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ICOS Controls the Pool Size of Effector-Memory and Regulatory T Cells[1]

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ICOS is an important regulator of T cell effector function. ICOS-deficient patients as well as knockout mice show severe defects in T cell-dependent B cell responses. Several in vitro and in vivo studies attributed this phenomenon to impaired up-regulation of cell surface communication molecules and cytokine synthesis by ICOS-deficient T cells. However, we now could show with Ag-specific T cells in a murine adoptive transfer system that signaling via ICOS does not significantly affect early T cell activation. Instead, ICOS substantially contributes to the survival and expansion of effector T cells upon local challenge with Ag and adjuvant. Importantly, the observed biological function of ICOS also extends to FoxP3$^+$ regulatory T cells, as can be observed after systemic Ag delivery without adjuvant. In line with these findings, absence of ICOS under homeostatic conditions of nonimmunized mice leads to a reduced number of both effector-memory and FoxP3$^+$ regulatory T cells. Based on these results, we propose a biological role for ICOS as a costimulatory, agonistic molecule for a variety of effector T cells with differing and partly opposing functional roles. This concept may reconcile a number of past in vivo studies with seemingly contradictory results on ICOS function. ***The Journal of Immunology,*** **2008, 180: 774–782.**

Costimulatory molecules play a central role in the regulation of T cell immune responses. Originally, costimulation was considered as a simple on/off switch provided by the CD28 molecule. With the identification of additional T cell costimulators belonging to the CD28 and TNF superfamilies, the situation became more complex (1). Costimulators significantly differ in their expression characteristics, and some of them deliver inhibitory signals. Constitutively expressed molecules like CD28 control the initial T cell activation, whereas costimulators like OX-40, only induced upon activation, are implicated in later T cell responses. Due to the complexity of molecular interactions, the individual contributions of various costimulators to T cell activation in vivo are still poorly delineated.

ICOS is a CD28 homolog with an expression restricted to activated T cells (2, 3). Its only interaction partner, ICOS ligand (ICOS-L),[4] is highly expressed on B cells and dendritic cells, and to a lesser degree on T cells and nonlymphoid cells like endothelial cells (1, 4), where it is up-regulated by inflammatory stimuli. ICOS knockout (KO) mice and ICOS-L KO mice have an identical phenotype (5–10).

In vitro, ICOS costimulation strongly supports T cell proliferation, up-regulation of cell surface molecules, and cytokine production. Early reports pointed to a specific role of ICOS in Th2 immune reactions. However, later it became apparent that ICOS is also important for Th1 reactions (11, 12). The biological function of ICOS has been investigated in various infection, autoimmunity, and allergy models in the mouse. Generally, blockade of the ICOS pathway resulted in reduced T cell effector functions at later stages of an immune response. This was attributed to reduced T cell proliferation, altered cytokine production (5, 13–16), or both, but the exact mechanisms of ICOS function in these models were usually not further resolved.

It has been firmly established that ICOS plays a critical role for T cell-dependent humoral immunity. ICOS KO mice show strongly impaired B cell responses, especially upon secondary challenge (6, 7). Human ICOS deficiency patients present with the clinical picture of common variable immunodeficiency and almost completely lack memory B cells (17). The importance of ICOS costimulation in T cell/B cell cooperation has been attributed to its involvement in the up-regulation of CD40L and CXCR5, a chemokine receptor homing T cells into B cell areas of lymphatic tissue (6, 18).

We have previously described high ICOS (ICOS^{high}) expression in nonimmunized mice on a small subpopulation of memory T cells. The majority of ICOS expressors were associated with the Th2 effector cytokines IL-4, IL-5, and IL-13, whereas T cells with very high ICOS expression were strongly correlated with IL-10 (19). In the present study, we demonstrate that this ICOS-expressing subpopulation consists of effector-memory T cells as well as FoxP3$^+$ regulatory T cells. We show that ICOS critically controls the pool size of both subpopulations in the steady state as well as in Ag-specific immune reactions by regulating the survival of T cells.

