SFC: Spot-forming cell
Tconv: Conventional T cell
Treg: Regulatory T cell

Contact hypersensitivity (CHS), also referred to as allergic contact dermatitis, is a major health issue and one of the most common occupational diseases in western countries.¹ CHS is a T-cell-mediated cutaneous inflammatory reaction caused by repeated skin exposure to contact allergens, that is organic and metallic chemicals called haptens.² Previous studies have emphasized that CHS is mediated by hapten-specific $CD8^+$ T cells and regulated by $CD4^+$ T cells.^{1,3} However, the precise nature of $CD4^+$ regulatory T (Treg) cells operating in CHS remains poorly defined.

$CD4^+CD25^+FoxP3^+$ (forkhead box protein 3) Treg cells make up 5% to 10% of $CD4^+$ T cells in human beings and mice. Naturally occurring Treg cells develop in the thymus, and adaptive (or induced) Treg cells can be generated in the periphery, although the stability and functional significance of these cells are still unclear.⁴ Treg cells have been involved in the downmodulation of numerous immune responses to self-antigens, pathogens, alloantigens, and tumors.⁵ Increasing evidence indicates that the development and the severity of skin inflammatory and allergic diseases such as psoriasis, CHS, and atopic dermatitis might result from a deficiency in regulatory mechanisms. In particular, $CD4^+CD25^+$ Treg cells have been implicated in the maintenance of tolerance and in the downmodulation of CHS responses to haptens in mice and human beings.^{6,7} Here, we show that CHS responses are controlled by hapten-specific Treg cells and that the inducible costimulator (ICOS) defines a highly suppressive Treg-cell population that produces IL-10 but also IL-17 and IFN- γ . Furthermore, $CD4^+FoxP3^+ICOS^+$ Treg cells originate from the pool of

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naturally occurring FoxP3⁺ cells in the context of active inflammation in response to the sensitizing hapten.

METHODS

Animals and reagents

Mice, allergens (2,4-dinitrofluorobenzene [DNFB], 2,4-dinitrobenzenesulfonic acid [DNBS], or oxazolone) or mAbs used in this study are described in the Online Repository, Methods section, at www.jacionline.org.

Animal experimental procedures were conducted in accordance with the local institutional review board (Comité Régional d'Ethique sur l'Expérimentation Animale).

Mouse ear swelling test

The mouse ear swelling test procedure was used to measure CHS responses, as previously described.⁸ Briefly, animals were sensitized epicutaneously on shaved abdomen with 25 μ L 0.5% DNFB or 100 μ L 2% oxazolone on day 0, and ear-challenged with 5 μ L 0.15% DNFB on day 5.

T-cell depletion *in vivo*

Animals were depleted of CD4⁺, CD8⁺, or CD25⁺ T cells using intraperitoneal injections of GK1.5, H35.17.2, and PC61 mAbs, respectively, as described elsewhere.^{7,9}

Flow cytometry analysis

Flow-cytometric experiments were conducted on draining and nondraining lymph node (LN) cells, harvested respectively from axillary/inguinal and mesenteric LNs. Stained cells were analyzed on a FACSCanto flow cytometer (BD Biosciences, Le Pont de Claix, France), and data were analyzed by using the FlowJo software (Treestar, Ashland, Ore).

T-cell purification and sorting

CD4⁺, CD4⁺CD25⁺, and CD8⁺ T-cell subsets were enriched by using isolation kits from Miltenyi Biotec (Bergish Gladbach, Germany). For purification of ICOS⁺ and ICOS⁻ T-cell subsets, enriched T cells were subsequently stained with anti-ICOS, anti-CD4, or anti-CD8 mAbs and sorted on a BD Biosciences FACS Vantage or FACS Aria. Purification of CD4⁺CD25⁻ ICOS⁺FoxP3⁻ or CD4⁺CD25⁻ICOS⁺FoxP3⁺ T cells was performed by using FoxP3-GFP (green fluorescent protein) animals. The purity of sorted cells was routinely >97%.

In vitro suppression assays

CD8⁺ T lymphocytes (5×10^4) were isolated from day 5 DNFB-sensitized mice and used as responder cells. They were cocultured in complete RPMI medium with mature BMDCs (1×10^4) generated and haptenized with DNBS (the soluble analog of DNFB) as described.¹⁰ Indicated CD4⁺ T-cell subsets from day 6 DNFB-sensitized mice were added at different suppressor-to-responder ratios. Triplicate cultures were pulsed with 1 μ Ci ³H-thymidine, and IFN- γ secretion was measured in culture supernatants by ELISA (R&D Systems, Lille, France).

Adoptive transfers

For *in vivo* proliferation experiments, dLN cells from day 6 DNFB-sensitized or oxazolone-sensitized or naive BALB/c mice were labeled with 1 μ mol/L CFSE. A total of 40×10^6 LN cells or a mixture of 1×10^6 CD4⁺CD25⁺ plus 5×10^6 CD8⁺ T cells was transferred intravenously into naive BALB/c mice. Recipient animals were sensitized 1 day later with DNFB. Proliferation of CD4⁺ T-cell subsets was measured 4 days later in dLNs.

For *in vivo* suppression experiments, recipients were injected with PBS, 15×10^6 CD4⁺ T cells, 1.5×10^6 CD4⁺CD25⁺ and 1.5×10^6 CD4⁺CD25⁻ T cells, or 0.7×10^6 FACS-sorted CD4⁺ T-cell subsets. Recipients were

sensitized 1 day later with DNFB, and the CHS or *in vitro* hapten-specific T-cell responses were measured 4 days later.

To detect generation of FoxP3⁺ cells, total splenocytes or sorted FoxP3/GFP splenocytes (2×10^6) from CD45.2 FoxP3-GFP mice were transferred intravenously into naive CD45.1 C57BL/6 animals. Recipient animals were sensitized the following day with DNFB. Six days later, dLN cells were recovered, and FoxP3 expression was analyzed.

Enzyme-Linked Immunospot Assay and ELISA

IFN- γ , IL-17a, or IL-4 Enzyme-Linked Immunospot Assays were performed on dLN cells as previously described.⁹ Results were expressed as the mean number of cytokine spot-forming cells (SFCs) $\pm$ SD/ 10^6 dLN cells. IFN- γ , IL-17a, IL-4, or IL-10 ELISA assays were conducted on supernatants of sorted CD4⁺ T-cell subsets cultured for 20 hours with unhaptenized BMDCs plus 2 μ g/mL anti-CD3 mAb (2C11).

Quantitative RT-PCR

cDNA was prepared by using standard protocols from sorted T-cell subsets. RT-PCR was performed with platinum SYBR Green kit (Invitrogen, Cergy Pontoise, France) on an Applied Biosystems 7000 machine. Relative gene expression was normalized to ubiquitin. Primers used in this study are reported in the Online Repository, Methods section, at www.jacionline.org.

Statistical analysis

Statistical analysis was performed by using a 1-way ANOVA test to compare groups of chemicals. $P < .01$ was used as the level of significance.

RESULTS

CHS is mediated by effector CD8⁺ T cells and controlled by hapten-specific CD4⁺CD25⁺ Treg cells

The CHS response was abrogated in mice depleted of CD8⁺ T cells (Fig 1, A), suggesting that CD8⁺ T cells mediate CHS to DNFB, as previously reported.^{3,9} Analysis of cytokine production by hapten-specific T cells in the dLNs of wild-type (WT) mice 5 days after sensitization with DNFB revealed a predominant IFN- γ response and the presence of IL-17-producing T cells, whereas IL-4-producing cells were barely detectable (Fig 1, B). In agreement with the upregulation of ICOS on T-cell activation and its costimulatory function in CHS,¹¹ IFN- γ -producing and IL-2-producing effector T cells predominantly displayed a CD8⁺ICOS⁺ phenotype, whereas IL-17-producing cells were both CD8⁺ICOS⁺ and CD4⁺ICOS⁺ T cells (see this article's Fig E1, A, in the Online Repository at www.jacionline.org). IFN- γ production peaked around day 3 to 5, and IL-2 production followed a similar but delayed pattern (Fig 1, C). Both the inflammatory response *in vivo* and the number of IFN- γ -producing CD8⁺ T cells were dramatically increased in mice depleted of CD4⁺ or CD25⁺ T cells (Fig 1, A and D, and data not shown), suggesting the involvement of CD4⁺CD25⁺ Treg cells in the regulation of CHS. Furthermore, recipient animals transferred with CD4⁺ or CD4⁺CD25⁺ T cells from DNFB-sensitized donors displayed an inhibition of DNFB-specific T-cell responses compared with PBS-treated controls, whereas responses were unaffected in recipients of CD4⁺CD25⁻ T cells (Fig 1, E). Moreover, CD4⁺ or CD4⁺CD25⁺ Treg cells collected from oxazolone-sensitized mice failed to control T-cell responses to DNFB (Fig E1; Fig 1, B), demonstrating that CD4⁺CD25⁺ Treg cells operate in an antigen-specific manner to control hapten-specific CD8⁺ T-cell responses.

