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Expression of ICOS In Vivo Defines CD4$^+$ Effector T Cells with High Inflammatory Potential and a Strong Bias for Secretion of Interleukin 10

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Abstract

The studies performed to date analyzed the overall participation of the inducible costimulator (ICOS) in model diseases, but did not yield information on the nature and function of ICOS-expressing T cells in vivo. We examined ICOS$^+$ T cells in the secondary lymphoid organs of nonmanipulated mice, in the context of an "unbiased" immune system shaped by environmental antigens. Using single cell analysis, ICOS^{low} cells were found to be loosely associated with the early cytokines interleukin (IL)-2, IL-3, IL-6, and interferon (IFN)-γ. ICOS^{medium} cells, the large majority of ICOS$^+$ T cells in vivo, were very tightly associated with the synthesis of the T helper type 2 (Th2) cytokines IL-4, IL-5, and IL-13, and these cells exhibited potent inflammatory effects in vivo. In contrast, ICOS^{high} T cells were highly and selectively linked to the anti-inflammatory cytokine IL-10. Overall, these data seem to indicate that ICOS cell surface density serves as a regulatory mechanism for the release of cytokines with different immunological properties. Further in vivo functional experiments with in vitro–activated T cells strongly suggested that the ICOS$^+$ population, although representing in vivo only around 10% of T cells bearing early or late activation markers, nevertheless encompasses virtually all effector T cells, a finding with major diagnostic and therapeutic implications.

Key words: T helper cell • T lymphocyte subsets • Th2 cells • cytokine • inflammation

Introduction

Inducible costimulator (ICOS)* is a T cell–specific cell surface molecule structurally related to CD28 and CTLA-4 (1, 2). Early in vitro functional studies revealed that ICOS had a costimulatory potential for the activation of T cells similar to CD28 (1), but also revealed striking differences. In contrast to the constitutive expression of CD28, up-regulation of ICOS requires prior activation of T cells (1, 2). Both costimulatory molecules can amplify the secretion of a broad spectrum of cytokines, but only CD28 coinduced substantial amounts of IL-2, whereas ICOS showed a certain preference for the coinduction of IL-10 (1). Early in vitro and in vivo experiments led to the paradigm that ICOS is preferentially involved in Th2 immune responses (3, 4), but subsequent work also demonstrated an essential contribution of ICOS to the pathogenesis of Th1-based murine models (5–7) and thus challenged this paradigm. Mice with a deleted ICOS gene revealed defects in the generation of IL-4 and IL-13, but have an intact production of IL-10 on stimulation in vitro (8). Thus, a number of studies suggested that ICOS is linked to the secretion of certain cytokines, but did not examine this important issue in detail.

Experiments performed to date on the function of ICOS in vivo were typically performed using reagents blocking the ICOS/ICOS-L interaction, and were designed to evaluate global parameters such as clinical scores, transplant survival rates, or cytokine levels in the body (3, 5, 6, 9–11). This approach yielded important information on the over-

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*Abbreviations used in this paper: BAL, bronchoalveolar lavage; Dig, digoxigenin; EC, endothelial cell; ICOS, inducible costimulator; MACS, magnetic cell sorting; pLN, peripheral LN; PP, Peyer's patch.

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all biological role of ICOS and its potential for therapeutic intervention, but did not provide any direct information on the nature of ICOS-bearing T cells in vivo. Therefore, the exact function of $ICOS^+$ T cells still remains ill defined.

In the presented work, we characterized for the first time ICOS-expressing cells obtained directly ex vivo. We chose to study the unmanipulated mouse in order to be able to analyze $ICOS^+$ cells in the context of an "unbiased" immune system at steady state. We examined ICOS-expressing T cells from central lymphoid compartments, where naive T cells encounter antigen and undergo a series of differentiation steps before becoming effector cells. The studies were performed at the single cell level, yielding maximal information on the expression of cell surface antigens and single cytokines. We observed for the first time an intriguing correlation between the expression levels of a cell surface molecule and the type of cytokine produced, suggesting a novel regulatory mechanism in vivo: in $CD4^+$ T cells, intermediate levels of ICOS were tightly correlated to the synthesis of the proinflammatory Th2 cytokines IL-4, IL-5, and IL-13, whereas high levels of ICOS were correlated to the synthesis of the antiinflammatory cytokine IL-10. In fact, $CD4^+$ T cells expressing high levels of ICOS predominantly secreted IL-10. Further cytokine expression and functional studies demonstrated that ICOS is clearly superior to the available early and late activation markers in defining effector T cells.

Materials and Methods

Mice. Mice transgenic for the OVA-specific DO11.10 T cell receptor (OVA-TCR; reference 12) were from Dr. D. Loh, Washington University School of Medicine, St. Louis, MO and maintained on the BALB/c background. BALB/c mice were bred under SPF conditions and kept under SPF conditions or under conventional conditions at two different facilities. All animal experiments were performed in accordance with institutional and state guidelines.

Generation of ICOS-specific mAb. Lewis rats were immunized with a murine ICOS-rabbit-Ig fusion protein (13). Spleen cells were fused with myeloma P3X63Ag8.653 (American Type Culture Collection) and the resulting hybridomas were screened by flow cytometry using L-cells transfected with murine ICOS (13). From 12 mAb obtained, MIC-280 (rat IgG2b) gave the brightest staining in flow cytometry and was used in this study.

Phenotypic Analysis of Cells from Secondary Lymphoid Tissue. Single cell suspensions from spleens, peripheral lymph nodes (pLN; axillary, inguinal, popliteal, illeosacral, and cervicalis superficialis), Peyer's patches (PP), and appendix-associated lymphoid follicles were prepared from normal, 6-mo-old BALB/c mice. The cells were stained with digoxigenized anti-murine ICOS mAb (MIC-280), FITC-labeled anti-CD4 mAb (GK1.5; American Type Culture Collection), and biotinylated mAb against activation markers CD69 (H1.2F3), CD25 (7D4), CD44 (IM7), CD45RB (23G2; all from BD Biosciences), and CD62L (MEL-14; American Type Culture Collection). To prevent unspecific binding of mAb, all samples were preincubated with blocking anti-FcγRII/III mAb (2.4G2; 100 μg/ml; American Type Culture Collection) and purified rat IgG (200 μg/ml; Nordic) 10 min before and during staining with the mAb. Cy5-conjugated anti-digoxigenin (Dig) Fab (Roche Biochemicals) and streptavidin-PE (BD Biosciences) were used as secondary reagents.

Specificity of staining with the digoxigenized anti-ICOS mAb MIC-280 was controlled by preincubating cells with a 100-fold excess of unconjugated MIC-280. Samples (~200,000 cells) were analyzed on a FACSCalibur™ using CELLQuest™ software (Becton Dickinson). Analysis gates were set on viable $CD4^+$ T cells, based on forward and side light scatter and exclusion of propidium iodide–positive cells (0.3 μg/ml; Sigma-Aldrich).

Magnetic Cell Sorting. Single cell suspensions from pLN, spleens, and PP of normal, 3–8-mo-old BALB/c mice or from in vitro cultures of DO11.10 spleen cells were stained with digoxigenized anti-ICOS mAb MIC-280 for 10 min on ice, followed by anti-Dig Fab conjugated to magnetic beads (Miltenyi Biotec) for 15 min, and anti-Dig-Cy5 for additional 5 min at 10°C. To prevent unspecific binding of the anti-ICOS mAb, the cells were preincubated with blocking anti-FcγRII/III mAb 2.4G2 (200 μg/ml) and purified rat IgG (500 μg/ml) as described above. Cells were washed and passed over a "mock" LS magnetic cell sorting (MACS®) column (Miltenyi Biotec), which was not inserted into the magnetic field, to remove cells that bound nonspecifically to the column. $ICOS^+$ cells were obtained by two consecutive passes over LS columns inserted into a MidiMACS® magnet (Miltenyi Biotec). To stringently deplete $ICOS^+$ cells from the cells that passed through the column, the cells of the first passage fraction were reapplied to a new LS column, to which a 26-gauge needle was attached to restrict the flow rate, thus removing also cells expressing ICOS only at a low level.