Materials and Methods

Mice

ICOS (15) and ICOS-L (9) KO mice were backcrossed for 10 generations to C57BL/6 mice. OVA TCR transgenic OT-II mice (20) were additionally backcrossed to B6.PL mice; this modeling allows the tracking of adoptively transferred cells using Thy-1.1 as a marker. For some experiments,

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[4] Abbreviations used in this paper: ICOS-L, ICOS ligand; KO, knockout; LN, lymph node; WT, wild type.

these mice were further crossed to ICOS KO mice. All mice were bred under specific pathogen-free conditions in the animal facility of the Federal Institute for Risk Assessment (Berlin, Germany). Experiments were performed according to state guidelines and approved by the local ethics committee.

Ag-specific adoptive transfer experiments

Naive T cells (2.5×10^6), isolated from spleens of OT-II mice by positive sorting with L-Selectin (CD62L) beads (Miltenyi Biotec), were transferred by i.v. injection into ICOS-L KO or C57BL/6 wild-type (WT) control mice. At 24 h later, recipients were immunized s.c. into both rear footpads with 50 μg of alum-precipitated OVA (haptenized with nitrophenol, allowing the tracking of hapten-specific B cell responses in subsequent experiments) and 5×10^7 heat-inactivated *Bordetella pertussis* (Chiron Behring) as adjuvant. For analysis, popliteal lymph nodes (LNs) were removed and mashed through sieves into PBS containing 0.5% BSA and 5 μg/ml DNase I (Roche). Cells were counted using a hemocytometer or a Personal Cell Analyzer with ViaCount Assay (Guava Technologies). For the analysis of regulatory T cells, 2.5×10^6 unsorted OT-II T cells were transferred and mice i.v. immunized with 2 mg endotoxin-free OVA (<5 pg of endotoxin/mg of protein by Limulus amebocyte lysate assay).

Flow cytometric analysis

Single-cell suspensions from LN or spleen were stained on ice with the following fluorophore-conjugated mAb: FITC-, Alexa Fluor 700-, or PE-Cy7-conjugated GK1.5 (anti-CD4), FITC-conjugated 53-6.72 (anti-CD8), FITC- or PE-conjugated IM7.8.1 (anti-CD44), Alexa Fluor 700- or PE-Cy7-conjugated MEL-14 (anti-CD62L), Cascade yellow-conjugated M5/114.15.2 (anti-MHC class II) are all from American Type Culture Collection and coupled to the respective fluorophore by standard procedures; Pacific blue- or Alexa Fluor 700-conjugated OX-7 (anti-Thy-1.1) are from European Collection of Cell Cultures; PE-conjugated MIC-280 (anti-ICOS) (19); PE- or biotin-conjugated MIC-2043 (anti-ICOS) are generated by immunization of BALB/c ICOS KO mice with ICOS transfectants. Biotinylated mAbs were followed by streptavidin-PE-Cy7. For intracellular staining of FoxP3, PE- or allophycocyanin-conjugated FJK-16s and a commercial buffer set (both from eBioscience) were used. To minimize unspecific binding, cells were preincubated with 100 μg/ml 2.4G2 (anti-FcγRII/III; American Type Culture Collection) and 50 μg/ml purified rat Ig (Nordic). A total of $1–3 \times 10^6$ cells were analyzed on a LSRII flow cytometer (BD Biosciences). Analysis gates were set on live T cells defined by scatter characteristics and exclusion of MHC class II-positive and DAPI (4′,6-diamidino-2-phenylindole) positive cells. Data were further analyzed with FlowJo software (Tree Star).

Analysis of cytokine production

LN cells were stimulated with PMA (10 ng/ml) and ionomycin (1 μg/ml) for 4.5 h. For intracellular cytokine staining, brefeldin A (5 μg/ml) was added for the last 3 h. Cells were fixed with FoxP3 fixation buffer (eBioscience) for 30 min, permeabilized, and stained for FoxP3 in combination with mAb against IFN-γ (Alexa Fluor 647-conjugated AN18.17.24) or IL-10 (digoxigenized JES5-2A5; American Type Culture Collection). IL-10 staining was followed by Alexa Fluor 647-conjugated anti-digoxigenin (Roche). Specificity of staining was controlled by preincubating the cells with a 100-fold excess of unlabeled Ab (cold blocking control). To improve the detection of IL-4-producing cells in correlation with FoxP3, we used a cytokine secretion assay (Miltenyi Biotec). The cells were stimulated for 3 h and 45 min followed by a 45-min secretion period. Cells were stained with PE-conjugated IL-4 detection Ab, fixed, and stained for FoxP3 as described. Dead cells were excluded from analysis using a fixable live stain (Aqua Fixable Dead Cell Stain kit; Invitrogen Life Technologies).