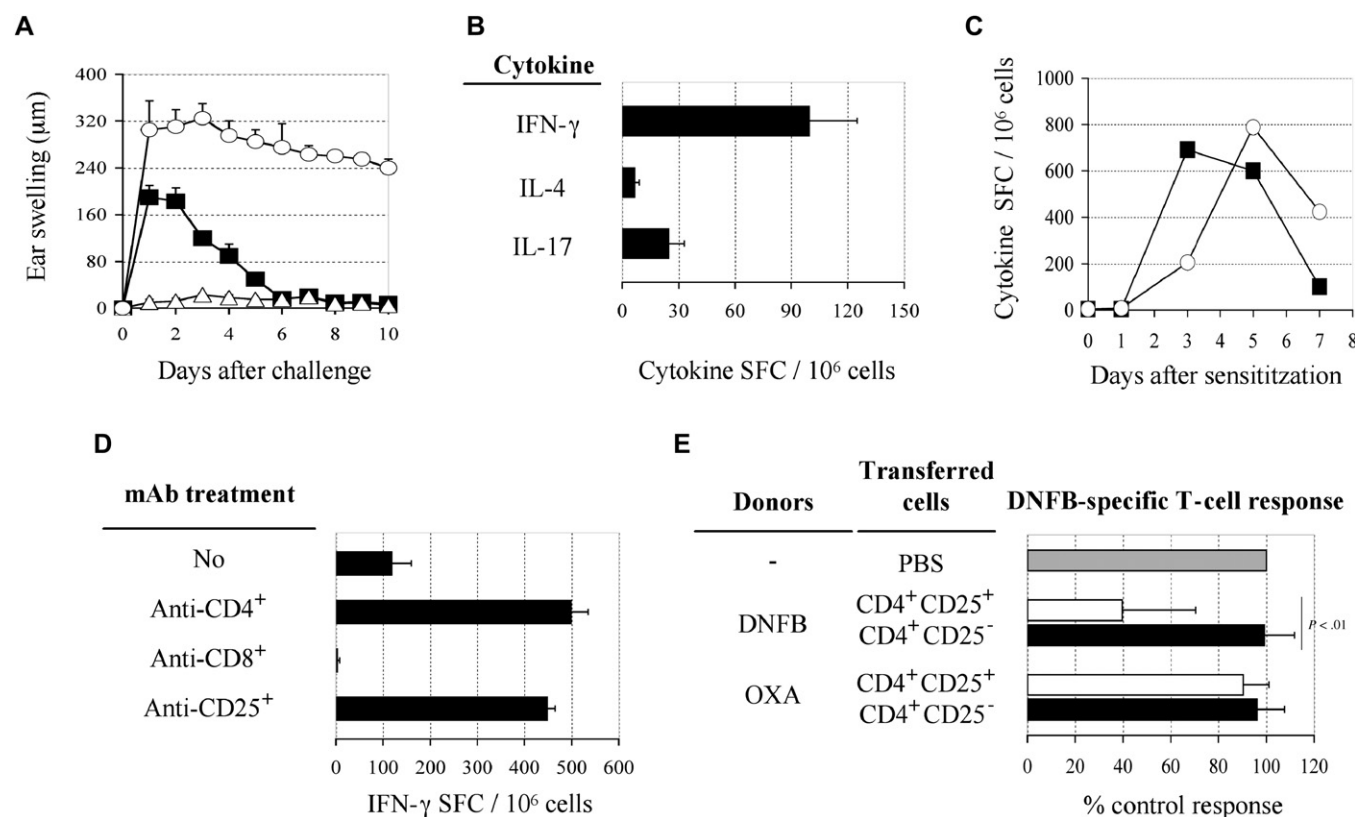


FIG 1. Effector and regulatory subsets in CHS to DNFB. **A**, BALB/c mice, either untreated (■) or injected with anti-CD8 (Δ) or anti-CD4 (○) mAb, were epicutaneously sensitized and challenged with DNFB. The CHS response was determined by measuring ear swelling at indicated times. **B**, Cells were collected from day 5 DNFB-sensitized mice, and the frequency of hapten-specific IFN-γ, IL-4, and IL-17 SFCs was determined by ELISPOT and expressed as the mean number of SFCs ± SD/10⁶ cells. **C**, Cells were collected from dLNs at indicated times, and the frequency of hapten-specific IFN-γ (■) or IL-2 (○) SFCs ± SD was determined as in **B**. **D**, CD4-depleted, CD8-depleted, or CD25-depleted and untreated mice were sensitized with DNFB, and the number of IFN-γ SFC was determined as in **B**. **E**, Naive mice were injected intravenously with PBS (gray bar) or transferred with 1.5×10^6 CD4⁺CD25⁺ (white bars) or CD4⁺CD25⁻ (black bars) T cells purified from DNFB-sensitized or oxazolone (OXA)-sensitized mice. Recipients were sensitized 1 day later with DNFB, and hapten-specific T-cell responses were assessed by IFN-γ ELISPOT 4 days later. Results are expressed as the percentage of the DNFB-specific control response obtained in PBS-treated mice (normalized to 100%). Results are representative of 3 experiments with 3 to 5 mice per group.

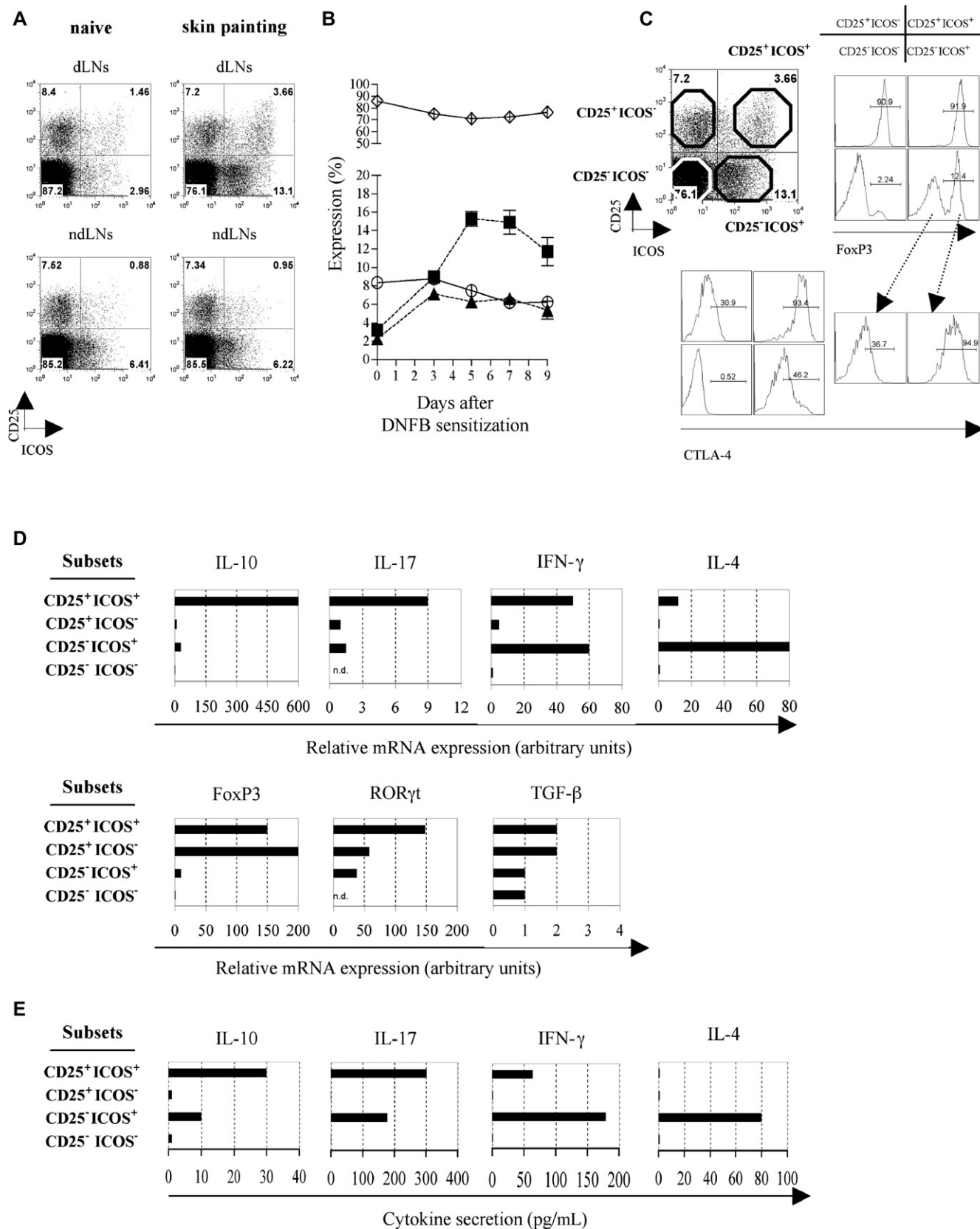
ICOS expression defines distinct populations of CD4⁺ Treg cells and conventional T cells after hapten sensitization

Flow cytometry analysis revealed that CD4⁺ cells could be subdivided into distinct subsets on the basis of the expression of CD25 and ICOS. In unsensitized naive mice, a modest frequency of CD4⁺CD25⁺ Treg cells or CD4⁺CD25⁻ conventional T (Tconv) cells expressing ICOS were found in LNs (Fig 2, A). Indeed, only a mean of $1.46 \pm 0.59\%$ of ICOS⁺ Treg cells and $2.76 \pm 0.47\%$ of ICOS⁺ Tconv cells were recorded in naive mice ($n = 14$). The percentage of ICOS⁺ CD4⁺ T cells increased in dLNs at day 6 postsensitization with DNFB, whereas no modification of ICOS expression was observed in non-dLNs (Fig 2, A). The percentage of ICOS⁺ Tconv cells increased to an average of $13.58 \pm 2.41\%$ ($n = 14$) in dLNs of DNFB-sensitized mice (Fig 2, A). The percentage of ICOS⁺ Treg cells also increased significantly in DNFB-sensitized animals (Fig 2, A), up to a mean of $3.24 \pm 0.99\%$ ($n = 14$) in dLNs. Importantly, the level of ICOS expression was much higher in ICOS⁺ Treg cells (average

mean fluorescence intensity [MFI] of 359 ± 37) compared with ICOS⁺ Tconv cells (169 ± 15 ; Fig 2, A). Kinetics studies revealed that the percentages of CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells expressing ICOS increased progressively to reach a plateau 3 to 5 days after sensitization (Fig 2, B) and were dependent on the dose of DNFB used for sensitization (see this article's Fig E2, A, in the Online Repository at www.jacionline.org).

