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

Marc Vocanson, PhD,[a,b,c] Aurore Rozieres, PhD,[a,b,c] Anca Hennino, PhD,[b,c] Gaelle Poyet, MSc,[a,b,c] Vincent Gaillard, BSc,[a,b,c] Sarah Renaudineau, MSc,[b,c] Amine Achachi, PhD,[b,c] Josette Benetiere, BSc,[a,b,c] Dominique Kaiserlian, PhD,[b,c] Bertrand Dubois, PhD,[b,c] and Jean-François Nicolas, MD, PhD[a,b,c,d] *Lyon, France*

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;126:280-9.)

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

From [a]the Université Lyon1, Faculté de Médecine Lyon Sud; [b]INSERM U851; [c]Université Lyon1, IFR128; and [d]Hospices Civils de Lyon, Service Allergologie et Immunologie Clinique, Centre hospitalier Lyon-Sud.
Supported by institutional grants from Université Claude Bernard Lyon1 and INSERM.
Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.
Received for publication November 9, 2009; revised April 16, 2010; accepted for publication May 5, 2010.
Available online July 12, 2010.
Reprint requests: Jean-François Nicolas, MD, PhD, INSERM U851, IFR 128, 21 av Tony Garnier, 69375 Lyon Cx 07, France. E-mail: jean-francois.nicolas@chu-lyon.fr.
0091-6749/$36.00

doi:10.1016/j.jaci.2010.05.022

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 μL 0.5% DNFB or 100 μL 2% oxazolone on day 0, and ear-challenged with 5 μL 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 Biotech (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 FACSVantage or FACSAria. 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 μCi ^{3}H-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 μmol/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) ± 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 μg/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.