The Journal of Immunology

BRIEF REPORT | SEPTEMBER 15 2009

Cutting Edge: TGF-β1 and IL-15 Induce FOXP3$^+$ γδ Regulatory T Cells in the Presence of Antigen Stimulation[1] FREE

Rita Casetti; ... et. al

J Immunol (2009) 183 (6): 3574–3577.
https://doi.org/10.4049/jimmunol.0901334

Cutting Edge

THE JOURNAL OF IMMUNOLOGY

Cutting Edge: TGF-β1 and IL-15 Induce FOXP3$^+$ γδ Regulatory T Cells in the Presence of Antigen Stimulation[1]

Rita Casetti,[2]* Chiara Agrati,* Marianne Wallace,† Alessandra Sacchi,* Federico Martini,* Angelo Martino,* Alessandra Rinaldi,* and Miroslav Malkovsky†

Several subsets of αβ regulatory T cells (Tregs) have been described and studied intensively, but the potential regulatory role of γδ T cells remains largely unclear. Lymphocytes expressing γδ TCR are involved in both innate and adaptive immune responses, and their major adult human peripheral blood subset (Vγ9Vδ2) displays a broad reactivity against microbial agents and tumors. In this study we report that γδ T lymphocytes with regulatory functions (Vδ2 Tregs) are induced in vitro in the presence of specific Ag stimulation and cytokines (TGF-β1 and IL-15). These cells express FOXP3 and, similarly as αβ Tregs, suppress the proliferation of anti-CD3/anti-CD28 stimulated-PBMC. Phenotypic and functional analyses of Vδ2 Tregs will very likely improve our understanding about the role of γδ T cells in the pathogenesis of autoimmune, infectious, and neoplastic diseases. ***The Journal of Immunology,*** **2009, 183: 3574–3577.**

Human γδ T cells are a lymphocyte population with unusual tissue distribution and Ag recognition pathways. Vγ9Vδ2 T cells are a major subset of the circulating γδ T cell pool and are involved in immunity against pathogens and malignancies (1–9). Unlike classical αβ T cells, Vγ9Vδ2 T cells recognize nonprocessed Ags such as pyrophosphomonoesters (10). This recognition is mediated by the TCR and is not restricted by MHC molecules (11, 12). A regulatory role for γδ T cells has already been shown in a number of studies and is demonstrated by their influence on dendritic cells (13), neutrophils (14), and B cells (15) in humans, and on neutrophils (16) and macrophages (17, 18) in mice.

The immune system is responsible for protective response against pathogens and tumors and maintains immune tolerance. Regulatory T cells (Tregs)[3] identified as Forkhead/winged helix transcription factor box P3 (FOXP3)-expressing CD4$^+$ CD25^{high} T cells represent a critical aspect in the balance of the immune response, preventing the development of autoimmune diseases (19) and preserving the efficiency of antitumor immune responses (20). To date, FOXP3 is the master gene that controls Treg development/function and is currently used as the major Treg marker (21, 22). In addition, IL-10-producing T regulatory type 1 (Tr1) cells and TGF-β-producing Th3 cells induced from uncommitted peripheral CD4$^+$ T cells possess suppressive functions similar to those of naturally occurring Tregs (nTregs) (23, 24). The potential regulatory role of γδ T cells has been recently suggested but not yet clarified (17, 25, 26). In this study, we show for the first time that a subset of FOXP3-expressing Vδ2 T cells (Vδ2 Tregs) is induced under opportune Ag stimulation and cytokines.

Materials and Methods

Cell isolation and culture conditions

PBMC from healthy donors were isolated from fresh buffy coats by Lympholyte (Cedarlane) density gradient centrifugation. Cells were cultured at a concentration of 4×10^6/ml in the presence of IL-2 (6.5 U/ml), IL-15 (10 ng/ml) (both from Sigma-Aldrich), TGF-β1 (1.7 ng/ml; Calbiochem) and isopentenyl pyrophosphate (IPP; 20 μg/ml; Sigma). On days 3, 6, and 9, half of the supernatant volume was discarded and replaced with fresh medium containing cytokines. Aliquots of PBMC and bead-sorted γδ T cells (Miltenyi Biotec) were used fresh or were frozen for use at later time points.