ICOS expression defines an IL-10-producing and IL-17/IFN-γ-producing FoxP3⁺ Treg-cell population

More than 90% of both ICOS⁺ and ICOS⁻ Treg cells expressed the lineage-specific transcription factor FoxP3 (Fig 2, C). Nevertheless, the MFI of FoxP3 was notably higher in ICOS⁺ Treg cells compared with ICOS⁻ Treg cells (MFI, 1063 ± 75 and 643 ± 48 , respectively). In addition, all ICOS⁺ Treg cells expressed high levels of CTLA-4, whereas only about 30% of ICOS⁻ Treg cells expressed it at low levels (Fig 2, C), indicating potential differences in suppressive activity. CD73 was expressed at comparable



levels in ICOS⁺ and ICOS⁻ Treg cells, and upregulation of CD44 and CD103 and downregulation of CD62L were observed predominantly in ICOS⁺ Treg cells (Fig E2, B). Although ICOS⁺ Tconv cells predominantly displayed an effector/memory phenotype characterized by the upregulation of CD44 and downregulation of CD62L (Fig E2, B), a small fraction (10% to 20%) expressed FoxP3 (Fig 2, C), although at notably lower levels than ICOS⁺ Treg cells (MFI, 772 ± 85 for ICOS⁺ Tconv cells compared with 1063 ± 75 for ICOS⁺ Treg cells). Further examination of CD4⁺CD25⁺ICOS⁺FoxP3⁺ cells showed that these cells expressed CTLA-4 and CD103 at higher levels than their FoxP3⁻ counterparts, but at lower levels than ICOS⁺ Treg cells, similarly to FoxP3 (Fig 2, C; Fig E2, B). As expected, ICOS⁻ Tconv cells displayed a naive phenotype and did not express FoxP3 or CTLA-4 (Fig 2, C; Fig E2, B).

Analysis of cytokine production by quantitative PCR revealed important differences between subsets sorted from dLNs of day 6 DNFB-sensitized animals. In agreement with their naive phenotype, ICOS⁻ Tconv cells did not express FoxP3 or effector cytokine mRNA (Fig 2, D). Whereas ICOS⁻ Treg cells expressed mainly FoxP3 mRNA, ICOS⁺ Treg cells expressed mRNA for FoxP3 and the immunomodulatory cytokine IL-10 but also for effector cytokines IFN- γ and IL-17. In contrast, ICOS⁺ Tconv cells predominantly expressed IFN- γ and IL-4 and low levels of FoxP3 and IL-17 (Fig 2, D). These features were confirmed at the protein level by ELISA after polyclonal restimulation *in vitro* (Fig 2, E). Expression of IL-17 mRNA correlated with expression of the transcription factor retinoic acid-related orphan receptor γ (ROR γ t), with high levels in ICOS⁺ Treg cells but low levels in ICOS⁻ Treg cells or ICOS⁺ Tconv cells (Fig 2, D). Interestingly, cytokine analysis in FoxP3⁺ and FoxP3⁻ ICOS⁺ Tconv cells sorted from FoxP3/GFP reporter mice revealed that the IL-17 and ROR γ t mRNA observed in ICOS⁺ CD4⁺CD25⁻ cells (Fig 2, D) originated from ICOS⁺ FoxP3⁻ Tconv cells and not from CD4⁺CD25⁺FoxP3⁺ cells (see this article's Fig E3 in the Online Repository at www.jacionline.org). Finally, levels of mRNA for TGF- β were comparable in all 4 subsets. Thus, ICOS⁺ Treg cells uniquely segregated from all other FoxP3⁺ Treg cells (CD4⁺CD25⁺ICOS⁻ and CD4⁺CD25⁺ICOS⁺) by their expression of IL-10, IL-17, and ROR γ t and from ICOS⁺FoxP3⁻ Tconv cells by their expression of IL-10.

ICOS⁺ Treg cells most efficiently inhibit DNFB-specific CD8⁺ T-cell responses

ICOS⁺ and ICOS⁻ Treg cells strongly inhibited both hapten-specific and polyclonal CD8⁺ T cell proliferation and IFN- γ secretion *in vitro* in comparison with ICOS⁺ and ICOS⁻ Tconv populations, which showed respectively weak and no suppressor capacities (Fig 3, A; see this article's Fig E4 in the Online Repository at www.jacionline.org). ICOS⁺ Treg cells proved much

more efficient to suppress CD8⁺ T-cell responses than their ICOS⁻ counterparts because 50% suppression was obtained with an average 8-fold ($\pm$ 2) lower Treg/effector cell ratio ($n = 6$) (Fig 3, A). Unexpectedly, a similar inhibition of DNFB-specific CD8⁺ T-cell responses *in vitro* was observed by using cells sorted from oxazolone-sensitized animals (data not shown). This result is in apparent discrepancy with our *in vivo* data showing that Treg cells controlled CHS in a hapten-specific manner (Fig 1) but is in keeping with previous reports of antigen nonspecific suppression by Tregs both *in vivo* and *in vitro*.¹²

Although ICOS⁺ Tconv cells failed to inhibit CD8⁺ T-cell proliferation, they significantly hampered IFN- γ secretion at a Teff:Treg-cell ratio of 1:1/2 (Fig 3, A). Because ICOS⁺ Tconv cells included 10% to 20% of FoxP3⁺ cells, we purified FoxP3⁺ and FoxP3⁻ T cells from the CD4⁺CD25⁺ICOS⁺ population by using FoxP3/GFP transgenic mice and tested their suppressive capacities. FoxP3⁺ but not FoxP3⁻ cells exhibited partial suppressive functions. Indeed, CD4⁺CD25⁺ICOS⁺FoxP3⁺ cells inhibited IFN- γ secretion but not proliferation of hapten-specific CD8⁺ T cells, whereas neither was suppressed by CD4⁺CD25⁺ICOS⁺FoxP3⁻ cells (Fig 3, B). Thus, all CD4⁺FoxP3⁺ T cells were capable of suppressing CD8⁺ T-cell responses to some extent *in vitro*. Importantly, recipient animals transferred with CD4⁺CD25⁺ICOS⁺ Treg cells, but not CD4⁺CD25⁺ICOS⁺ or CD4⁺CD25⁺ICOS⁻ cells, displayed a significant inhibition of DNFB-specific T-cell responses *in vivo* (Fig 3, C). Taken together, these results indicated that CD4⁺CD25⁺ICOS⁺ Treg cells clearly represent the most efficient suppressor population.

FoxP3⁺ICOS⁺ Treg cells proliferate in an antigen-dependent manner *in vivo*

We next monitored the *in vivo* proliferation of Treg cells obtained from DNFB-sensitized recipients after adoptive transfer into naive recipients. First, CD4⁺CD25⁺ Treg cells were isolated from unsensitized, DNFB-sensitized, or oxazolone-sensitized mice, labeled with CFSE and transferred into naive recipients together with CD8⁺ effector T cells obtained from DNFB-sensitized mice. On DNFB immunization, we observed intense proliferation of DNFB-induced, but not oxazolone-induced, CD4⁺CD25⁺ Treg cells (Fig 4, A), along with activation of DNFB-specific CD8⁺ T cells in both cases (data not shown). These data showed that Treg cells proliferated in a hapten-specific manner *in vivo* rather than nonspecifically as a consequence of the local inflammation and/or cytokines produced by effector CD8⁺ T cells. To examine the phenotype of hapten-specific T cells proliferating on antigenic stimulation *in vivo*, cells from skin dLNs of naive, DNFB-sensitized, or oxazolone-sensitized mice were labeled with CFSE and transferred into naive recipients that were subsequently immunized with DNFB. Four days after immunization, both FoxP3⁺ and FoxP3⁻ CD4⁺

FIG 2. ICOS expression distinguishes a specific population of Treg cells. **A**, Expression of CD25 and ICOS is shown in gated CD4⁺ T cells from dLNs of naive or day 6 DNFB-sensitized mice. **B**, The proportion of cells expressing CD25⁺ICOS⁻ ($\circ$), CD25⁺ICOS⁺ ($\blacktriangle$), CD25⁻ICOS⁻ ($\diamond$), and CD25⁻ICOS⁺ ($\blacksquare$) phenotype in CD4⁺ cells in dLNs is shown at indicated times after DNFB sensitization. Results are expressed as mean values $\pm$ SDs. **C**, Cells were obtained as in A, and the percentage of cells expressing FoxP3 and CTLA-4 in indicated subsets is shown. **D** and **E**, Indicated CD4⁺ cell subsets from day 6 DNFB-sensitized mice were FACS-purified, and mRNA expression (**D**) or cytokine secretion (**E**) was analyzed by quantitative RT-PCR or ELISA, respectively. *nd*, Not detected. Data are shown as means of triplicate PCR values. Results are representative of 3 experiments with 3 to 6 mice per experiment.