Assays for cell proliferation

To analyze proliferation, cells were labeled with CFSE (Molecular Probes) before adoptive transfer, according to standard protocols. To assess cell proliferation in a defined time frame, mice received 1 mg of BrdU i.v. 2 h before sacrifice. Cells were fixed, permeabilized and stained with an allophycocyanin-conjugated anti-BrdU mAb using a commercial staining set (BD Biosciences).

Assays for apoptosis detection

For detection of early apoptotic cells, we either used PE-conjugated annexin V (Invitrogen Life Technologies/Nexins Research) in combination with DAPI or a fluorochrome-labeled inhibitor of caspases (FLICA; Immunochemistry Technologies).

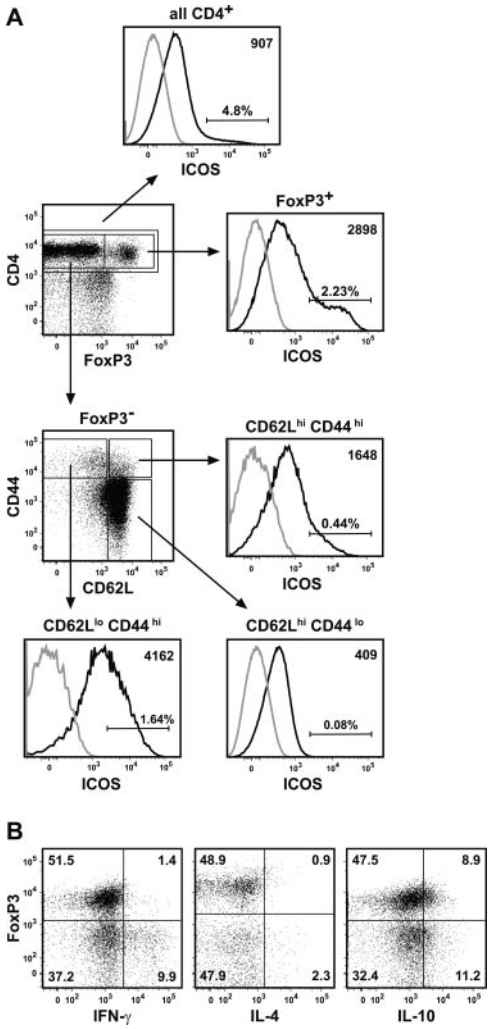

FIGURE 1. Characterization of ICOS expression in the steady state. *A*, Analysis of T cell subsets. Peripheral LN cells from WT C57BL/6 mice (black line histogram) were stained for ICOS, cells from ICOS KO mice served as a specificity control (gray line histogram). The mean fluorescence intensity of the ICOS staining is indicated for each histogram (*upper right*). Shown is the gating strategy to define different T cell subpopulations (starting with a $CD3^+$ population). The vertical bar in the histograms indicates cells with high ICOS expression. In addition, their frequency as a percentage of all $CD4^+$ T cells is shown, to illustrate how $ICOS^{high}$ cells are distributed within the T cell subpopulations. Histograms represent cells in the indicated gate (arrow). Identical results were obtained with LN cells from BALB/c mice. *B*, Cytokine profile of $ICOS^{high}$ $CD4^+$ T cells. LN cells from C57BL/6 mice were stimulated with PMA and ionomycin for 4.5 h. Cells were gated on the $ICOS^{high}$ cell population as defined by the vertical bar in *A*, and different cytokines were correlated with FoxP3 expression (intracellular staining for IFN-γ and IL-10, cytokine secretion assay for IL-4). Number in each quadrant dot plots indicates percentage of cells expressed.

Statistical analysis

The frequency of cell populations in adoptive transfer experiments is either described in relation to the recipient endogenous $CD4^+$ T cells (as a stable reference population) or as a proportion of the transgenic T cells ($Thy\text{-}1.1^+$). All statistical analysis was performed with Prism software (GraphPad software). Data are presented by a single symbol for each mouse. The median value for all animals within one experimental group is shown. Differences between groups of mice were analyzed by Mann-Whitney *U* test. A value for $p < 0.05$ was considered significant.