Flow cytometry

Cells were surface stained with anti-Vδ2, anti-CD25, anti-CD69, anti-CD45RA, anti-CD45RO, anti-CD27, and anti-CD127 (BD Biosciences), fixed and permeabilized with saponin or Perm/Fix solution, and finally stained intracellularly with anti-CTLA-4 (BD Biosciences) or FOXP3 (Biolegend) according to the manufacturer's instructions. The cells were analyzed using a FACScalibur instrument with CellQuest software (BD Biosciences). Because FOXP3 is expressed intracellularly, it is not possible to use it as a marker of the Treg phenotype for purification by sorting Vδ2$^+$FOXP3$^+$ cells. Therefore, Vδ2$^+$ cells were purified from PBMC cultured to the point where the Vδ2$^+$FOXP3$^+$ population represented 30% or more of the Vδ2$^+$ cells (35.6 ± 9.7; range, 30.1–50.4), using a FACSVantage-SE cell sorter (95% purity; BD Biosciences).

*Laboratory of Cellular Immunology, National Institute for Infectious Diseases "Lazzaro Spallanzani" Instituto di Ricovero e Cura a Carattere Scientifico, Rome, Italy; and †Department of Medical Microbiology and Immunology and University of Wisconsin Comprehensive Cancer Center, University of Wisconsin School of Medicine and Public Health, Madison, WI 53792

Received for publication May 7, 2009. Accepted for publication July 16, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This study was supported by grants from the National Institutes of Health, U. S. Department of Defense, and the Italian Ministry of Health.

[2] Address correspondence and reprint requests to Dr. Rita Casetti, Laboratory of Cellular Immunology, National Institute for Infectious Diseases "Lazzaro Spallanzani" Instituto di Ricovero e Cura a Carattere Scientifico, Via Portuense 292, 00149 Rome, Italy. E-mail address: casetti@inmi.it

[3] Abbreviations used in this paper: Treg, regulatory T cell; CFDA-SE, carboxyfluorescein diacetate succinimidyl ester; FOXP3, Forkhead box P3; IPP, isopentenyl pyrophosphate.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0901334

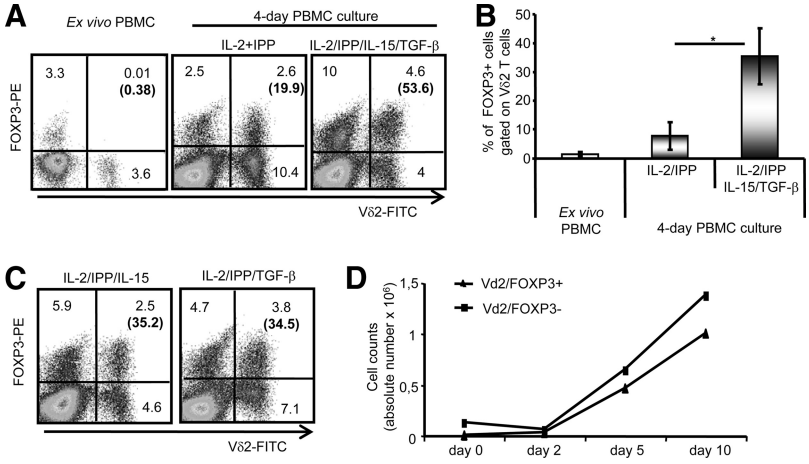

FIGURE 1. Induction and expansion of Vδ2 T cells expressing FOXP3. *A* and *C*, Cytometric dot plots from a representative experiment are shown. The percentage of FOXP3$^+$ cells among the total Vδ2 T cells is given (the bold numbers in parentheses). *B*, Mean ± SD of 14 independent experiments is shown. *D*, Kinetic of proliferation of Vδ2/FOXP3$^-$ and Vδ2/FOXP3$^+$ T cells from a representative experiment is shown. *, $p < 0.05$.