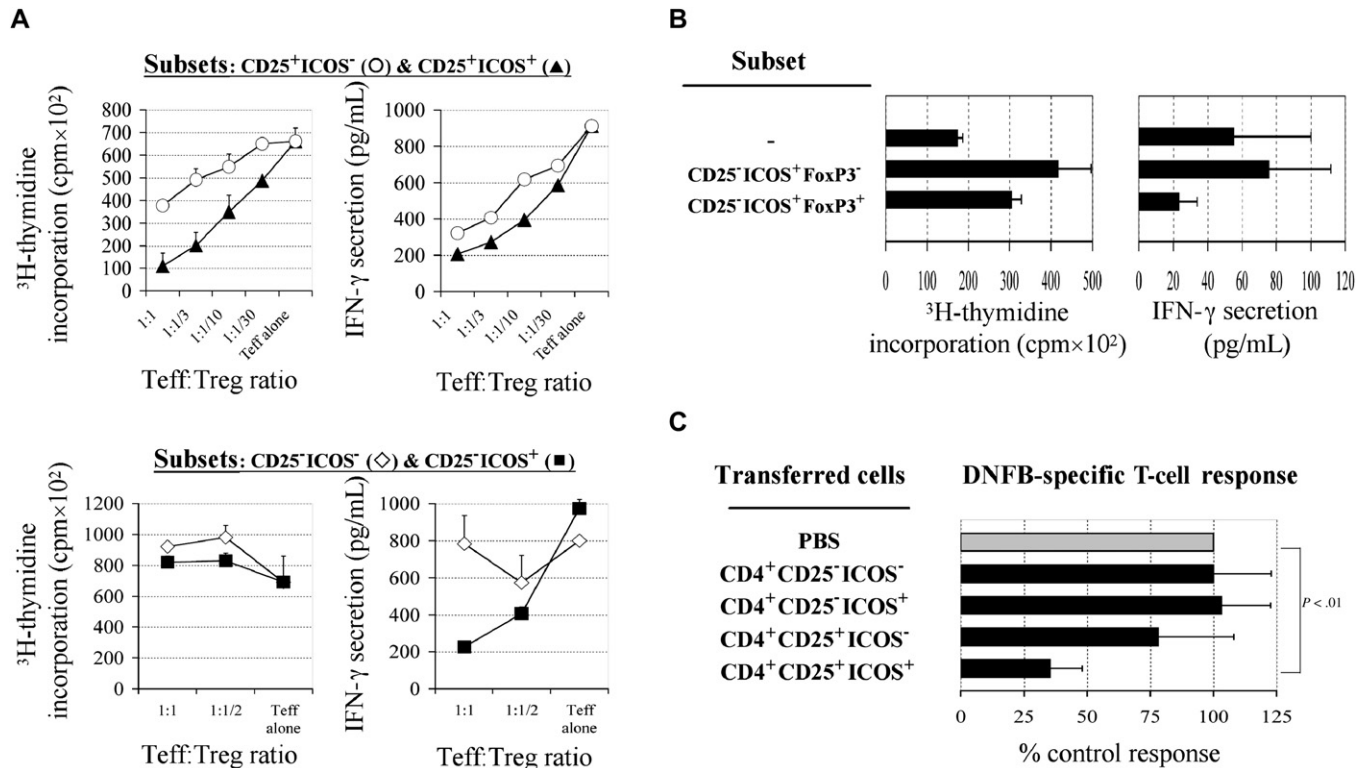


FIG 3. ICOS⁺ Treg cells display the highest suppressive capacities *in vitro* and *in vivo*. **A**, CD8⁺ effector T cells (5×10^4) were isolated from day 5 DNFB-sensitized mice and cultured with mature DNBS-haptenized BMDCs (1×10^4) in the absence or presence of different ratios of CD4⁺CD25⁺ICOS⁺ (▲), CD4⁺CD25⁺ICOS⁻ (○), CD4⁺CD25⁻ICOS⁺ (■), and CD4⁺CD25⁻ICOS⁻ (◇) T cells sorted from day 6 DNFB-sensitized animals. Proliferation was measured by ³H-thymidine incorporation, and IFN-γ secretion was measured by ELISA. Data are representative of 3 experiments with 3 to 6 mice per experiment. **B**, CD8⁺ T cells were cultured as in **A** with CD4⁺CD25⁻ICOS⁺FoxP3⁺ or CD4⁺CD25⁻ICOS⁺FoxP3⁺ cells sorted from DNFB-sensitized FoxP3/GFP transgenic animals. **C**, Naive mice were injected intravenously with PBS or transferred with 0.7×10^6 CD4⁺CD25⁻ICOS⁺, CD4⁺CD25⁻ICOS⁻, CD4⁺CD25⁺ICOS⁺, or CD4⁺CD25⁺ICOS⁻ T cells purified from day 6 DNFB-sensitized mice. Subsequent treatment and measure of IFN-γ responses are as in Fig 1, E. Results are representative of experiments with 3 to 4 mice per group.

T cells proliferated strongly in mice transferred with LN cells from DNFB-sensitized mice, but not in mice transferred with LN cells from naive or oxazolone-sensitized donors (Fig 4, B). FoxP3⁺ T cells that diluted CFSE mostly displayed a CD25^{low}ICOS⁺ phenotype typical of activated Tconv cells (Fig 4, B). Importantly, the large majority of CFSE^{low}CD4⁺FoxP3⁺ Treg cells that divided after stimulation with their cognate antigen expressed ICOS and CD25, in contrast with nonproliferating CD4⁺FoxP3⁺ Treg cells that contained a low frequency of CD25⁺ICOS⁺ cells (respectively, 82% and 13% of cells; Fig 4, B). Of note, we observed that expanding CD4⁺FoxP3⁺ Treg cells in these adoptive transfer experiments derived chiefly from ICOS⁺ precursors (data not shown), confirming that a ICOS⁺ Treg population collected at day 6 after DNFB sensitization encompassed a significant number of antigen-specific Treg cells.

ICOS⁺ Treg cells are generated by expansion of natural Treg cells rather than generation of adaptive Treg cells

It is unclear whether adaptive Tregs can be induced from naive CD4⁺CD25⁻ T cells during a hapten-specific inflammatory

response. Thus, we tested whether naive FoxP3⁻ T cells could convert into FoxP3⁺ cells after DNFB sensitization. Total splenocytes or sorted GFP⁺ cells from FoxP3/GFP transgenic reporter donor animals (CD45.2) were transferred into congenic CD45.1 C57BL/6 hosts that contained normal numbers of FoxP3⁺ cells. Recipients were then sensitized with DNFB or left unsensitized, and dLN cells were analyzed for FoxP3/GFP expression in the CD45.2⁺ donor population on day 6. A distinct CD4⁺ FoxP3/GFP⁺ population could be detected in CD45.2⁺ donor cells when recipients received unsorted splenocytes (Fig 5, A). In contrast, FoxP3/GFP expression was not induced in donor cells when FoxP3/GFP⁺ splenocytes were transferred, whether animals were sensitized with DNFB or not (Fig 5, A). Of note, we detected CD45.2⁺ T cells expressing a CD25⁻ICOS⁺ phenotype on immunization with DNFB (see this article's Fig E5 in the Online Repository at www.jacionline.org), showing that DNFB sensitization induced the proliferation of Tconv cells but not the generation of adaptive Treg cells. Together, these data show that FoxP3⁻ naive T cells were not converted into FoxP3⁺ Treg cells after DNFB sensitization. Moreover, adoptive transfer experiments using sorted ICOS⁻ cells showed that ICOS⁺ Treg cells could be generated, at least partly, from ICOS⁻ precursors (data not shown). To