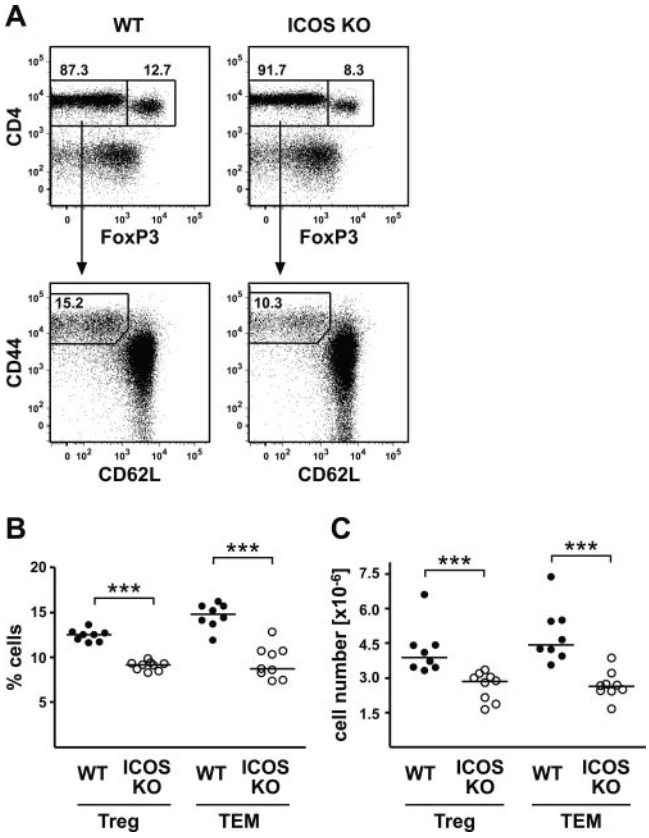

FIGURE 2. Regulatory and memory T cells in ICOS KO mice. Spleens of WT and ICOS KO mice (12-wk-old) were analyzed for the frequency of regulatory T cells ($FoxP3^+$) and effector-memory T cells ($CD62L^{low}CD44^{high}$). *A*, Representative staining shows the gating strategy for the different subpopulations. The value shown at gated region indicates the frequency of the respective T cell subset in relation to all $CD3^+CD4^+$ T cells. $FoxP3^-$ $CD4^+$ T cells were further subdivided by expression of $CD62L^{low}CD44^{high}$ (arrow). Frequency of effector-memory T cells (TEM) and regulatory T cells (Treg) within the $CD4^+$ T cell population (*B*) and absolute cell numbers (*C*). Horizontal line indicates the median value for all animals within one experimental group. A representative experiment of four conducted is shown. ***, $p < 0.001$. Comparable results were also obtained with LN cells (data not shown). Similar results were seen in ICOS KO mice on a BALB/c background.