Functional immunosuppressive assay

The suppressive effect of the FOXP3$^+$-rich Vδ2 T cells on autologous PBMC was analyzed. First, PBMC were stained with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE; 6 μg/ml; Molecular Probes and Invitrogen) in PBS-FCS (5%) and stimulated with plate-bound anti-CD3 (5 μg/ml; BD Pharmingen) and anti-CD28 (10 μg/ml; eBioscience) Abs. The flow-sorted FOXP3$^+$-rich Vδ2 T cells were then added at different ratios, and the mixed lymphocyte cultures were incubated for 5 days at 37°C in a 5% CO_2-containing atmosphere. Bead-sorted γδ T cells were used as FOXP3-negative controls. IL-2 (6.5 U/ml; Sigma-Aldrich) was present in all cultures. Some cultures contained an anti-TGF-β1-blocking Ab (10 μg/ml; R&D Systems) or indomethacin (1 μg/ml; Sigma-Aldrich). In some experiments, PBMC and "effector cells" were separated by a semipermeable membrane (0.4-μm pore size, Falcon; Costar). Cell proliferation was measured by CFDA-SE through decreasing relative fluorescence intensity by half during each round of cell division by flow cytometry and analyzed by Flow Jo software (Tree Star).

Cytokine profile

Sorted FOXP3$^+$-rich Vδ2 T cells were stimulated with IPP for 18 h and then supernatants were collected. The cytokine measurements were performed using a capture ELISA (R&D Systems) or cytometric bead assays for Th1/Th2 cytokines (BD Biosciences).

Statistical analysis

Nonparametric Mann-Whitney *U* test and Wilcoxon matched pairs test were used to evaluate the statistical significance. Differences were considered significant at $p < 0.05$.

Results and Discussion

Expression of FOXP3 in Vδ2 T cells

In the presence of TGF-β1, CD4$^+$CD25$^-$ lymphocytes may differentiate into FOXP3$^+$ Tregs, suppressing T cell proliferation (27). As a first step, we analyzed the expression of FOXP3 in Vδ2 T cells. Fig. 1 shows that the frequency of FOXP3$^+$ Vδ2 T cells in freshly isolated PBMC is very low (1.6 ± 0.7%). Because Vδ2 T cells can be selectively stimulated using phosphoantigen and IL-2 (10), we analyzed the expression of FOXP3 on Vδ2 T cells under IPP/IL-2 stimulation in the presence of Treg-promoting cytokines such as IL-15 and TGF-β1 (28, 29). After 4 days of culture, IL-2/IPP-stimulated Vδ2 T cells expressed higher levels of FOXP3 in the presence of TGF-β1 and IL-15 (35.6 ± 9.7%; range, 30.1–50.4%) than in their absence (7.9 ± 4.9%) (Fig. 1, *A* and *B*). Interestingly, IL-15 or TGF-β1 alone (Fig. 1*C*) may induce a considerable expression of FOXP3 on Vδ2 T cells, but lower than that obtained in combination. Experiments using only cytokines without IPP were also performed, showing that FOXP3 induction requires Ag stimulation (data not shown). Furthermore, FOXP3$^+$ Vδ2 T cells were able to proliferate as well as FOXP3$^-$ Vδ2 T cells (Fig. 1*D*). These data suggest that the conventional Treg cytokine milieu may induce the expression of FOXP3 in Vδ2 T cells.

TGF-β1/IL-15-stimulated Vδ2 T cells stably express FOXP3 and activation-related molecules

FOXP3 may be transiently expressed in activated nonregulatory T cell populations (30, 31). To assess the transient-vs-stable expression of the FOXP3 in Vδ2 T cells, we performed FOXP3 staining daily during a 10-day period. Fig. 2*A* illustrates the kinetics of FOXP3 expression, which started to increase after 1 day of culture (day 1, 3.6 ± 0.9 vs day 0, 1.6 ± 0.7; $p < 0.05$), peaked on day 3 (day 3, 38.5 ± 10.7 vs day 1, 3.6 ± 0.9; $p < 0.05$), and remained high over 10 days.