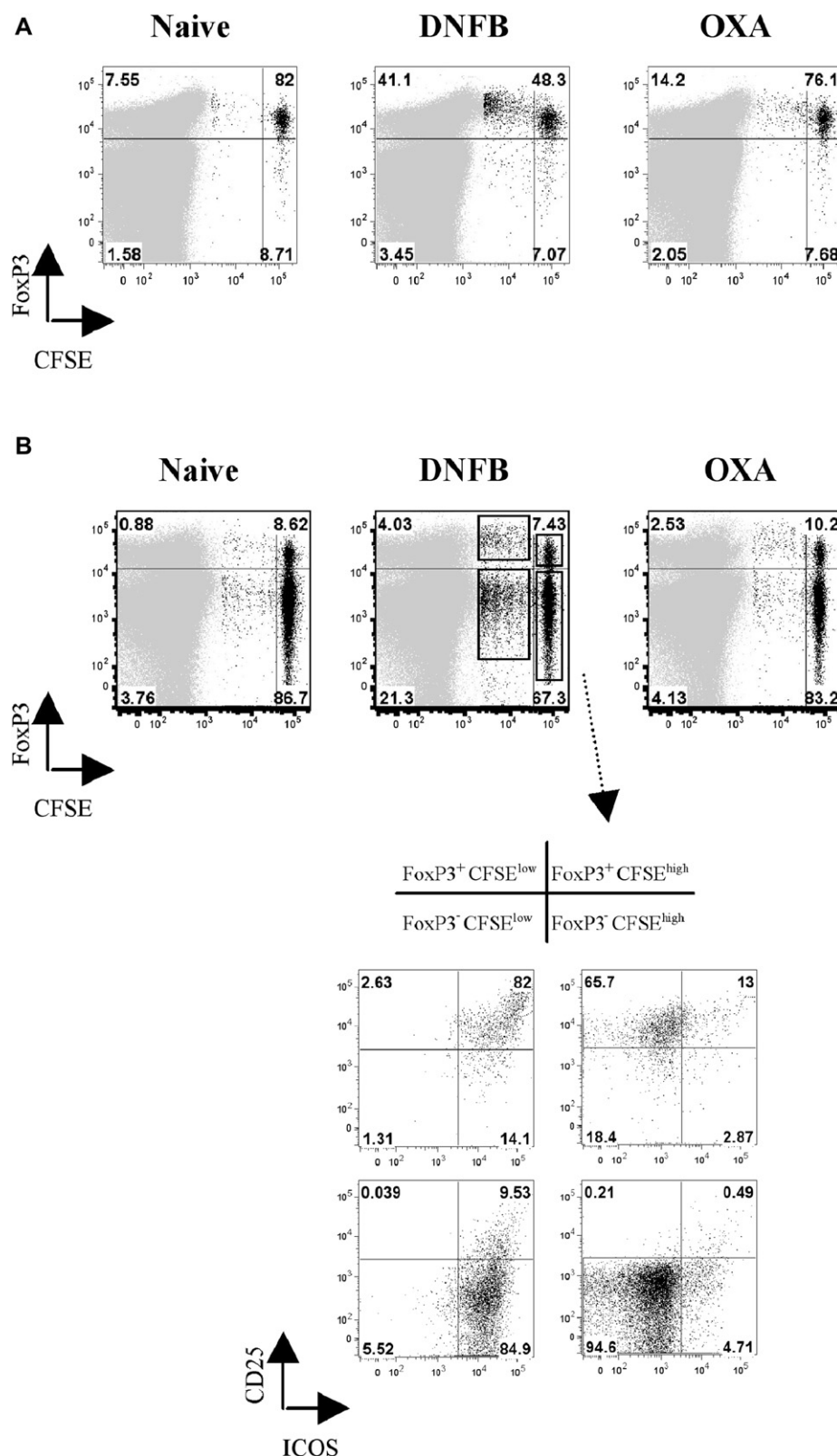


FIG 4. CD4⁺CD25⁺ICOS⁺FoxP3⁺Treg cells proliferate in an antigen-specific way *in vivo*. **A**, Naive mice were transferred with CD8⁺ T cells collected from DNFB-sensitized mice and CFSE-labeled CD4⁺CD25⁺ T cells collected from naive, DNFB-sensitized, or oxazolone (OXA)-sensitized mice. Recipients were sensitized with DNFB 1 day later, and CFSE dilution was analyzed in dLN cells after 4 days. The percentage of cells expressing CFSE and FoxP3 in gated CD4⁺ cells is shown. CFSE⁺ cells (*black dots*) were discriminated from endogenous cells (*gray dots*) for clarity. **B**, Naive mice were transferred with CFSE-labeled total LN cells collected from naive, DNFB-sensitized, or oxazolone-sensitized mice. Recipients were sensitized and analyzed as in **A**. The percentage of cells expressing CD25 and ICOS in proliferating and nonproliferating FoxP3⁺ or FoxP3⁻ cells is shown. Results are representative of 3 experiments with 3 mice per group.

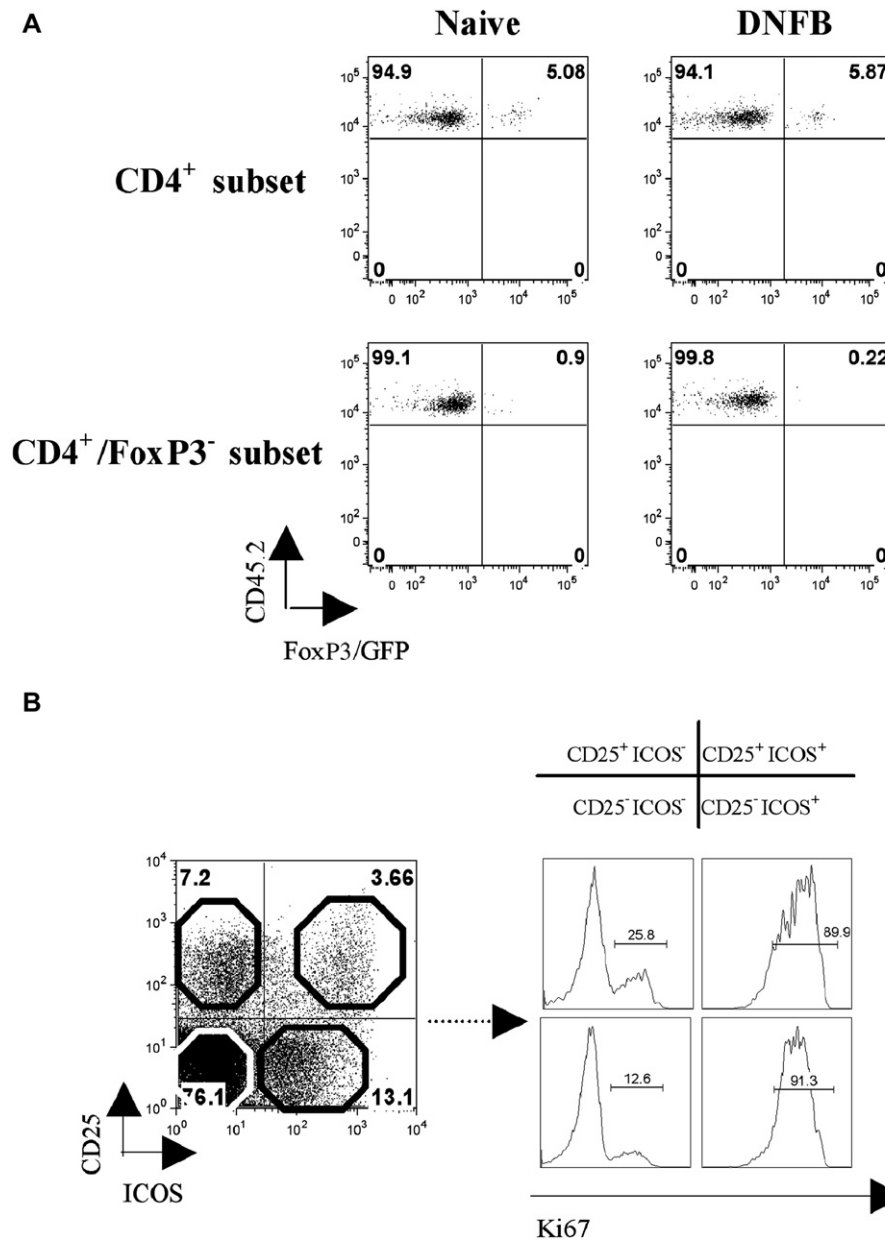


FIG 5. CD4⁺CD25⁺ICOS⁺ Treg cells expand from the pool of natural Treg cells. **A**, Naive CD45.1 mice were transferred with 2×10^6 unsorted splenocytes or FoxP3/GFP⁺ splenocytes sorted from naive CD45.2 FoxP3-GFP transgenic donors. Recipients were sensitized with DNFB 1 day after transfer. dLN cells were recovered 6 days later, and expression of FoxP3 in the CD4⁺CD45.2⁺ population was analyzed. **B**, Cells were harvested from dLNs of day 6 DNFB-sensitized mice, and the percentage of indicated CD4⁺ T cells expressing the proliferation marker Ki67 was determined. Representative results out of 3 experiments with 3 mice per groups are shown.

examine whether Treg cells could instead result from expansion of an existing pool, mice were sensitized with DNFB, and the proliferation of different populations of Treg cells was assessed on day 6 by examining expression of the Ki67 marker for cell division. Ninety percent of ICOS⁺ Treg cells and ICOS⁺ Tconv cells expressed Ki67 after sensitization with DNFB compared with only 25% of ICOS⁻ Treg cells and 12% of ICOS⁻ Tconv cells (Fig 5, B). Taken together, our results suggest that ICOS⁺ Treg cells expand from the pool of naturally occurring FoxP3⁺ Treg cells on hapten sensitization.

DISCUSSION

In this study, we identify an antigen-specific CD4⁺CD25⁺ICOS⁺FoxP3⁺ Treg population that plays an essential role in controlling skin inflammation in a mouse model of contact hypersensitivity to haptens. CD4⁺CD25⁺FoxP3⁺ T cells upregulated ICOS expression on *in vivo* sensitization and specifically suppressed hapten-reactive CD8⁺ T cells both *in vivo* and *in vitro*, confirming that ICOS expression is important for antigen-specific suppression.¹³ We found that whereas FoxP3 was expressed in most CD4⁺CD25⁺ T cells and a subset of CD4⁺CD25⁻

T cells, high levels of ICOS expression distinguished a population of Treg cells endowed with superior suppressive capabilities. Because ICOS is a T-cell activation marker and TCR (T cell-receptor)-mediated stimulation is necessary for Treg-cell suppressive function, it is likely that the increased suppressive ability of ICOS⁺ Treg cells is related to their activated status. Furthermore, elevated ICOS expression segregated with higher levels of FoxP3 and CTLA-4 expression compared with ICOS⁻ Treg cells or CD25⁺ICOS⁺FoxP3⁺ T cells. This could explain the increased immunoregulatory properties of ICOS⁺ Treg cells given the role of these 2 molecules in the suppressive function of Treg cells.^{14,15} Finally, we also observed that ICOS⁺ Tregs are derived from the pool of naturally occurring FoxP3⁺ Treg cells on hapten sensitization and yet display T_H17/T_H1 cell features and express IFN- γ and IL-17 in addition to FoxP3 and IL-10.