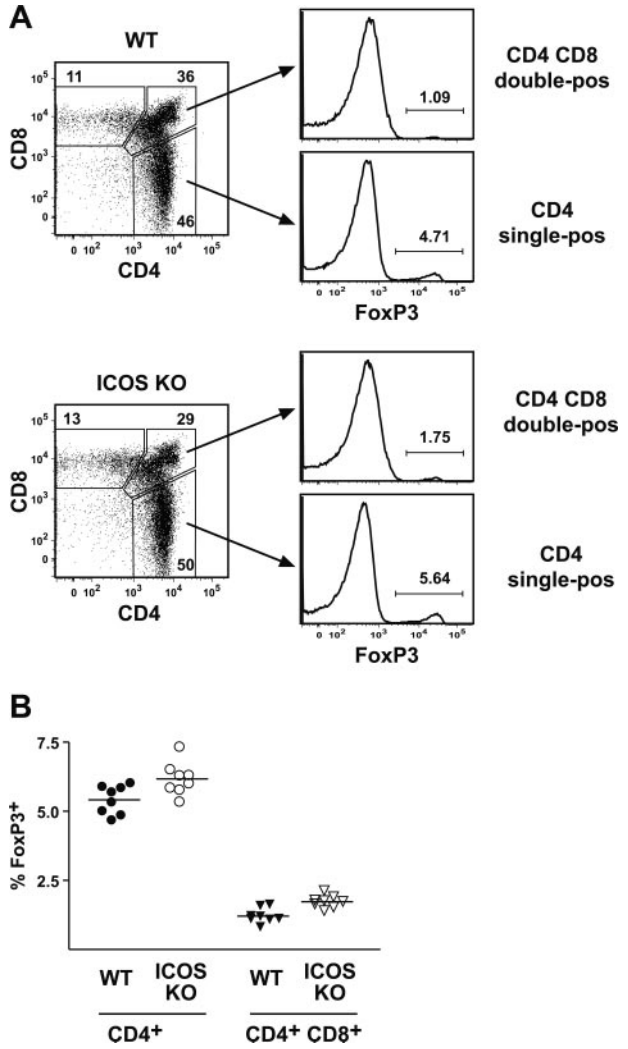

FIGURE 3. Regulatory T cells in the thymus of ICOS-deficient mice. Thymocytes from ICOS KO and WT mice were analyzed by flow cytometry for the frequency of $FoxP3^+$ cells. *A*, $CD3^+$ cells were further subdivided into CD4 single positive and CD4/CD8 double positive T cell populations. A representative staining is shown for both animal groups. Number in dot plot is the frequency of the thymic subpopulations, number in histograms is the frequency of $FoxP3^+$ cells. *B*, Statistical analysis of two independent experiments performed. Horizontal line indicates the median value for all animals within one experimental group. Identical results were obtained with ICOS KO mice on a BALB/c background.

Results

ICOS is highly expressed on effector-memory as well as on regulatory T cells

In nonimmunized mice, $ICOS^{high}$ expression can be found on ~5% of all $CD4^+$ T cells, according to Löhning et al. (19) and Fig. 1. The remaining 95% of $CD4^+$ T cells just exhibit a low basal expression that can only be visualized with high-affinity staining reagents. For further analysis of $ICOS^{high}$ expressors, we used markers defining functional T cell subpopulations. FoxP3 staining (~15% of total $CD4^+$ T cells) showed that almost all regulatory T cells express ICOS (Fig. 1*A*) with highest expression on $FoxP3^+$ $CD44^{high}$ cells (data not shown). $FoxP3^-$ cells were further subdivided by expression of CD62L and CD44, markers for memory T cells (21). The highest expression of ICOS was found on $CD62L^{low}CD44^{high}$ effector-memory T cells (~4% of all $CD4^+$ T cells). $CD62L^{high}CD44^{high}$ T cells (~2.5% of all $CD4^+$ T cells), which have been termed central-memory T cells, show an intermediate expression of ICOS. The main population of $CD62L^{high}CD44^{low}$ naive T cells (~95%) did not contain any cells expressing ICOS at a high level (Fig. 1*A*). Taken together, in nonimmunized mice, significant ICOS expression is restricted to regulatory T cells and memory T cells. Interestingly, all of these cells are almost uniformly positive for ICOS, which differs from other activation-induced costimulators like OX-40, 4-1BB, or CD30 found only on subpopulations of memory T cells (data not shown).

We recently described a striking correlation of ICOS expression with the potential of T cells to produce IL-10 (19). The expression of ICOS on almost all regulatory T cells taken together with their preferential production of IL-10 now raises the question whether $ICOS^{high}$ IL-10 producers might represent regulatory T cells. To analyze this question, we correlated cytokine production of $ICOS^{high}$ $CD4^+$ T cells after short-term in vitro stimulation with expression of FoxP3. Approximately 50% of $ICOS^{high}$ T cells are $FoxP3^+$ and almost all effector T cells producing cytokines are found within this small subpopulation (Fig. 1*B* and data not shown). Whereas IFN-γ and IL-4 are almost exclusively produced