Moreover, FOXP3$^+$ Vδ2 T cells expressed significantly higher levels of CD45RA (17.9 ± 5.3 vs 6.45 ± 2.4; $p < 0.05$) and CD69 (16.1 ± 6.7 vs 6.3 ± 1.5; $p < 0.05$) than FOXP3$^-$ Vδ2 T cells. Higher levels of CTLA-4 (215 ± 77.2 vs 113 ± 61.3), CD25 (113.6 ± 54.8 vs 74.4 ± 39.3), and HLA-DR (36.5 ± 6.3 vs 23.7 ± 11.6) were also observed, although the median fluorescence intensity values were not significantly different. The expression of CD27 (13.5 ± 8.0 vs 12 ± 7.9) and CD45R0 (20.2 ± 10.5 vs 15.9 ± 5.1) was similar in both populations. CD127, which has been reported to be inversely correlated with FOXP3 expression in human CD4$^+$ Treg cells (32), was comparably expressed by FOXP3$^+$ (11.9 ± 3.4) and FOXP3$^-$ (15 ± 4) Vγ9Vδ2 T cells (Fig. 2*B*). Thus, FOXP3$^+$ Vδ2 T cells display an activated phenotype with an increased expression of CTLA-4 molecules that are also present on the cell surface of both adaptive and natural αβ Tregs (33).

FOXP3$^+$-rich Vδ2 T cells inhibit PBMC proliferation

We then evaluated the functional ability of cultured Vδ2$^+$ cells, of which a high proportion (>30%) express FOXP3, to inhibit PBMC proliferation. The stimulation of CFDA SE-labeled PBMC with anti-CD3/anti-CD28 Ab-induced cell proliferation (Fig. 3*A*) was significantly reduced in the presence of flow-sorted, FOXP3$^+$-rich Vδ2 T cells (Fig. 3*C*). The addition of ex vivo bead-purified Vδ2$^+$ γδ T cells,

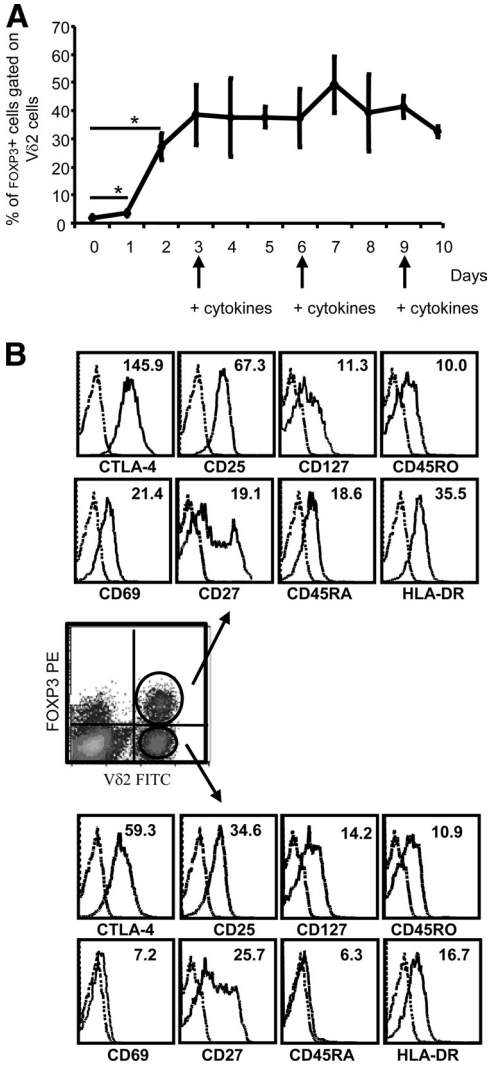

FIGURE 2. Time course of FOXP3 expression in Vδ2 T cells and phenotypic analyses. *A*, The percentage of FOXP3$^+$ Vδ2 T cells during a 10-day culture period is shown. Means ± SD of three independent experiments are shown ($p < 0.05$). *B*, Phenotypic analysis of FOXP3$^+$ (*upper histograms*) and FOXP3$^-$ (*lower histograms*) Vδ2 T cells on day 4 of culture. Dotted histograms indicate the cells stained with isotype control Abs. Numbers in each panel show the median fluorescence intensity. One of three independent experiments is shown.