Flow Cytometric Analysis for Intracellular Cytokines. Cells were suspended at 10^6/ml in complete RPMI 1640, supplemented with 10% fetal calf serum, 0.3 mg/ml glutamine, 10 μM 2-mercaptoethanol, 100 U/ml penicillin, and 0.1 mg/ml streptomycin and stimulated with phorbol 12-myristate 13-acetate (PMA; 10 ng/ml) and ionomycin (1 μg/ml), with Brefeldin A (5 μg/ml; all from Sigma-Aldrich) added at 2 h. After 4.5–5 h, the cells were washed in PBS and fixed with 2% formaldehyde in PBS for 15 min at RT. Fixed cells were washed in PBS supplemented with 0.5% BSA and 0.02% sodium azide (PBS/BSA/azide), and stained with biotinylated anti-CD4 mAb (GK1.5), followed by streptavidin-PerCP (BD Biosciences). For intracellular cytokine detection, cells were permeabilized with 0.5% saponin (Sigma-Aldrich) in PBS/BSA/azide and stained with two of the following rat anti–mouse cytokine mAb conjugated to PE or FITC for 15 min at RT: PE-coupled anti–IL-3 (8F8), anti–IL-4 (11B11), anti–IL-6 (20F3), anti–IL-10 (JES5–16E3; all from BD Biosciences), and anti–IL-13 (38213.11; R&D Systems), and FITC-coupled anti–IFN-γ (AN18.17.24; ref. 14), anti–IL-2 (S4B6; American Type Culture Collection), anti–IL-5 (TRFK5), anti–TNF-α (MP6-XT22), and anti–GM-CSF (22E9; all from BD Biosciences). PE- or FITC-labeled isotype control mAb (BD Biosciences) were used at the same concentrations as the respective anti-cytokine mAb. Unspecific binding of the mAb was blocked by preincubating the fixed cells with blocking anti-FcgRII/III mAb 2.4G2 (50 μg/ml) and purified rat IgG (200 μg/ml) in 0.5% saponin in PBS/BSA/azide. After intracellular cytokine staining, cells were washed twice in 0.5% saponin in PBS/BSA/azide. Samples (~200,000 cells) were analyzed by four-color flow cytometry on a FACSCalibur™.

Analysis of the Correlation Between ICOS Expression and Cytokine Production. Cells from pLN of normal BALB/c mice were stained for ICOS, stimulated, and further stained for CD4 and intracellular cytokines. ICOS surface expression (gated on $CD4^+$ cells) was plotted against the staining intensity of single intracellu-

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lar cytokines. In this plot, adjacent analysis regions containing at least 1,000 $CD4^+$ cells with a distinct ICOS staining intensity were defined. The percentage of cytokine-positive cells in each analysis region was determined and plotted against the mean fluorescence intensity of ICOS expression. To cope with the extreme rareness of ICOS-bright cells in unseparated pLN cells obtained directly ex vivo, data for cells with an ICOS fluorescence intensity ≥100 were obtained from ICOS-enriched $CD4^+$ pLN cells stimulated and stained in the same experiment.

Cytokine ELISA. Supernatants from cell cultures stimulated for 20–24 h with PMA and ionomycin were analyzed for different cytokines by sandwich ELISA. For the quantitation of IL-2, IFN-γ, IL-4, IL-5, and IL-10 we used JES6–1A12, R4–6A2, 11B11, TRFK5, and SXC-1 as coating mAb and biotinylated JES6–5H4, AN18.17.24, BVD6–24G2, TRFK4, and JES5–2A5 as detection mAb, respectively.

In Vitro Stimulation of OVA-specific T Cells. Spleen cells from DO11.10 mice, which had been magnetically depleted of $CD8^+$ cells, were stimulated with the peptide $OVA_{323–339}$ (0.2 μM; synthesized by Dr. P. Henklein, Charité, Berlin, Germany) at 2×10^6/ml in complete RPMI. Before magnetic sorting, dead cells were removed by Ficoll-gradient centrifugation (Ficoll Histopaque®-1083; Sigma-Aldrich).

Design and Analysis of the Adoptive Transfer Animal Model. Unseparated, ICOS-depleted, and ICOS-enriched fractions containing identical numbers of $CD4^+KJ1–26^+$ T cells (1.5×10^6/mouse) were injected intravenously into the tail vein of recipient BALB/c mice (6–8 animals per group, 4 animals in control group). The recipient mice had been exposed to an aerosol (for 20 min) of 1% OVA (Sigma-Aldrich) in PBS or, as a control, PBS without OVA 24 h before. 24 h and 48 h after cell transfer, the recipients were again exposed to OVA or PBS nebulization. Mice were killed on day 4. Lungs were washed via the trachea with 2 ml of PBS, and the cells analyzed by flow cytometry or centrifuged onto slides. Cells on slides were stained with Diff-Quick® (Dade AG) and differentiated by counting at least 200 cells. Lungs were inflated through the trachea with 1.6 ml of 50% Tissue-Tek (Miles Diagnostics) in PBS, and the right lower lobe was snap-frozen in cold isopentane. Lung cryostat sections (8 μm) were stained with mAb against CD3 (KT3; reference 15), CD45R/B220 (RA3–6B2; reference 16), F4/80 (F4/80; American Type Culture Collection), and Ly-6G (RB6–8C5; ref. 17), using the APAAP technique (18), and analyzed by light microscopy. Major basic protein (MBP; reference 19) was stained with a polyclonal rabbit anti–mouse Ab (provided by Drs. G. Gleich and J. Lee, Mayo Clinic, Scottsdale, AZ, and Rochester, MN; reference 20), followed by anti–rabbit-Ig-FITC, and analyzed on a LSM-510 laser scanning microscope (Carl Zeiss Microimaging, Inc.). Cells obtained from peribronchial or inguinal lymph nodes or bronchoalveolar lavage (BAL) were stained with a combination of mAb directed to ICOS (MIC-280-PE), OVA-TCR (KJ1–26-Dig, followed by anti-Dig-Cy5), CD62L (MEL-14-FITC), or CD69 (H1.2F3-FITC).

Results

ICOS Is Expressed on a Subpopulation of Antigen-experienced $CD4^+$ T Cells In Vivo. We generated mAb MIC-280, an antibody with optimal flow cytometry staining properties, and analyzed the expression of ICOS in vivo. Around 2.5% of $CD4^+$ T cells from pLN, 6% of $CD4^+$ T cells from PP, and 1.1% of splenic $CD4^+$ T cells from non-immunized mice expressed ICOS, but only a very small fraction of $CD8^+$ T cells (less than 0.3% in pLN). We therefore focused our further analysis on $CD4^+$ T cells. Using the expression density of CD44 and CD45RB as criteria, >95% of $ICOS^+$ $CD4^+$ cells in vivo were T cells with previous antigen exposure (Fig. 1 A). However, ICOS was expressed only on around 10% of $CD4^+$ pLN cells previously exposed to antigen ($CD44^{high}$, $CD45RB^{low}$, or $CD62L^{low}$). More surprisingly, again only around 10% of T cells bearing CD69 or CD25 in vivo, markers typically expressed on recently activated T cells, coexpressed ICOS (Fig. 1 A). Similar data were obtained for PP and splenic $CD4^+$ T cells (unpublished data). Collectively, these results suggested that ICOS expression in vivo characterizes a subpopulation of antigen-experienced T cells with a particular biological role.