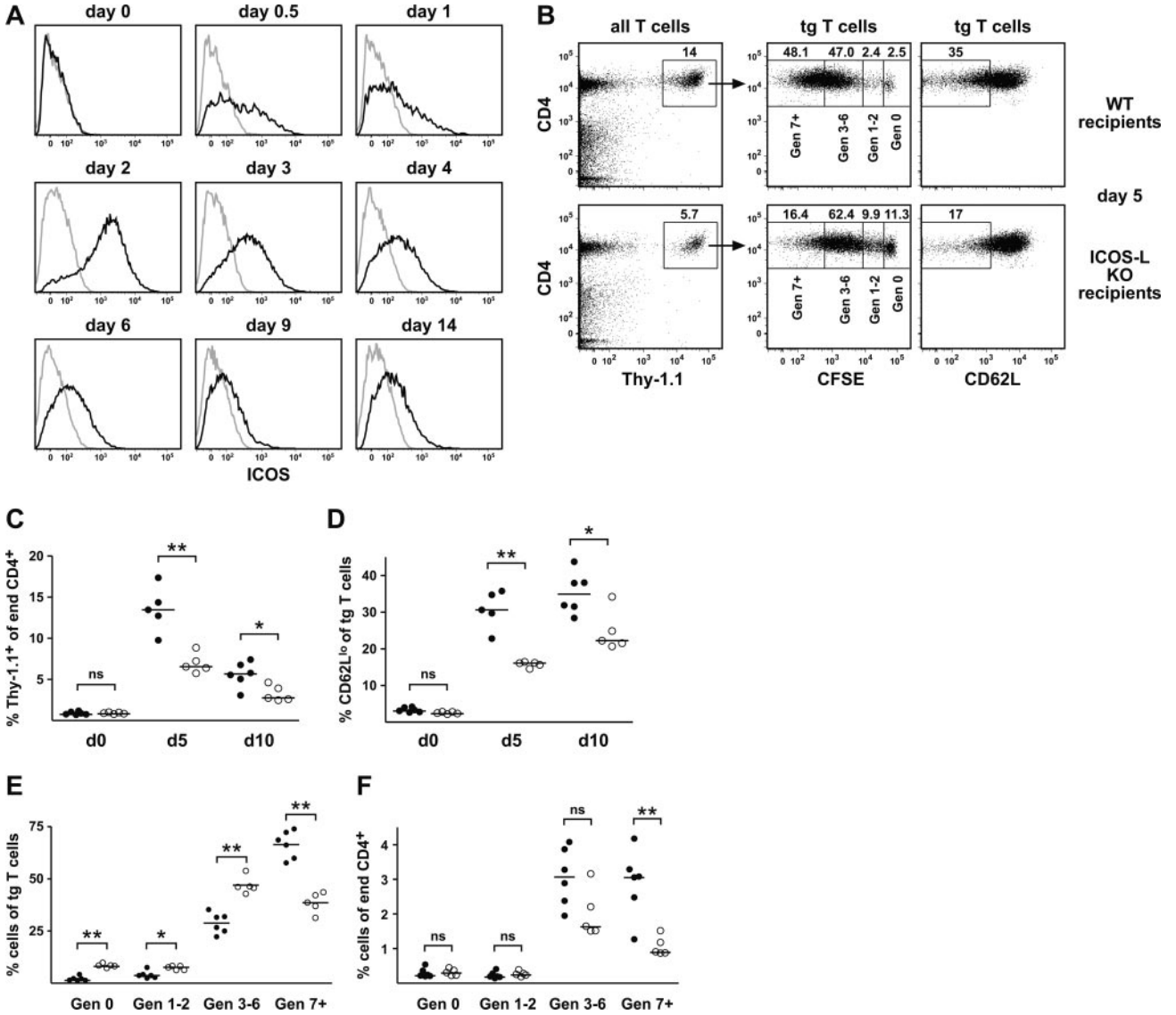

FIGURE 4. Analysis of ICOS function in an Ag-specific adoptive transfer system. Transfer of OVA-specific OT-II TCR transgenic T cells and local immunization into footpads with OVA plus adjuvant. *A*, ICOS expression kinetics on transgenic T cells from the draining popliteal LN. Staining with PE-conjugated anti-ICOS mAb MIC-280 (black line histogram); as a control for the specificity of staining, cells were preincubated with a 100-fold excess of unlabeled MIC-280 (gray line histogram). *B*, Transgenic (tg) T cells were labeled with CFSE and transferred into WT or ICOS-L KO mice. Cells from popliteal LNs were analyzed by flow cytometry at different time points after immunization. Ag-specific $CD4^+$ T cells were identified by Thy-1.1^+ staining. The number at each gate indicates their frequency in relation to recipients' $CD4^+$ T cells. Cells were further subdivided into different T cell division generations (Gen) based on CFSE staining intensity. At the gated generation divisions, the number indicates the relative frequency within the Thy-1.1^+ population. Furthermore, the proportion of transgenic T cells with low expression of CD62L was determined. A representative analysis for day 5 is shown. *C*, Overall frequency of transgenic T cells in relation to endogenous (end) $CD4^+$ T cells after transfer into WT (●) or ICOS-L KO (○) recipients. *D*, Proportion of $CD62L^{low}$ transgenic T cells within the transgenic T cell population. Frequency of transgenic cells in defined division generations on day 10 shown either within the transgenic population (*E*) or in relation to recipient $CD4^+$ T cells (*F*). Horizontal line indicates the median value for all animals within one experimental group. ∗, $p < 0.05$; ∗∗, $p < 0.01$. ns, Not significant. A representative experiment of five is shown.

by $FoxP3^-$ effector T cells, IL-10 production divides more or less equally between $FoxP3^+$ and $FoxP3^-$ $ICOS^{high}$ T cells.