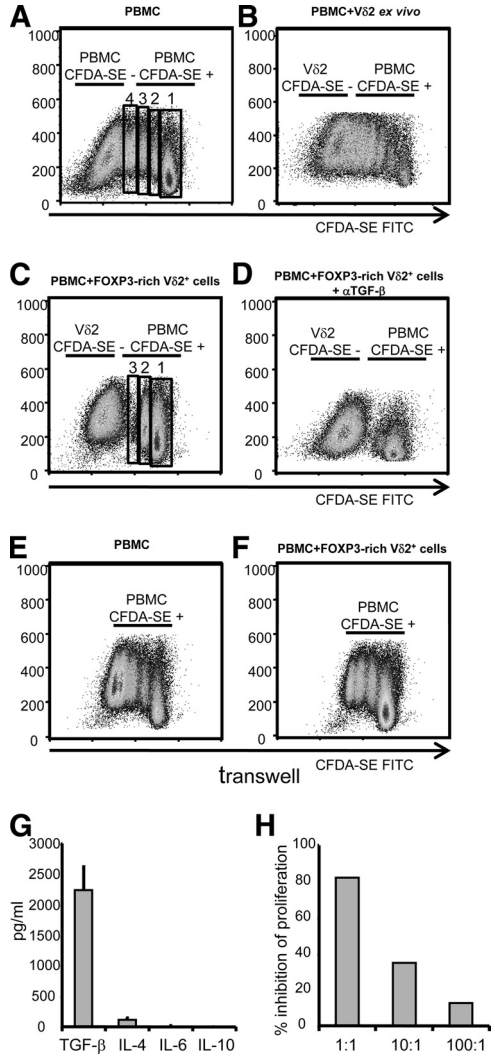

FIGURE 3. Inhibition assay of FOXP3-rich Vδ2$^+$ cells and cytokine profile. Autologous CFDA SE-labeled PBMC were stimulated with anti-CD3/CD28 Abs and cultured with unlabeled PBMC (*A*), unlabeled bead-purified ex vivo Vδ2 T cells (*B*), unlabeled FOXP3-rich Vδ2$^+$ cells (*C*), or unlabeled FOXP3-rich Vδ2$^+$ cells in the presence of anti-TGF-β mAb (*D*). CFDA SE-labeled PBMC were separated from unlabeled PBMC (*E*) or unlabeled FOXP3-rich Vδ2$^+$ cells (*F*) by a semipermeable membrane. PBMC:Vδ2 ratios were 1:1 in all the conditions. The numbers in *A* and *C* represent the percentage of cells in each round of cell division: *A*, 1 = 21.9, 2 = 9.7, 3 = 9.7, 4 = 16.5; *C*, 1 = 33.3, 2 = 18, 3 = <2. One of 10 independent experiments is shown. *G*, The cytokine levels in supernatants from IPP-stimulated sorted FOXP3-rich Vδ2$^+$ cells were analyzed. Means ± SD of three independent experiments are shown. *H*, Different PBMC:Vδ2 ratios (1:1, 10:1, and 100:1) are shown.