ICOS Expression on $CD4^+$ T Cells In Vivo Is Highly Correlated with the Capacity to Secrete IL-13, IL-4, IL-5, and in Particular IL-10. To define the functional properties of $CD4^+ICOS^+$ cells in vivo, we examined whether ICOS expression is correlated with a specific cytokine pattern. Upon stimulation with PMA and ionomycin in the presence of Brefeldin A, the majority of the unseparated $CD4^+$ pLN T cells (58.7%) generated TNF-α, as determined by single cell analysis (Fig. 1 B, left column, and Table I). The frequency of unseparated pLN $CD4^+$ T cells capable of secreting IL-3, IL-6, IFN-γ, IL-2, GM-CSF, IL-13, IL-4, IL-5, or IL-10 was substantially lower and was in the 0.1-4.8% range (Fig. 1 B, left column, and Table I). Coexpression analysis of cytokines and ICOS determined that only around 9% of TNF-α–secreting cells, but 21-42% of cells secreting IL-3, IL-6, IFN-γ, IL-2, or GM-CSF, and even 46–58% of cells secreting IL-13, IL-4, IL-5, or IL-10 expressed ICOS (Table I). These results demonstrated that $ICOS^+$ T cells, although representing only a small fraction of antigen-experienced cells in normal mice, nevertheless encompass a large proportion of cells capable of secreting effector cytokines, in particular IL-13, IL-4, IL-5, and IL-10.

The Degree of ICOS Cell Surface Density on $CD4^+$ T Cells Is Directly Correlated with the Cytokine Pattern Secreted. For an extended cytokine pattern analysis we magnetically sorted $ICOS^+$ T cells from pLN cells. This procedure resulted in a pure population of $ICOS^+$ T cells ("ICOS-enriched"), with around 30% of cells expressing low amounts, and 70% of $CD4^+$ cells expressing intermediate to high amount of ICOS on the cell surface (Fig. 1 C). The negative population ("ICOS-depleted," Fig. 1 C) was stringently depleted of ICOS-bearing cells by two rounds of magnetic separation. In relation to the ICOS-depleted cell population, enrichment for $ICOS^+$ cells resulted in a relative reduction of TNF-α–generating cells and in a slight enrichment of T cells secreting IL-3, IL-6, IFN-γ, IL-2, and GM-CSF (1.3 to 2.6-fold, Fig. 1 B, middle and right column, and Table II). At the same time, a strong enrichment for cells secreting IL-13, IL-4, and IL-5 (13-fold), and a dramatic enrichment for cells generating IL-10 (28-fold) were observed (Fig. 1 B and Table II), resulting ap-

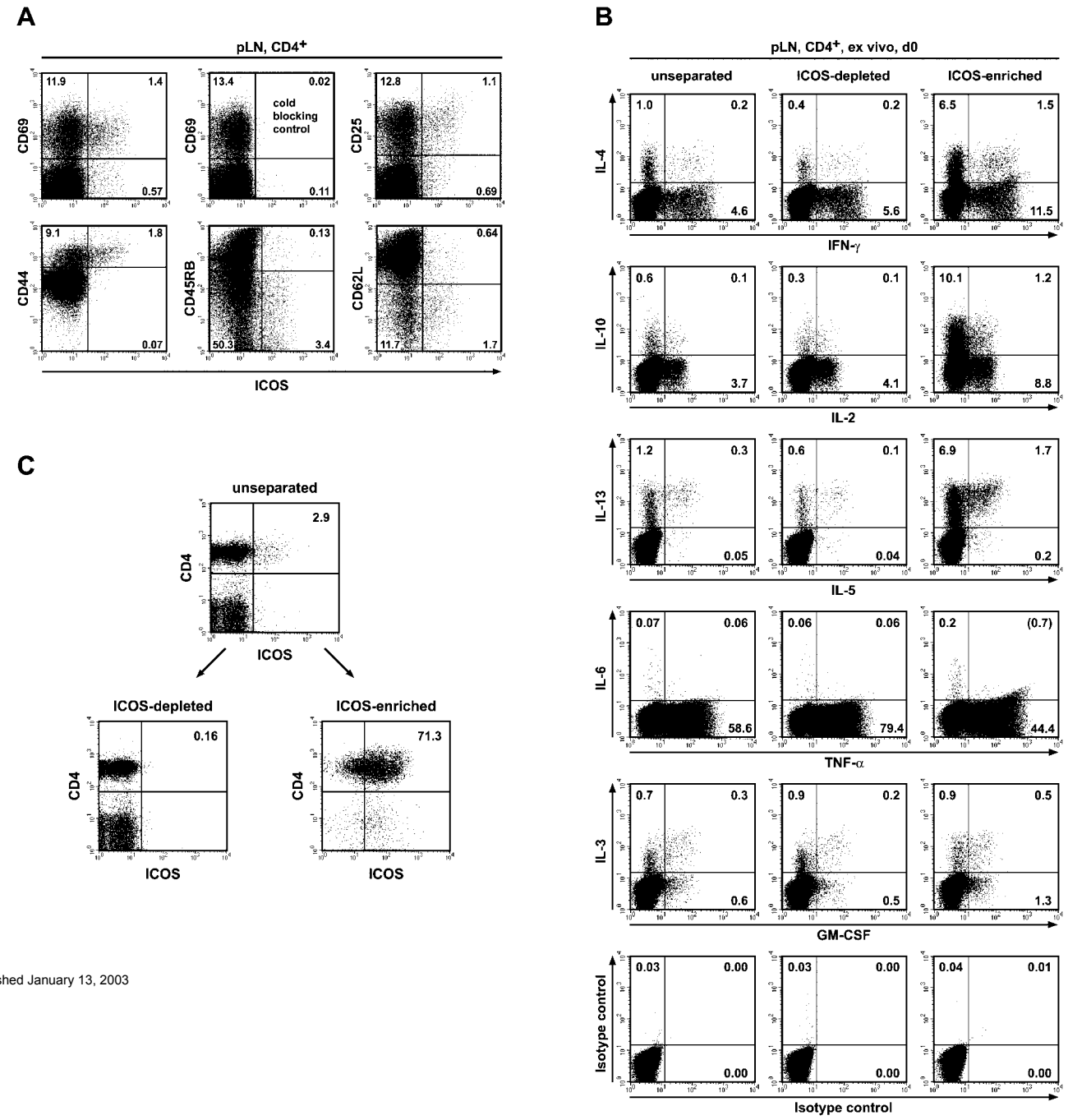

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Figure 1. Phenotype and cytokine pattern of ICOS-expressing $CD4^+$ T cells in vivo. (A) ICOS is expressed on a subpopulation of antigen-experienced $CD4^+$ T cells in vivo. Cells from pLN of 6-mo-old BALB/c mice were stained with mAb against CD4, ICOS, and a panel of T cell activation markers, as indicated. In each case, the specificity of ICOS staining was controlled by preincubating the cells with an excess of unconjugated anti-ICOS mAb (cold blocking control, only shown for CD69 coexpression analysis). Analysis gates were set on live $CD4^+$ T lymphocytes. The inserted numbers represent the net percentage of ICOS-positive and/or activation marker-positive $CD4^+$ cells after subtraction of the respective cold-blocking controls. (B) ICOS expression on $CD4^+$ T cells in vivo correlates with a Th2-biased, IL-10–dominated cytokine pattern. Unseparated, ICOS-depleted, and ICOS-enriched (compare panel C) pLN cells were stimulated with PMA and ionomycin for 5 h in the presence of Brefeldin A for the last 3 h. After fixation, cells were stained for CD4, and simultaneously for the indicated intracellular cytokines. The inserted numbers represent percentages of $CD4^+$ cells generating a given cytokine. The results shown are representative of three independent experiments. (C) Separation of ex vivo T cells into ICOS-depleted and ICOS-enriched populations. Cells from pLN of BALB/c mice were stained with digoxigenized anti-ICOS mAb MIC-280, followed by anti-Dig MACS microbeads and anti-Dig-Cy5. The cells were separated by MACS and stained also for CD4. Similar numbers of $CD4^+$ T cells of the unsorted and sorted fractions are shown. Percentages of $ICOS^+$ cells in the $CD4^+$ population are indicated. The specificity of enrichment for $ICOS^+$ cells is reflected by the selective accumulation of $CD4^+$ cells in the ICOS-enriched fraction.