ICOS KO mice have reduced numbers of effector-memory and regulatory T cells

To examine the functional consequences of ICOS expression, we analyzed $CD4^+$ T cells from ICOS-deficient mice (Fig. 2). ICOS KO mice showed no abnormalities regarding the absolute number of $CD4^+$ and $CD8^+$ T cells, B cells, or dendritic cells (5–7 and data not shown). However, a more detailed analysis revealed a substantial reduction of effector-memory T cells ($CD62L^{low}CD44^{high}$) in ICOS KO mice (Fig. 2). This effect was even more pronounced in older animals, which have a higher number of memory T cells (data not shown). Moreover, $FoxP3^+$ regulatory T cells were also reduced by ~30%. This reduction was counterbalanced by an increased frequency of naive T cells. To further substantiate these results, we also analyzed T cell subpopulations in ICOS-L KO mice. Because ICOS/ICOS-L is a monogamous receptor/ligand pair, these mice should have an identical phenotype. Indeed, we found the same reduction in memory and regulatory T cells in ICOS-L KO mice (data not shown).

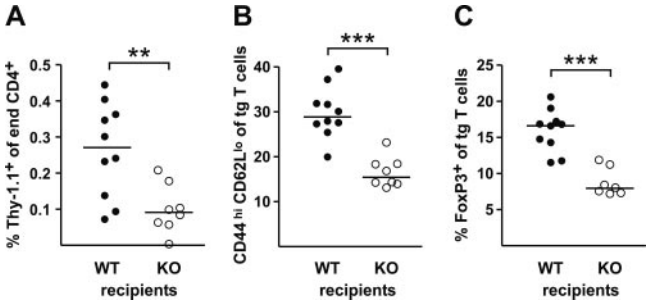

FIGURE 5. Analysis of Ag-specific regulatory T cells. Same experimental setup as in Fig. 4. However, immunization was systemic with 2 mg of endotoxin-free OVA i.v. Splenocytes were analyzed on day 12 by flow cytometry. *A*, Frequency of transgenic T cells in relation to endogenous $CD4^+$ T cells. **, $p < 0.01$. *B*, Frequency of effector-memory T cells (*B*) and $FoxP3^+$ T cells within the transgenic population (*C*). Horizontal line indicates the median value for all animals within one experimental group. ***, $p < 0.001$. A representative experiment of three is shown.

Reduction of regulatory T cells in the periphery is not caused by impaired generation in the thymus

The reduction of $FoxP3^+$ regulatory T cells in ICOS KO mice raised the question whether their generation in the thymus is impaired or whether their homeostasis in the periphery is affected. The thymus of ICOS KO mice was of normal size, and the thymocytes did not exhibit any abnormalities in their differentiation (5, 6 and data not shown). As in normal mice, $FoxP3^+$ cells were mainly found in the CD4 single positive but also in the CD4/CD8 double positive population. The number of FoxP3-expressing T cells was not lower and even slightly enhanced in ICOS KO mice, when compared with controls (Fig. 3). This result excludes a diminished generation of regulatory T cells in the thymus as a cause for their reduced frequency in the periphery.

ICOS costimulation contributes to the expansion and differentiation of Ag-specific effector T cells

The reduced number of memory and regulatory T cells represents end points of differentiation. To investigate the mechanisms behind this phenomenon, we used an adoptive transfer system allowing us to track the de novo generation of effector and memory cells from naive Ag-specific T cells. In this system OVA-specific T cells were transferred into WT vs ICOS-L KO recipients followed by local immunization with OVA.