which are almost entirely FOXP3-negative, had no inhibitory effect (Fig. 3*B*). Surprisingly, the TGF-β1-neutralizing Ab was not able to totally abrogate the inhibitory activity of Vδ2 Tregs (Fig. 3*D*), whereas FOXP3$^+$-rich Vδ2 T cells produced high amounts of TGF-β1 (2,231.2 ± 390.2 pg/ml) (Fig. 3*G*). Furthermore, FOXP3$^+$-rich Vδ2 T cells produced moderate amounts of IL-4 (108.3 ± 39.1 pg/ml) and low levels of IL-6 (7.6 ± 7.4 pg/ml) and IL-10 (1.8 ± 2.1 pg/ml) (Fig. 3*G*). Given that, to assess the role of cell contact in the inhibitory activity we performed Transwell experiments, which showed that PBMC proliferation was restored when FOXP3$^+$-rich Vδ2 T cells were physically separated from effector PBMC (Fig. 3, *E* and *F*). These results suggest that, differently than T regulatory type 1 (Tr1) and Th3 regulatory cells, which suppress largely by cytokines, the feature of FOXP3$^+$-rich Vδ2 T cells appear to be similar to that of CD4$^+$CD25^{high} naturally occurring Tregs (nTregs) (34, 35). Moreover, the level of the proliferation-blocking effect was dependent on the ratio of PBMC to FOXP3$^+$-rich Vδ2 T cells; the strongest effect was observed at a 1:1 target: FOXP3$^+$-rich Vδ2 T cell effector ratio (Fig. 3*H*).

We conclude that the TGF-β1 signaling pathway, known to regulate many immune processes, appears to be also involved in inducing γδ Tregs similarly as it does for αβ Tregs. Differently from αβ, the combination of TGF-β1, IL-15, and TCR stimulation may be central in this process, whose detailed molecular mechanisms nevertheless remain to be clarified. Vδ2 Tregs may be expanded and potentially exploited therapeutically in a variety of clinical situations.

Acknowledgments

We dedicate this work in memoriam to our dearest friend Fabrizio Poccia, who was ne plus ultra of Vγ9Vδ2 T cell research. It was a great privilege and an immense pleasure for us to have been associated with Fabrizio. We will miss forever his friendship and infectious enthusiasm for immunological investigations.

Disclosures

The authors have no financial conflict of interest.