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Table I. *Coexpression Analysis of Cytokines and ICOS in CD4$^+$ T Cells*

pLN CD4$^+$ T cells ex vivo	TNF-α	IL-3	IL-6	IFN-γ	IL-2	GM-CSF	IL-13	IL-4	IL-5	IL-10
Total frequency of cells secreting a given cytokine[a]	58.7%	1.0%	0.13%	4.8%	3.8%	0.9%	1.5%	1.2%	0.35%	0.7%
Proportion of ICOS$^+$ cells in the population of CD4$^+$ T cells secreting a given cytokine[a]	9%	22%	21%	29%	23%	42%	56%	57%	58%	46%

[a]pLN cells were activated with PMA and ionomycin for 5 h in the presence of Brefeldin A for the last 3 h and analyzed for intracellular cytokines by flow cytometry.

parently from a preferential accumulation of ICOS "high expressors" in the ICOS-enriched population. Similar data were obtained with T cells recovered from PP and spleen (unpublished data). The dramatic increase in IL-10$^+$ T cells in the ICOS-enriched fraction was independent of whether the IL-10 producer did or did not coexpress IL-2 (Fig. 1 B), IFN-γ, IL-4, or IL-5 (unpublished data).

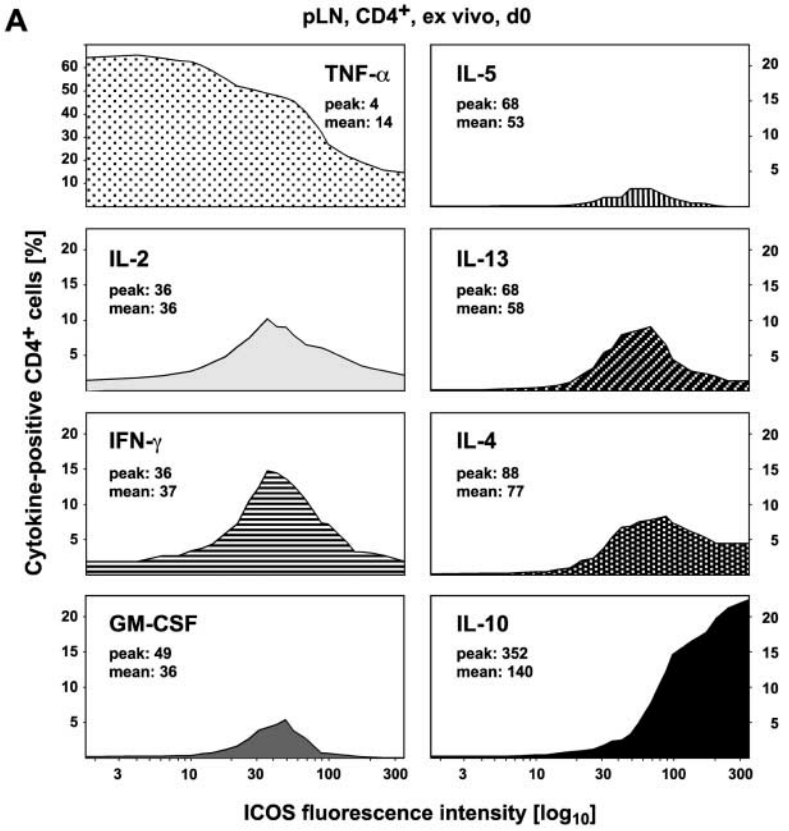
The high enrichment of T cells secreting certain cytokines in the ICOS$^+$ cell fraction suggested that the amount of ICOS expressed on the cell surface might be directly correlated to the type of cytokine secreted. To test this hypothesis, we plotted the percentages of cells secreting a given cytokine against the density of ICOS expressed on the cell surface, measured as relative fluorescence intensity. The result of this analysis is shown for each single cytokine (Fig. 2 A), as well as cumulatively for all cytokines measured (Fig. 2 B). This analysis revealed that T cells negative for ICOS almost exclusively secreted TNF-α and thus exhibited a cytokine pattern typical of naive T cells (21). T cells with low levels of ICOS showed a reduced TNF-α secretion, significant IL-2 and IFN-γ secretion, and the capacity to generate IL-3 and GM-CSF (Fig. 2, A and B). Intermediate ICOS expression was associated with the production of IL-5, IL-13, and IL-4. High levels of ICOS on the cell surface were very strongly correlated with the generation of IL-10. In fact, in CD4$^+$ T cells with the highest expression of ICOS, IL-10 was the dominant cytokine produced (Fig. 2, A and B).

ICOS$^+$ T Cells Isolated Ex Vivo Exhibit a Stable Cytokine Pattern. To determine whether the capacity to secrete certain cytokines is a stable feature of cells expressing ICOS in vivo, ICOS-depleted and ICOS-enriched CD4$^+$ T cells

Table II. *Intracellular Cytokine Profiles of Sorted ICOS$^+$ and ICOS$^-$CD4$^+$ T Cells*

pLN CD4$^+$ T cells ex vivo, freshly isolated	TNF-α	IL-3	IL-6	IFN-γ	IL-2	GM-CSF	IL-13	IL-4	IL-5	IL-10
ICOS-depleted										
Frequency of cells secreting a given cytokine[a]	79.5%	1.1%	0.12%	5.8%	4.2%	0.7%	0.7%	0.6%	0.14%	0.4%
ICOS-enriched										
Frequency of cells secreting a given cytokine[a]	45.1%	1.4%	0.22%	13%	10%	1.8%	8.6%	8%	1.9%	11.3%
Factor of increase[b]	0.6×	1.3×	1.8×	2.2×	2.4×	2.6×	12.3×	13.3×	13.6×	28.3×
pLN CD4$^+$ T cells ex vivo, cultured for 6 d in vitro[c]										
ICOS-depleted										
Frequency of cells secreting a given cytokine[a]	84.1%	0.3%	n.d.	12.5%	7.2%	n.d.	0.21%	0.5%	0.01%	0.2%
ICOS-enriched										
Frequency of cells secreting a given cytokine[a]	56.2%	0.5%	n.d.	18.4%	12.5%	n.d.	6.7%	9.6%	0.56%	14.9%
Factor of increase[b]	0.7×	1.7×	n.d.	1.5×	1.7×	n.d.	31.9×	19.2×	56×	74.5×

n.d., not determined.
[a]pLN cells were activated with PMA and ionomycin for 5 h in the presence of Brefeldin A for the last 3 h and analyzed for intracellular cytokines by flow cytometry.
[b]Obtained when comparing ICOS-depleted to ICOS-enriched cell populations.
[c]pLN cells were magnetically sorted into ICOS-depleted and ICOS-enriched cell fractions, stimulated with PMA and ionomycin for 36 h, transferred into fresh medium, and further cultured for 6 d. At the end of culture, CD4$^+$ T cells were restimulated and analyzed for intracellular cytokines as above.



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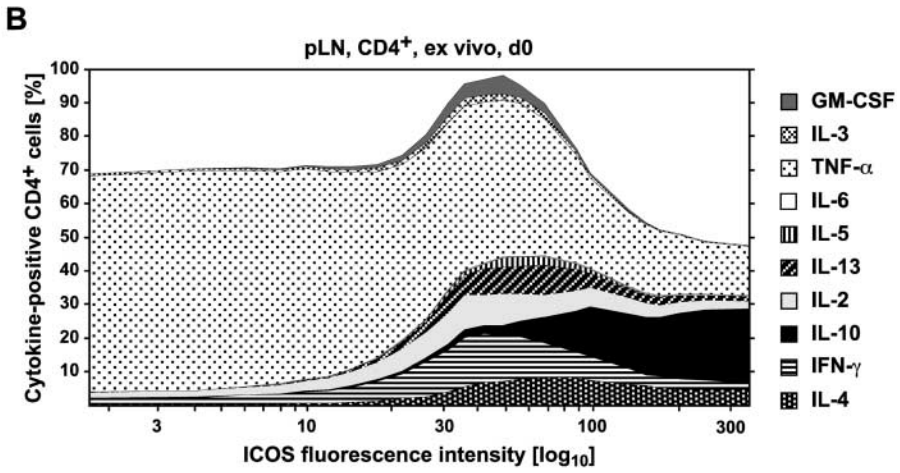