The ICOS pathway has been shown to be important for the development of mucosal and respiratory tolerance that is mediated by CD4⁺CD25⁺ Treg cells.^{13,16} Furthermore, high levels of ICOS expression have been found in Treg cells involved in the control of autoimmune diabetes and atherosclerosis.^{17,18} Although the molecular mechanisms underlying the role of the ICOS pathway in Treg function are still unclear, IL-10 production has been associated with immunoregulation by ICOS-dependent Treg cells.^{16,19} In this regard, we found that ICOS⁺ Treg cells were the only CD4⁺ T-cell population to express high levels of IL-10 mRNA. This finding is in agreement with the report that high levels of ICOS correlated with synthesis of IL-10, whereas ICOS^{medium} T cells predominantly produced T_H2 effector cytokines.²⁰ In our model, CD4⁺CD25⁻ Tconv cells expressed intermediate levels of ICOS and were associated with high levels of effector cytokines IL-4 but also IFN- γ , in agreement with the role of ICOS in costimulation of T_H1 and T_H2 effector T cells depending on the immunologic context.²¹ Furthermore, examination of FoxP3⁺ and FoxP3⁻ subpopulations in the CD4⁺CD25⁻ subset showed that effector cytokines IL-4, IFN- γ , and IL-17 were mainly produced by CD4⁺CD25⁻FoxP3⁻ cells, revealing the presence of an effector FoxP3⁻ subset and a weakly suppressive FoxP3⁺ subset within the CD4⁺CD25⁻ population.

There is still some controversy regarding cytokines involved in effector and regulatory responses in CHS to haptens. Immunoregulatory cytokines IL-10 and TGF- β have been involved in the downregulation of CHS responses and induction of oral tolerance to haptens.²² In particular, IL-10 is believed to be critical for the inhibition of hapten-induced inflammation in mice and human beings.^{23,24} Although we cannot exclude a role for TGF- β , low levels of TGF- β mRNA were found in all CD4⁺ T-cell subsets, whereas highly suppressive ICOS⁺ Treg cells produced IL-10, suggesting the possible involvement of IL-10. Conversely, Treg-cell activity is intimately linked to cytokines produced during ongoing effector immune responses, particularly IL-2.²⁵ It was reported that DNFB-specific effector CD8⁺ T cells (but not CD4⁺ T cells) produce large amounts of IL-2 on DNFB sensitization and that IL-2 is essential for CD4⁺CD25⁺ T-cell regulation in CHS.²⁶ Here, we observed that the DNFB-specific IL-2 response displayed a slightly delayed kinetic relatively to IFN- γ production in dLNs of DNFB-sensitized mice, supporting the hypothesis of a sequential activation of effector and Treg-cell responses. However, experiments conducted in CD8⁺ T cell depleted mice revealed that ICOS⁺ Treg cells emerged independently of the effector CD8⁺ T-cell response (see this article's Fig E6 in the Online Repository at www.jacionline.org), suggesting

that signals necessary for the generation of ICOS⁺ Treg cells were predominantly provided by innate cells. The possible involvement of plasmacytoid dendritic cells (pDCs) in CHS in human beings²⁷ and the preferential induction of IL-10-producing Treg cells by plasmacytoid DCs expressing ICOS-ligand in inflammatory conditions²⁸ position this DC subset as a potential inducer of IL-10-producing ICOS⁺ Treg cells in CHS.

A striking characteristic of ICOS⁺FoxP3⁺ Treg cells in DNFB-sensitized mice was their production of effector cytokines IFN- γ and IL-17. Although IFN- γ and IL-17 were also expressed in ICOS⁺ Tconv cells, ICOS⁺ Treg cells could be distinguished from all other FoxP3⁺ Treg cells by their expression of IL-17 and ROR γ t. Kinetic studies of cytokine mRNA expression on naive or day 3, 6, 9, or 15 DNFB-sensitized animals showed that IL-17 and ROR γ t mRNA (along with IL-10 mRNA) were expressed at all time points, suggesting that IL-17 (and IL-10) expression is a stable feature of ICOS⁺ Treg cells. In contrast, IFN- γ expression was more transient because IFN- γ mRNA transcripts were detected only at days 3 and 6 postsensitization (data not shown). Although we could not demonstrate that effector and regulatory cytokines (IL-17/IFN- γ vs IL-10) were produced by the same cells, their concomitant expression at the population level in highly suppressive ICOS⁺ Treg cells raises important questions on its origin and function. The lack of conversion of CD4⁺FoxP3⁻ T cells into CD4⁺FoxP3⁺ T cells (Fig 5, A) suggests that IL-17-producing ICOS⁺ Treg cells do not originate from ICOS⁺ Tconv cells. Alternatively, recent findings support that Treg cells adapt to the cytokine environment created by effector T cells by expressing transcription factors, enabling Treg cells to suppress the corresponding effector T-cell subset.²⁹⁻³¹ Activation of STAT3 (signal transducer and activator of transcription 3), a T_H17-specific transcription factor, in Treg cells endows them with the ability to suppress T_H17 responses.³⁰ Similarly, Treg-cell expression of T-bet (T cell-specific T-box transcription factor) is required for Treg-cell homeostasis under conditions of induced T_H1 inflammation.³¹ In this respect, IL-17/IFN- γ -producing ICOS⁺ Treg cells could regulate specifically the generation and activation of DNFB-specific IFN- γ and IL-17-producing effector CD8⁺ T cells (Fig 1, B). Finally, it has become clear that a certain degree of plasticity exists between Treg cells and T_H17 cells.^{32,33} A subset of FoxP3⁺ Treg cells demonstrates some instability in normal homeostatic conditions, accompanied by a loss of FoxP3 and CD25 expression and the production of effector cytokines such as IFN- γ and IL-17,^{34,35} a conversion that was amplified in inflammatory settings.³⁵ Thus, in our study, one can hypothesize that on DNFB-induced inflammation, some of the suppressive IL-17-producing ICOS⁺ Treg cells are progressively turning into cells with effector phenotype, which might limit their suppressive function and their ability to control CD8⁺ effector T-cell responses fully.

In conclusion, our report identifies the existence of a highly suppressive ICOS⁺ Treg-cell population that selectively expresses high levels of FoxP3 and CTLA-4 but also IL-10, IL-17, and IFN- γ . Our findings highlight the plasticity of T-cell lineages and the need for a careful characterization of these subsets at a time when clinical trials using Treg cells are being initiated in patients.

Online Repository

Additional Methods information appears in the Online Repository. Fig E1 shows the CD8⁺ effector and CD4⁺ Treg-cell

subsets in CHS to DNFB. Fig E2 shows the kinetic and phenotypic characterization of CD4⁺ T-cell subsets defined by CD25 and ICOS expression. Fig E3 shows cytokine mRNA expression by CD4⁺ T-cell subsets. Fig E4 shows *in vitro* suppression by CD4⁺ T-cell subsets on polyclonal stimulation. Fig E5 shows ICOS expression in CD4⁺FoxP3/GFP⁺ transferred cells. Fig E6 shows the induction of ICOS⁺ Treg cells independent of effector CD8⁺ T cells *in vivo*.

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Clinical implications: The identification of ICOS as a marker for a highly suppressive antigen-specific Treg cell population that controlled skin inflammation opens new avenues for the characterization of Treg cells in patients and clinical trials.

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METHODS

mAbs and reagents

Anti-CD25, anti-ICOS, anti-CD4, anti-CD8 α , anti-CD44, anti-CD103, anti-CD62L, anti-CTLA-4, and anti-Ki67 were from BD Biosciences (Le Pont de Claix, France). Anti-FoxP3 was from eBiosciences (Clinisciences, Montrouge, France). CFSE was from Invitrogen (Cergy Pontoise, France).

Allergens

2,4-Dinitrofluorobenzene, DNBS, and oxazolone were from Sigma. DNFB and oxazolone were diluted in acetone/olive oil (AOO) vehicle (4:1 vol/vol).

Animals

BALB/c females and congenic CD45.1 and CD45.2 C57BL/6 mice were from Charles River Laboratories (L'Arbresle, France). FoxP3-GFP transgenic

mice were kindly provided by B & M Malissen (Centre d'Immunologie, Marseille-Luminy, France). All mice were housed at the institute's animal facility under specific pathogen-free conditions and used between 6 and 10 weeks of age.

Quantitative RT-PCR

The primers used in this study are as follows: ubiquitin, 5'-AAG AAT TCA GAT CGG ATG ACA CAC T-3' and 5'-GCC ACT TGG AGG TTG ACA CTT T-3'; FoxP3, 5'-CAC TGG GCT TCT GGG TAT GT-3' and 5'-TTT TAG CTT GCG GCT CCTAA-3'; IFN- γ , 5'-CCA GCG CCA AGC ATT CAATGA G-3' and 5'-GAC AAT CTC TTC CCC ACC CCG-3'; IL-4, 5'-GCA TTT TGA ACG AGG TCA CAG GAG-3' and 5'-CCT TGG AAG CCC TAC AGA CGA G-3'; IL-10, 5'-CAG AGC CAC ATG CTC CTA GA-3' and 5'-TGT CCA GCT GGT CCT TTG TT-3'; RoR γ T, 5'-CCG CTG AGA GGG CTT CAC-3' and 5'-TGC AGG AGT AGG CCA CAT TAC A-3'; and IL-17a or TGF- β , Invitrogen commercial quantitative PCR primers.