References

1. Poccia, F., M. L. Gougeon, M. Bonneville, M. Lopez-Botet, A. Moretta, L. Battistini, M. Fallace, V. Colizzi, and M. Malkovsky. 1998. Innate T-cell immunity to nonpeptidic antigens. *Immunol. Today* 19: 253–256.
2. Poccia, F., C. Agrati, G. Ippolito, V. Colizzi, and M. Malkovsky. 2001. Natural T cell immunity to intracellular pathogens and nonpeptidic immunoregulatory drugs. *Curr. Mol. Med.* 1: 137–151.
3. Ferrarini, M., E. Ferrero, L. Dagna, A. Poggi, and M. R. Zocchi. 2002. Human γδ T cells: a nonredundant system in the immune-surveillance against cancer. *Trends Immunol.* 23: 14–18.
4. Poccia, F., C. Agrati, F. Martini, M. R. Capobianchi, M. Wallace, and M. Malkovsky. 2005. Antiviral reactivities of γδ T cells. *Microbes Infect.* 7: 518–528.
5. Choudhary, A., F. Davodeau, A. Moreau, M. A. Peyrat, M. Bonneville, and F. Jotereau. 1995. Selective lysis of autologous tumor cells by recurrent γδ tumor-infiltrating lymphocytes from renal carcinoma. *J. Immunol.* 154: 3932–3940.
6. Kunzmann, V., E. Bauer, J. Feurle, F. Weissinger, H. P. Tony, and M. Wilhelm. 2000. Stimulation of γδ T cells by aminobisphosphonates and induction of anti-plasma cell activity in multiple myeloma. *Blood* 96: 384–392.
7. Kato, Y., Y. Tanaka, F. Miyagawa, S. Yamashita, and N. Minato. 2001. Targeting of tumor cells for human γδ T cells by nonpeptide antigens. *J. Immunol.* 167: 5092–5098.
8. Viey, E., G. Fromont, B. Escudier, Y. Morel, S. Da Rocha, S. Chouaib, and A. Caignard. 2005. Phosphostim-activated γδ T cells kill autologous metastatic renal cell carcinoma. *J. Immunol.* 174: 1338–1347.
9. Dieli, F., D. Vermijlen, F. Fulfaro, N. Caccamo, S. Meraviglia, G. Cicero, A. Roberts, S. Buccheri, M. D'Asaro, N. Gebbia, et al. 2007. Targeting human γδ T cells with zoledronate and interleukin-2 for immunotherapy of hormone-refractory prostate cancer. *Cancer Res.* 67: 7450–7457.
10. Tanaka, Y., C. T. Morita, Y. Tanaka, E. Nieves, M. B. Brenner, and B. R. Bloom. 1995. Natural and synthetic non-peptide antigens recognized by human γδ T cells. *Nature* 375: 155–158.
11. Bukowsky, J. F., C. T. Morita, Y. Tanaka, B. R. Bloom, M. B. Brenner, and H. Band. 1995. Vγ2Vδ2 TCR-dependent recognition of non-peptide antigens and Daudi cells analyzed by TCR gene transfer. *J. Immunol.* 154: 998–1006.
12. Morita, C. T. 1995. Direct presentation of nonpeptide prenyl pyrophosphate antigens to human γδ T cells. *Immunity* 3: 495–507.
13. Martino, A., R. Casetti, A. D'Alessandri, A. Sacchi, and F. Poccia. 2005. Complementary function of γδ T-lymphocytes and dendritic cells in the response to isopentenyl-pyrophosphate and lipopolysaccharide antigens. *J. Clin. Immunol.* 25: 230–237.
14. Agrati, C., E. Cimini, A. Sacchi, V. Bordoni, C. Gioia, R. Casetti, F. Turchi, M. Tripodi, and F. Martini. 2009. Activated Vγ9Vδ2 T cells trigger granulocyte functions via MCP-2 release. *J. Immunol.* 182: 522–529.
15. Caccamo, N., L. Battistini, M. Bonneville, F. Poccia, J. J. Fournié, S. Meraviglia, G. Borsellino, R. A. Kroczek, C. La Mendola, E. Scotet, et al. 2006. CXCR5 identifies a subset of Vγ9Vδ2 T cells which secrete IL-4 and IL-10 and help B cells for antibody production. *J. Immunol.* 177: 5290–5295.
16. D'Souza, C. D., A. M. Cooper, A. A. Frank, R. J. Mazzaccaro, B. R. Bloom, and I. M. Orme. 1997. An anti-inflammatory role for γ δ T lymphocytes in acquired immunity to *Mycobacterium tuberculosis*. *J. Immunol.* 158: 1217–1221.
17. Egan, P. J., and S. R. Carding. 2000. Downmodulation of the inflammatory response to bacterial infection by γδ T cells cytotoxic for activated macrophages. *J. Exp. Med.* 191: 2145–2158.