Figure 2. The degree of ICOS cell surface expression on $CD4^+$ T cells is correlated with the cytokine pattern secreted. Cells from pLN of BALB/c mice were stained for ICOS and sorted according to ICOS expression. Unseparated or ICOS-enriched cells were activated with PMA and ionomycin for 5 h in the presence of Brefeldin A for the last 3 h, stained for CD4 and various intracellular cytokines, and analyzed by flow cytometry. ICOS surface expression on $CD4^+$ T cells, measured as relative fluorescence intensity, was correlated to the percentage of cells capable of secreting a given cytokine. The percentage of $CD4^+$ T cells secreting the indicated cytokines is plotted in correlation to their ICOS cell surface expression density (A). Peak and mean ICOS fluorescence intensities of $CD4^+$ T cells secreting a given cytokine are indicated. Note the different scale for TNF-α. The same data are plotted for all cytokines in a cumulative fashion in B.

were activated, cultured in vitro for 6 d, and reexamined for their cytokine generation. Remarkably, after this culture period the cytokine patterns of T cells in the ICOS-depleted and ICOS-enriched fractions closely resembled the cytokine patterns of ICOS-depleted and ICOS-enriched T cells directly obtained ex vivo, before culture (Table II). In particular, virtually all T cells secreting IL-13, IL-4, IL-5, and IL-10 were found in the $ICOS^+$ fraction on day 6 of culture. These experiments clearly demonstrated that $ICOS^+$ T cells exhibit a memory for the cytokines generated, at least in the short term.

In Vitro–activated Splenic $CD4^+$ Cells from DO11.10 Mice with Stable ICOS Expression Preferentially Secrete IL-4, IL-13, IL-5, and IL-10. As $ICOS^+$ T cells are capable of synthesizing a variety of cytokines with partly opposing biological roles (e.g., IFN-γ and IL-4), we were interested to test the overall biological effects of ICOS-expressing $CD4^+$ T cells in vivo using an adoptive transfer model. To this end, we chose an airway inflammation model which allows a functional testing of both Th1- and Th2-biased immune responses (neutrophilic versus eosinophilic lung inflammation). However, one limitation for this type of experiment

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was the low total number of ICOS$^+$CD4$^+$ T cells which could be isolated from pLN of TCR-transgenic mice. We therefore undertook in vitro studies to identify conditions for the isolation of preactivated ICOS-expressing cells with the characteristic cytokine profile observed in vivo. When DO11.10 CD4$^+$ T cells were activated with $OVA_{323–339}$, we noticed that virtually all CD4$^+$ T cells expressed ICOS on day 3, but only a fraction of cells maintained ICOS surface expression at a high level beyond day 5 (Fig. 3 A). When the cultured cells were separated on day 6 into ICOS$^+$ and ICOS$^-$ fractions (Fig. 3 B) and restimulated, the vast majority of cells from the ICOS$^+$ fraction continued to express ICOS at high levels and thus exhibited memory for the expression of ICOS. In contrast, only a minority of cells from the ICOS-depleted fraction transiently expressed ICOS (Fig. 3 C, top panel). On the other hand, both populations up-regulated and down-regulated CD69 in a similar fashion (Fig. 3 C, bottom panel). The ICOS$^+$ T cell fraction, when compared with the ICOS$^-$ cell fraction, exhibited a slightly reduced capacity to produce TNF-α and IL-3, a modestly increased capacity to produce IFN-γ, IL-2, and GM-CSF, and a substantially increased capacity to generate IL-4, IL-13, IL-5, and in particular IL-10 (Fig. 4 A), and thus exhibited a cytokine pattern similar to T cells expressing ICOS in vivo. These data on cytokine secretion were also confirmed by ELISA (Fig. 4 B). Additional experiments revealed that this cytokine pattern remained stable in vitro for at least further 7 d (unpublished data).

The ICOS$^+$ T Cell Fraction Contains Virtually All Inflammatory Effector Cells. In a next step we assessed the overall biological effect of ICOS$^+$ T cells in vivo. CD4$^+$ T cells from DO11.10 mice were cultured in the presence of $OVA_{323–339}$ for 6 d in vitro and magnetically separated into ICOS$^+$ and ICOS$^-$ cell fractions, as before (compare Fig. 3 B). ICOS$^+$, ICOS$^-$, and unseparated cell fractions containing equal numbers of OVA-TCR transgenic KJ1–26$^+$ cells (22) were transferred into BALB/c recipients, which had been exposed to OVA via the airways 24 h before (Fig. 5 A). Recipient mice were then reexposed to OVA