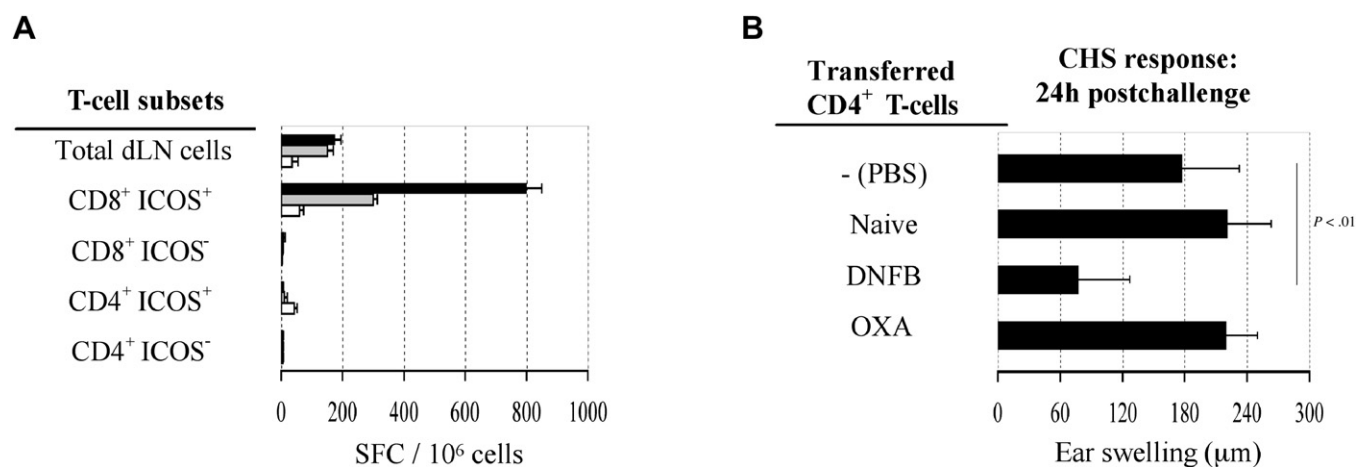


FIG E1. Effector and regulatory subsets in CHS to DNFB. **A**, Cells were collected from dLNs of day 5 DNFB-sensitized BALB/c mice, and the frequency of hapten-specific IFN- γ (black bars), IL-2 (gray bars), and IL-17 (white bars) SFCs in purified T-cell subsets was determined by ELISPOT and expressed as the mean number of SFC/10⁶ cells. **B**, Naive BALB/c mice were transferred with PBS or with 15×10^6 CD4⁺ LN T cells from naive, DNFB-sensitized, or oxazolone (OXA)-sensitized donor mice. Recipients were subsequently sensitized and challenged with DNFB, and the CHS response was determined by measuring ear swelling 24 hours postchallenge. Results are representative of 3 independent experiments with 3 to 5 mice/group.

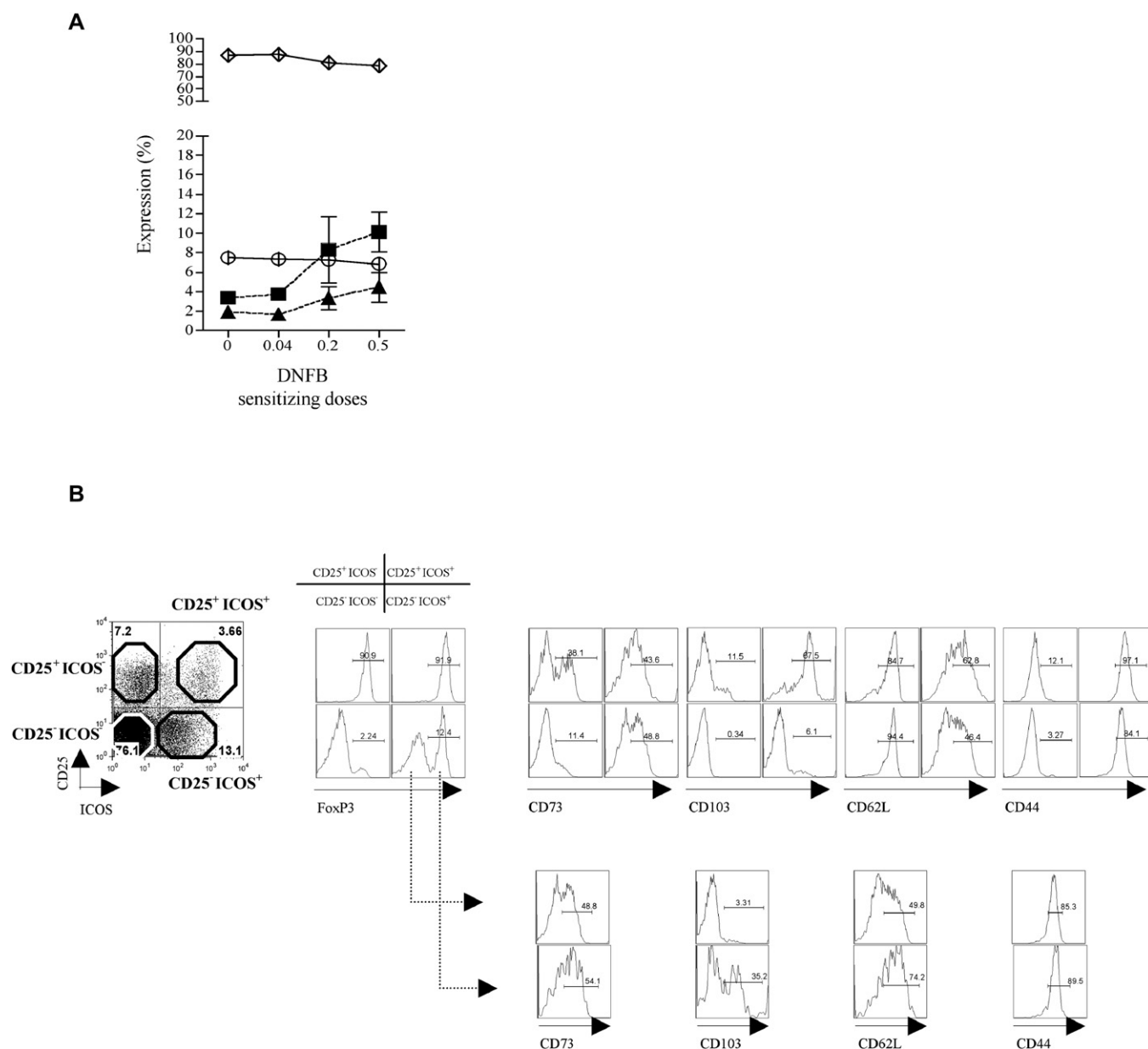


FIG E2. Characterization of CD4⁺ T-cell subsets defined by CD25 and ICOS expression. **A**, Cells were purified from dLNs at day 6 after sensitization with different doses of DNFB (0.5%, 0.2%, 0.04%) or after acetone/olive oil vehicle application. The proportion of cells expressing the CD25⁺ICOS⁻ (○), CD25⁺ICOS⁺ (▲), CD25⁻ICOS⁻ (◇), and CD25⁻ICOS⁺ (■) phenotype in the gated CD4⁺ population was then analyzed by FACS. Results are expressed as the mean values ± SDs, obtained with 3 mice per group, and they are representative of 3 independent experiments. **B**, Cells were purified from non-dLNs and dLNs of naive or BALB/c mice sensitized with 0.5% DNFB for 6 days. The percentages of cells expressing the surface markers CD73, CD103, CD44, and CD62L in electronically gated CD4⁺CD25⁺ICOS⁺, CD4⁺CD25⁺ICOS⁻, CD4⁺CD25⁻ICOS⁺, and CD4⁺CD25⁻ICOS⁻ populations were determined by FACS analysis. Results are representative of 3 independent experiments.