18. Tagawa, T., H. Nishimura, T. Yajima, H. Hara, K. Kishihara, G. Matsuzaki, I. Yoshino, Y. Maehara, and Y. Hoshikai. 2004. Vδ1$^+$ γδ T cells producing CC chemokines may bridge a gap between neutrophils and macrophages in innate immunity during *Escherichia coli* infection in mice. *J. Immunol.* 173: 5156–5164.
19. Lu, L. F., E. F. Lind, D. C. Gondek, K. A. Bennett, M. W. Gleeson, K. Pino-Lagos, Z. A. Scott, A. J. Coyle, J. L. Reed, J. Van Snick, et al. 2006. Mast cells are essential intermediaries in regulatory T cell tolerance. *Nature* 442: 997–1002.
20. Wang, R. F. 2006. Regulatory T cells and Toll-like receptors in cancer therapy. *Cancer Res.* 66: 4987–4990.
21. Schubert, L. A., E. Jeffery, Y. Zhang, F. Ramsdell, and S. F. Ziegler. 2001. Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation. *J. Biol. Chem.* 276: 37672–37679.
22. Khattri, R., D. Kasprowicz, T. Cox, M. Mortrud, M. W. Appleby, M. E. Brunkow, S. F. Ziegler, and F. Ramsdell. 2003. An essential role for Scurfin in CD4$^+$CD25$^+$ T regulatory cells. *Nat. Immunol.* 4: 337–342.
23. Bergmann, C., L. Strauss, Y. Wang, M. J. Szczepanski, S. Lang, J. T. Johnson, and T. L. Whiteside. 2008. T regulatory type 1 cells in squamous cell carcinoma of the head and neck mechanisms of suppression and expansion in advanced diseased. *Clin. Cancer Res.* 14: 3706–3715.
24. Faria, A. M., and H. L. Winer. 2006. Oral tolerance and TGF-β-producing cells. *Inflamm. Allergy Drug Targets* 5: 179–180.
25. Lahn, M., A. Kanehiro, K. Takeda, A. Joetham, J. Schwarze, G. Köhler, R. O'Brien, E. W. Gelfand, and W. Born. 1999. Negative regulation of airway responsiveness that is dependent on γδ T cells and independent of αβ T cells. *Nat. Med.* 5: 1150–1156.
26. Nagaeva, O. L. Jonsson, and L. Micheva-Nilsson. 2002. Dominant IL-10 and TGF-β mRNA expression in γδ T cells of humans early pregnancy deciduas suggests immunoregulatory potential. *Am. J. Reprod. Immunol.* 48: 9–17.
27. Chen, W., W. Jin, N. Hardegen, K. J. Lei, L. Li, N. Marinos, G. McGrady, and S. M. Wahl. 2003. Conversion of peripheral CD4$^+$CD25$^-$ naive T cells to CD4$^+$CD25$^+$ regulatory T cells by TGF-β induction of transcription factor Foxp3. *J. Exp. Med.* 198: 1875–1886.
28. Imamichi, H., I. Sereti, and H. C. Lane. 2008. IL-15 acts as a potent inducer of CD4$^+$CD25^{hi} cells expressing FOXP3. *Eur. J. Immunol.* 38: 1621–1630.
29. Rao, P. E., A. L. Petrone, and P. D. Ponath. 2005. Differentiation and expansion of T cells with regulatory function from human peripheral lymphocytes by stimulation in the presence of TGF-β. *J. Immunol.* 174: 1446–1455.
30. Wang, J., A. Ioan-Facsinay, E. I. van der Voort, T. W. Huizinga, and R. E. Toes. 2007. Transient expression of FOXP3 in human activated nonregulatory CD4$^+$ T cells. *Eur. J. Immunol.* 37: 129–138.
31. Pillai, V., S. B. Ortega, C. K. Wang, and N. J. Karandikar. 2007. Transient regulatory T-cells: a state attained by all activated human T-cells. *Clin. Immunol.* 123: 18–29.
32. Liu, W., A. L. Putnam, Z. Xu-Yu, G. L. Szot, M. R. Lee, S. Zhu, P. A. Gottlieb, P. Kapranov, T. R. Gingeras, B. Fazekas de St Groth, et al. 2006. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4$^+$ Treg cells. *J. Exp. Med.* 203: 1701–1711.
33. Rallón, N. I., M. López, V. Soriano, J. García-Samaniego, M. Romero, P. Labarga, P. García-Gasco, J. González-Lahoz, and J. M. Benito. 2009. Level, phenotype and activation status of CD4$^+$FoxP3$^+$ regulatory T cells in patients chronically infected with human immunodeficiency virus and/or hepatitis C virus. *Clin. Exp. Immunol.* 155: 35–43.
34. Gregori, S., R. Bacchetta, L. Passerini, M. K. Levings, and M. G. Roncarolo. 2007. Isolation, expansion, and characterization of human natural and adaptive regulatory T cells. *Methods Mol. Biol.* 380: 83–105.
35. Faria, A. M., and H. L. Weiner. 2006. Oral tolerance and TGF-β-producing cells. *Inflamm. Allergy Drug Targets* 5: 179–190.