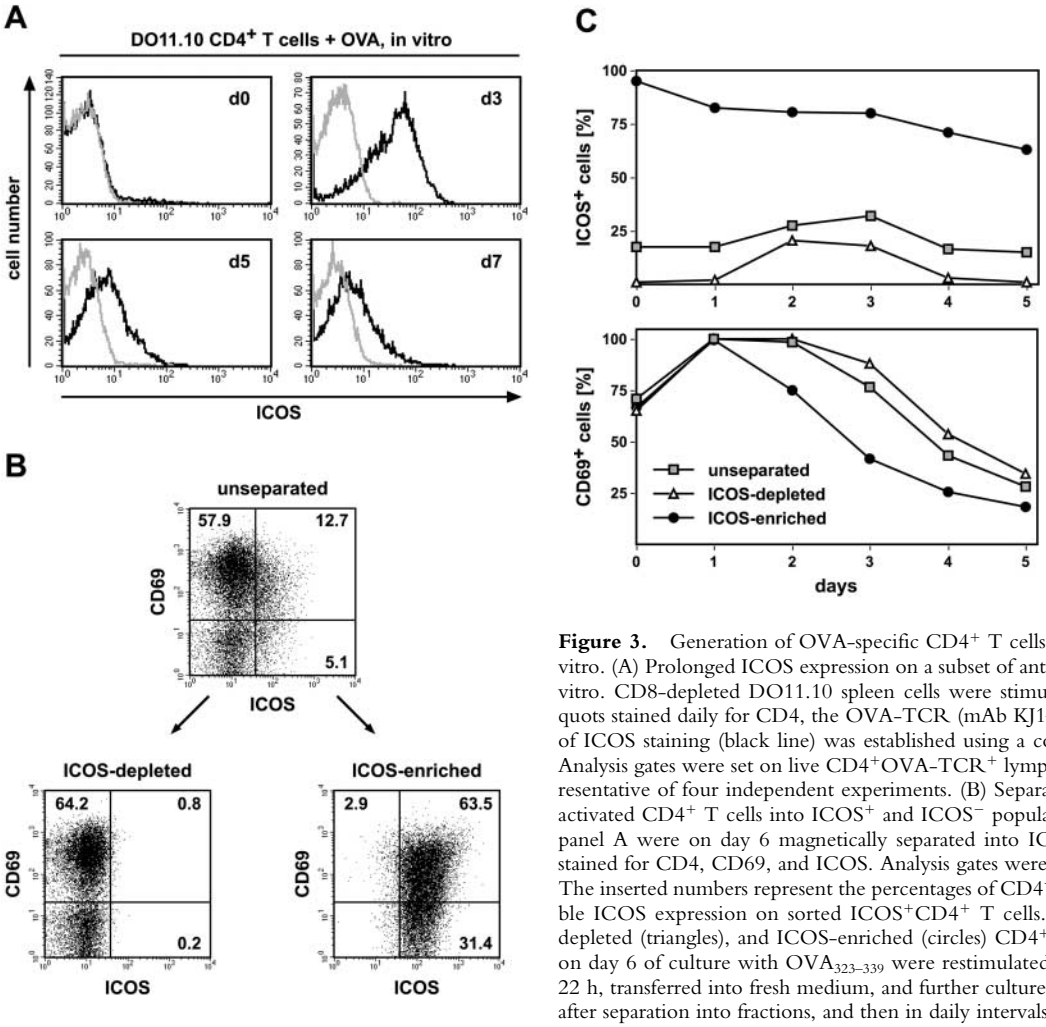

Figure 3. Generation of OVA-specific CD4$^+$ T cells with stable ICOS expression in vitro. (A) Prolonged ICOS expression on a subset of antigen-stimulated CD4$^+$ T cells in vitro. CD8-depleted DO11.10 spleen cells were stimulated with $OVA_{323–339}$, and aliquots stained daily for CD4, the OVA-TCR (mAb KJ1–26), and ICOS. The specificity of ICOS staining (black line) was established using a cold blocking control (gray line). Analysis gates were set on live CD4$^+$OVA-TCR$^+$ lymphocytes. Results shown are representative of four independent experiments. (B) Separation of OVA-specific, in vitro–activated CD4$^+$ T cells into ICOS$^+$ and ICOS$^-$ populations. The cells stimulated as in panel A were on day 6 magnetically separated into ICOS$^+$ and ICOS$^-$ fractions and stained for CD4, CD69, and ICOS. Analysis gates were set on live CD4$^+$ lymphocytes. The inserted numbers represent the percentages of CD4$^+$ cells in each quadrant. (C) Stable ICOS expression on sorted ICOS$^+$CD4$^+$ T cells. Unseparated (squares), ICOS-depleted (triangles), and ICOS-enriched (circles) CD4$^+$ OVA-specific T cells obtained on day 6 of culture with $OVA_{323–339}$ were restimulated with PMA and ionomycin for 22 h, transferred into fresh medium, and further cultured for a total of 5 d. Immediately after separation into fractions, and then in daily intervals, aliquots of the cell populations were stained for CD4, CD69, and ICOS. In each case, the specificity of the ICOS staining was established using a cold blocking control. Analysis gates were set on live CD4$^+$ lymphocytes. Percentages of ICOS$^+$ and CD69$^+$ cells in the CD4$^+$ populations are indicated. Similar results were obtained in two independent experiments.

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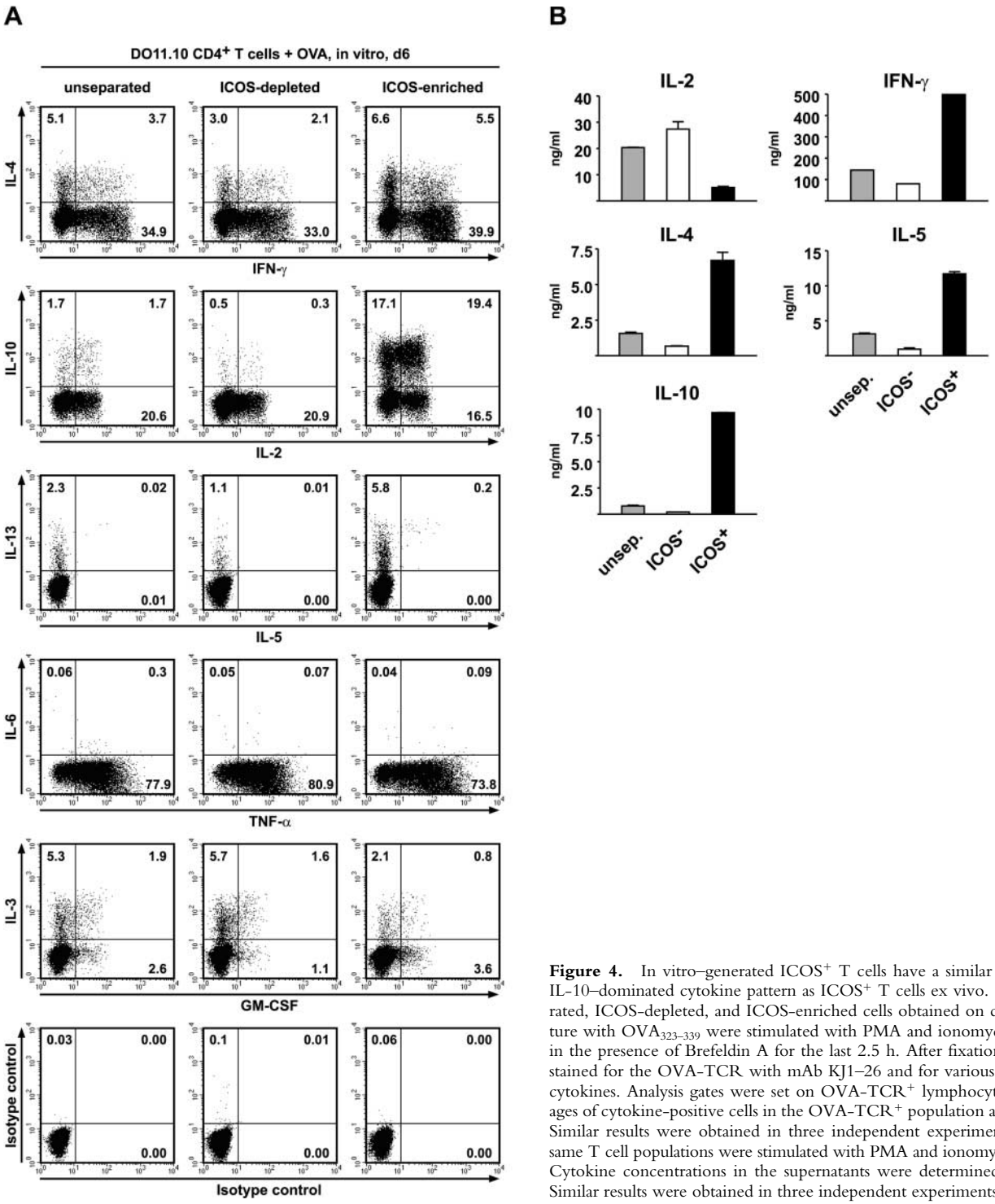

Figure 4. In vitro–generated ICOS$^+$ T cells have a similar Th2-biased, IL-10–dominated cytokine pattern as ICOS$^+$ T cells ex vivo. (A) Unseparated, ICOS-depleted, and ICOS-enriched cells obtained on day 6 of culture with $OVA_{323–339}$ were stimulated with PMA and ionomycin for 4.5 h in the presence of Brefeldin A for the last 2.5 h. After fixation, cells were stained for the OVA-TCR with mAb KJ1–26 and for various intracellular cytokines. Analysis gates were set on OVA-TCR$^+$ lymphocytes. Percentages of cytokine-positive cells in the OVA-TCR$^+$ population are indicated. Similar results were obtained in three independent experiments. (B) The same T cell populations were stimulated with PMA and ionomycin for 20 h. Cytokine concentrations in the supernatants were determined by ELISA. Similar results were obtained in three independent experiments.

on days 1 and 2 after transfer, killed on day 4, and analyzed. In all three groups of mice we found a local accumulation of OVA-TCR$^+$ T cells in peribronchial LN (1.2–3.6% of T cells; Fig. 5 B) and BAL fluid (4.5–6.2%), but not inguinal LN (0.2–0.4%), demonstrating a successful homing of OVA-specific T cells to the site of antigen delivery. In agreement with our findings of stable ICOS expression in vitro (see Fig. 3 C, top panel), only in recipients of ICOS$^+$ cells a significant proportion of OVA-TCR$^+$ cells in peribronchial LN and BAL fluid expressed ICOS on the cell surface (Fig. 5 B and Table III). In contrast, similar frequencies of CD69$^+$ and CD62L^{low} cells in the OVA-TCR$^+$ populations were found in all three groups of mice that were exposed to OVA (Table III), but not in the PBS control mice (unpublished data). This indicates that in all transferred animal groups a substantial pro-