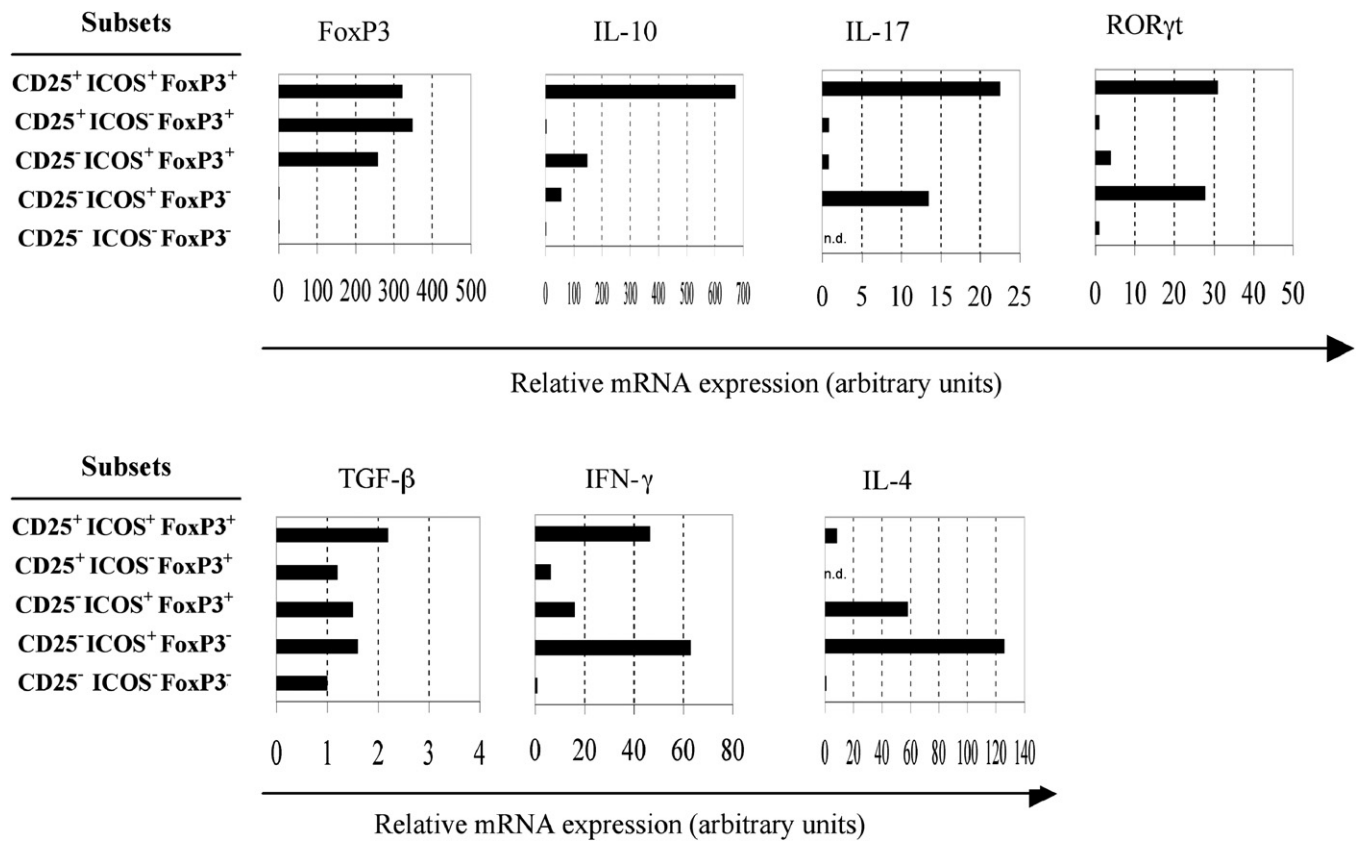


FIG E3. mRNA transcripts revealed by quantitative RT-PCR in FoxP3^{+/+} T-cell subsets. Indicated CD4⁺ cell subsets from day 6 DNFB-sensitized FoxP3-GFP transgenic mice were FACS-purified, and mRNA expression was analyzed by quantitative RT-PCR. Data are shown as mean of triplicate PCR values. Results are representative of experiments with 6 mice per group. *nd*, Not detected.

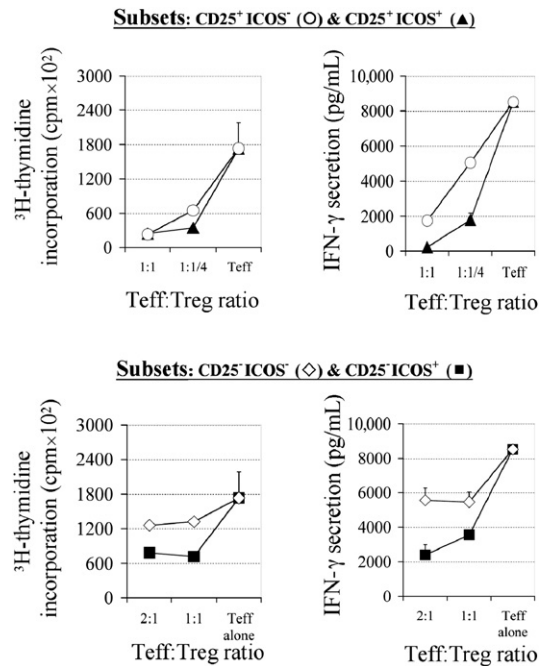


FIG E4. ICOS⁺ Treg cells display the highest suppressive capacities *in vitro* on anti-CD3 mAb stimulation. CD8⁺ effector T cells (5×10^4) were isolated from day 5 DNFB-sensitized mice and cultured with mature BMDCs (1×10^4) plus anti-CD3 mAb ($1 \mu\text{g/mL}$) in the absence or presence of different ratios of CD4⁺CD25⁺ICOS⁺ (▲), CD4⁺CD25⁺ICOS⁻ (○), CD4⁺CD25⁻ICOS⁺ (■), and CD4⁺CD25⁻ICOS⁻ (◇) T cells sorted from day 6 DNFB-sensitized animals. Proliferation was measured by ³H-thymidine incorporation, and IFN-γ secretion was measured by ELISA. Data are representative of independent experiments with 3 to 6 mice.

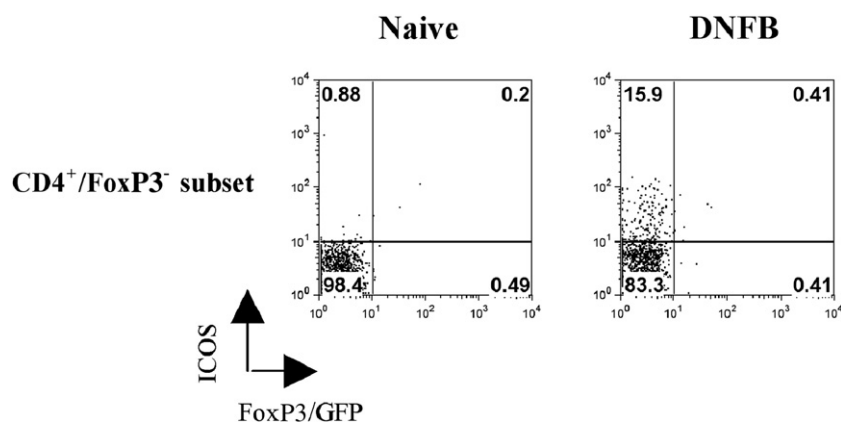


FIG E5. Naive CD4⁺FoxP3/GFP⁻ cells failed to convert in FoxP3/GFP⁺ cells on DNFB painting. Naive CD45.1 mice were transferred with 0.8×10^6 CD4⁺FoxP3/GFP⁻ splenocytes sorted from naive CD45.2 FoxP3-GFP transgenic donors. Recipients were sensitized with DNFB 1 day after transfer. dLN cells were recovered 6 days later, and expression of FoxP3 and ICOS markers in the CD4⁺CD45.2⁺ population was analyzed.

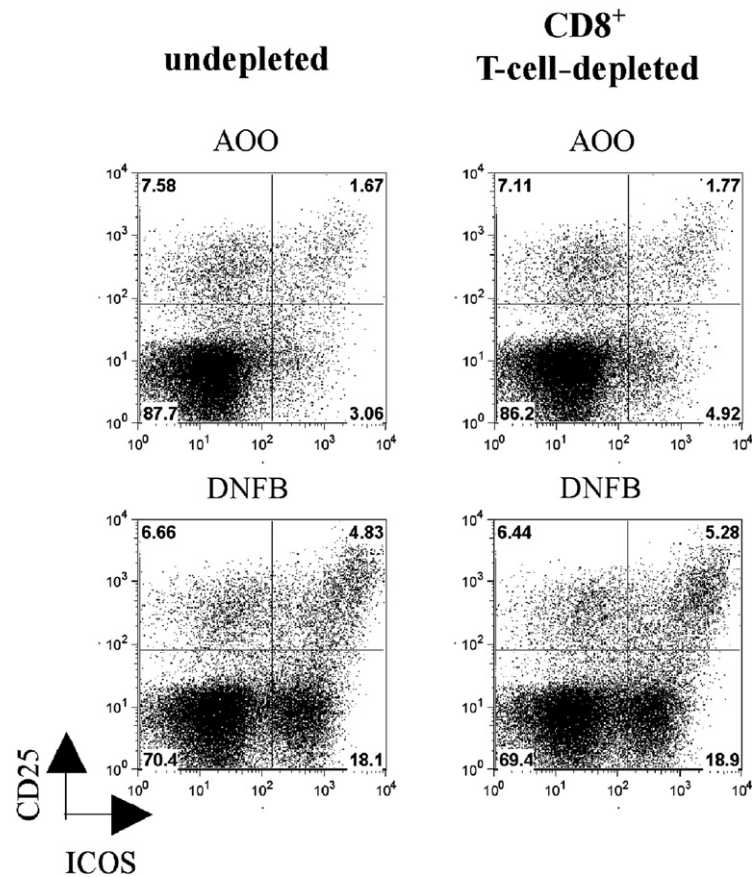


FIG E6. Induction of CD4⁺CD25⁺ICOS⁺ Treg cells occurs independently of effector CD8⁺ T cells *in vivo*. CD8⁺ T cell-depleted or undepleted BALB/c animals were sensitized with 0.5% DNFB or with the vehicle for 6 days. Cells were purified from dLNs, and expression of CD25 and ICOS in gated CD4⁺ population was analyzed by FACS. Results are representative of 3 different experiments. AOO, Acetone/olive oil.