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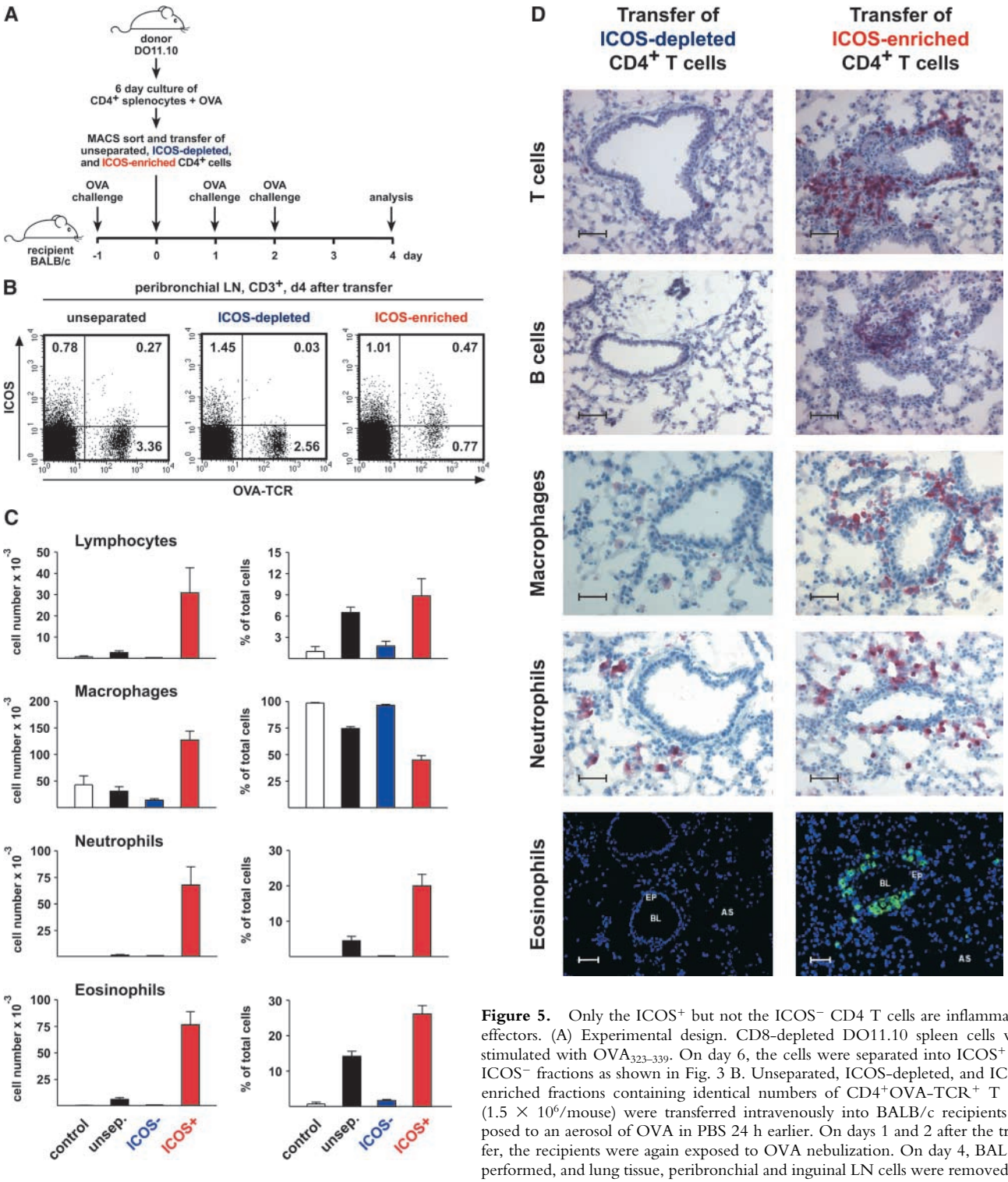

Figure 5. Only the ICOS$^+$ but not the ICOS$^-$ CD4 T cells are inflammatory effectors. (A) Experimental design. CD8-depleted DO11.10 spleen cells were stimulated with $OVA_{323–339}$. On day 6, the cells were separated into ICOS$^+$ and ICOS$^-$ fractions as shown in Fig. 3 B. Unseparated, ICOS-depleted, and ICOS-enriched fractions containing identical numbers of CD4$^+$OVA-TCR$^+$ T cells (1.5×10^6/mouse) were transferred intravenously into BALB/c recipients exposed to an aerosol of OVA in PBS 24 h earlier. On days 1 and 2 after the transfer, the recipients were again exposed to OVA nebulization. On day 4, BAL was performed, and lung tissue, peribronchial and inguinal LN cells were removed and analyzed. (B) Stable ICOS expression in vivo. On day 4 after transfer, peribronchial LN cells from the three experimental groups were stained for ICOS, OVA-TCR, and CD3. Analysis gates were set on CD3$^+$ T cells. The inserted numbers represent the percentages of CD3$^+$ cells in each quadrant. Note that the vast majority of transgenic T cells in the ICOS-enriched recipients express ICOS, albeit to varying degree. (C) Accumulation of inflammatory effector cells in the airway lumen is strictly dependent on the presence of ICOS$^+$ T cells. On day 4 after cell transfer, the total number and frequency of lymphocytes, macrophages, neutrophils, and eosinophils in the BAL fluid were determined. The mice of the control group had been transferred with unseparated CD4$^+$OVA-TCR$^+$ T cells but had been exposed to PBS instead of OVA aerosol. Data are shown as the mean ± SEM of 6–8 animals. Similar results were obtained in two independent experiments. (D) Peribronchial infiltration of inflammatory effector cells in the lung is highly dependent on the presence of ICOS$^+$ T cells. Lung sections of animals transferred with ICOS$^+$ and ICOS$^-$ cell fractions were stained for T cells (CD3), B cells (CD45R/B220), macrophages (F4/80), neutrophils (Ly-6G), and eosinophils (major basic protein, MBP), and analyzed by conventional and confocal laser microscopy (original magnification 20×, bar scale indicates 50 μm; AS, alveolar space; BL, bronchial lumen; EP, epithelium).

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Table III. *Expression of Activation Markers on Adoptively Transferred T Cells in Lung Compartments of Recipient Mice*

	ICOS$^+$	CD69$^+$	CD62L^{low}
Peribronchial LN			
Unseparated	7.4%	38.1%	81.1%
ICOS-depleted	1.2%	43.9%	78.4%
ICOS-enriched	37.9%	27.3%	77.2%
BAL			
Unseparated	4.8%		
ICOS-depleted	1.1%		
ICOS-enriched	27.2%		

Mice were adoptively transferred with T cell fractions as shown in Fig. 5 A. On day 4 of transfer, peribronchial LN cells and cells from the BAL fluid were prepared and stained with mAb against CD3, the clonotypic OVA-TCR (KJ1-26), ICOS, CD69, and CD62L. Percentages of ICOS$^+$, CD69$^+$, and CD62L^{low} cells in the CD3$^+$OVA-TCR$^+$ populations are given.

portion of OVA-specific T cells became activated by the antigen in vivo.

We also tested whether the cytokine pattern of the transferred cells remained stable in vivo. For this, we activated peribronchial LN cells from recipient mice and analyzed the cytokine production of OVA-TCR$^+$ T cells (Table IV). The differences in the cytokine profiles between transferred ICOS-depleted and ICOS-enriched T cells were comparable to the results obtained before transfer.

We next analyzed the three groups of recipient mice for signs of airway inflammation. In mice transferred with the ICOS$^+$ cell fraction, the total numbers of lymphocytes, macrophages, neutrophils, and eosinophils in the BAL fluid increased dramatically (Fig. 5 C). The relative proportion of lymphocytes, neutrophils, and eosinophils also increased, and was accompanied by a relative decline of macrophages, the most abundant cell population in the BAL (Fig. 5 C). In stark contrast, the ICOS$^-$ cell fraction did not elicit any measurable biological effects, when compared with control animals transferred with the unseparated cells and exposed to PBS instead of OVA (Fig. 5 C). Finally, we determined the distribution of the infiltrating cells in the lungs by immunohistology. This analysis revealed high numbers of infiltrating T cells, B cells, macrophages, neutrophils, and eosinophils in the peribronchial and perivascular regions of small airways in animals transferred with ICOS$^+$ cells (Fig. 5 D), and thus resembled the pattern observed in murine and human asthma (23, 24). In animals which had received the unseparated cell fraction there were only low numbers of infiltrating cells, but with a similar pattern (unpublished data). In animals transferred with ICOS$^-$ cells, no infiltrates were found with the exception of a modest number of neutrophils in the peribronchial tissue (Fig. 5 D). Collectively, the transfer experiments clearly demonstrated that within the population of antigen-specific CD4$^+$ T cells virtually all biological effects were exerted by cells bearing ICOS on the cell surface.

Discussion

Our study reveals that expression of ICOS is an integral part of a T cell differentiation program leading to the synthesis of various effector cytokines, whereas ICOS$^-$ T cells, typical of naive T cells (21), predominantly produce the early inflammatory mediator TNF-α. This general observation is not unexpected, given the fact that ICOS is only significantly expressed on the surface after T cell activation (1, 2). A more detailed analysis, however, reveals a number of intriguing findings. First of all, there is a clear link between ICOS cell surface density and the type of cytokines produced. A low expression of ICOS was associated with the generation of IL-2, IL-3, IL-6, and GM-CSF, effector cytokines used in the early phase of an immune response (25–29). At the same time, ICOS$^+$ T cells represented only 20–30% (GM-CSF: 40%) of T cells capable of generating these early cytokines in vivo. Thus, at least in an immune system at steady state, ICOS expression is only loosely associated with early effector cytokines. In contrast, intermediate expression of ICOS was found to be tightly linked to the production of IL-4, IL-5, and IL-13 (Fig. 2). Here, ICOS$^+$ cells represented around 50% of all T cells capable of producing these cytokines in lymphatic

Table IV. *Stability of Intracellular Cytokine Profiles of OVA-TCR$^+$ T Cells from Peribronchial LN after Adoptive Transfer*

OVA-TCR$^+$ T cells ex vivo	TNF-α	IFN-γ	IL-2	IL-13	IL-4	IL-10
ICOS-depleted						
Frequency of cells secreting a given cytokine[a]	46.6%	37.7%	21.4%	0.44%	3.3%	0.07%
ICOS-enriched						
Frequency of cells secreting a given cytokine[a]	40.3%	42.6%	29.5%	1.9%	15.2%	4.2%
Factor of increase[b]	0.9×	1.1×	1.4×	4.3×	4.6×	60×

[a]Mice were adoptively transferred with T cell fractions as shown in Fig. 5 A. On day 4 of transfer, peribronchial LN cells were prepared. Cells were activated with PMA and ionomycin for 5 h in the presence of Brefeldin A for the last 3 h and analyzed for intracellular cytokines by flow cytometry. Percentages of cytokine-producing cells in the CD4$^+$OVA-TCR$^+$ populations are given.
[b]Obtained when comparing ICOS-depleted to ICOS-enriched cell populations.

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organs (Table I). These observations are highly significant, as only very few percent of $CD4^+$ T cells in an immune system at steady state express ICOS. Functionally, the link to Th2 cytokines was demonstrated by induction of a massive Th2-type eosinophilic lung inflammation upon adoptive transfer of intermediate-to-high $ICOS^+$ T cells. Thus, we can demonstrate a Th2 bias for the bulk of $ICOS^+$ T cells in vivo. We interpret our findings as a basic functional link between ICOS and effector cytokines, with a preference for the Th2 cytokines IL-4, IL-5, and IL-13, but do not exclude that under strong Th1-polarizing conditions, this basic link can be temporarily overruled in favor of Th1 cytokines (5–7).

ICOS, however, is not only associated with the prototypical Th2 cytokines. An even stronger association could be observed between ICOS and IL-10. First of all, around 50% of all T cells capable of generating IL-10 in vivo expressed ICOS (Table I). Second, and in a quite unique fashion, IL-10 was the dominant cytokine produced by T cells with high ICOS expression (Fig. 2). We thus demonstrate for the first time a tight link between ICOS and IL-10 in central lymphoid organs, and this statement also holds true for the human immune system (unpublished data). In fact, ICOS is the first surface molecule which can be correlated to this central immune regulator (30). The observation that $CD4^+$ T cells from mice with a deleted ICOS gene are fully capable of secreting IL-10 on CD3 stimulation in vitro (8) seems to indicate that ICOS is not directly involved in the differentiation of T cells to IL-10–secreting cells, but rather controls the release of this cytokine.

The linkage between low ICOS expression and "early" cytokines, and between intermediate/high ICOS expression and "late" cytokines is intriguing and could mean that ICOS is gradually up-regulated in the course of progressing T cell differentiation. First, T cells would utilize ICOS for the release of proinflammatory Th2 cytokines, later a population of T cells would differentiate to produce IL-10 and would utilize the high ICOS expression for an effective down-regulation of the immune response. In an alternative scenario, T cells $ICOS^{medium}$/Th2 and T cells $ICOS^{high}$/IL-10 would develop in parallel and then migrate as effector cells to the periphery. Many peripheral tissues, among them endothelial cells (ECs), constitutively express low levels of ICOS-L in the resting state (31). Our recent results indicate that upon inflammation, endothelial cells strongly up-regulate ICOS-L (31). Under these conditions, ligation of ICOS-L by ICOS is the main pathway for the release of cytokines by $CD4^+$ T cells interacting with ECs (31) presumed to present antigen (32, 33), as the inflamed ECs remain negative for CD80 and CD86 (31–33). ECs expressing low levels of ICOS-L can be presumed to preferentially trigger $ICOS^{high}$ T cells releasing predominantly IL-10, which has downmodulatory effects on the production of cytokines by inflamed ECs (34, 35). In contrast, ECs with higher levels of ICOS-L could also activate the majority of T cells expressing lower levels of ICOS and thus trigger the release of IL-4, IL-5, and IL-13, which have proinflammatory actions on ECs (34, 36–39). Regulated by a threshold expression of ICOS-L, this scenario would result in an antigen-specific, localized opening up of the endothelial barrier for the influx of inflammatory cells into tissues. In fact, we have preliminary evidence that such a mechanism is operative in vivo. In an abstract sense, the quantitative differences in ICOS expression on $CD4^+$ T cells would translate into qualitatively different biological effects, depending on the level of ICOS-L expression on the partner cell.

It seems that ICOS expression not only defines certain cytokine patterns but altogether demarcates effector $CD4^+$ T cells in vivo. Our data with stimulated DO11.10 spleen cells clearly demonstrated that only $CD4^+$ T cells with prolonged expression of ICOS exhibit memory for the reexpression of ICOS. Based on their cytokine profile, it is very probable that these cells were reactivated $CD4^+$ T cells with previous antigen exposure and a history of differentiation to effector cells in vivo. In line with this interpretation were the dramatic biological effects exerted by these T cells upon transfer into syngeneic hosts. Even more impressive, however, was the observation that in our studies the T cell fraction depleted of $ICOS^+$ T cells did not exert any measurable biological effects upon adoptive transfer. Of note, this $ICOS^-$ cell fraction was derived from an in vitro culture, in which virtually all T cells were fully activated and expressed high levels of ICOS a few days before separation into fractions (see Fig. 3 A). This $ICOS^-$ cell population was fully capable of synthesizing TNF-α and IL-2 on restimulation in vitro (Fig. 4) and exhibited high levels of CD69 in the lung-draining peribronchial LN as a sign of activation after exposure to OVA, their nominal antigen (Table III). Nevertheless, no influx of cells into the lung or any other sign of inflammation was induced by this $ICOS^-$ T cell population.

Collectively, these data strongly suggest an association between stable ICOS expression and T cell effector capacity. This conclusion is corroborated by our initial finding that only some 10% of $CD4^+$ T cells expressing in vivo early (CD69 or CD25) or late ($CD44^{high}$, $CD45RB^{low}$, or $CD62L^{low}$) activation markers coexpress ICOS, but nevertheless represent the bulk of the T cell population capable of secreting late effector cytokines (Fig. 1 and Table I). Thus, ICOS seems to be a highly specific marker for effector T cells in vivo, an observation with high diagnostic and therapeutic potential.

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