Local immunization strongly increases the total cellularity of the draining LN. Specifically in ICOS KO mice, on immunization with CFA, a reduced recruitment of (Ag-unspecific) B cells to the LN

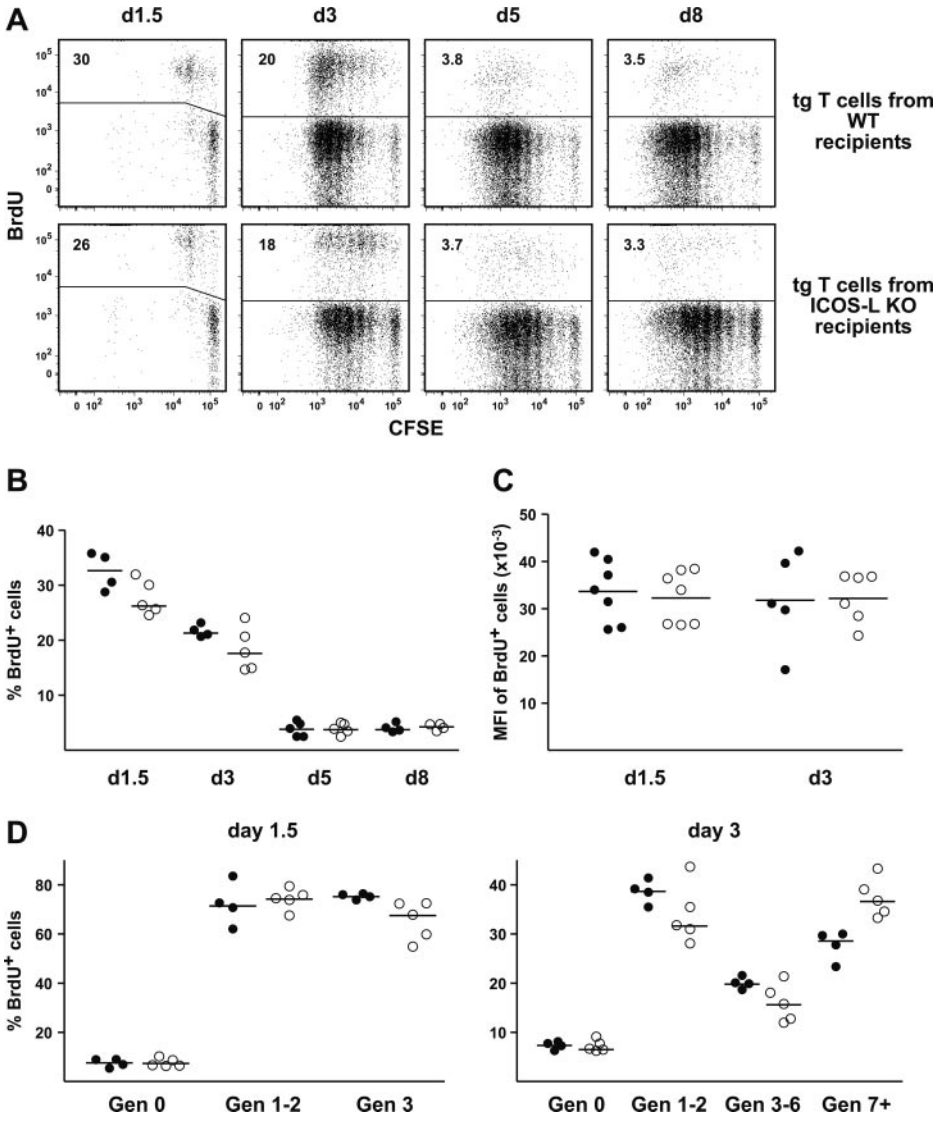

FIGURE 6. Analysis of short-term proliferation by BrdU incorporation. OVA-specific T cells were labeled with CFSE and were transferred into WT (●) or ICOS-L KO (○) mice. Mice were immunized with OVA plus adjuvant into footpads, and cells from popliteal LN were analyzed by flow cytometry for cell division at the indicated time points. Mice also received BrdU 2 h before analysis. Ag-specific T cells were identified by staining using the same gating strategy as in Fig. 4. The number in the gate indicates their frequency in relation to all transgenic T cells. *A*, Representative stainings for each time point. *B*, Percentage of BrdU-positive cells at different time points. *C*, Mean fluorescence intensity (MFI) of anti-BrdU staining. *D*, Percentage of BrdU-positive cells in early (generations 1 and 2), intermediate (generations 3–6), and late (generation 7+) stages of cell division as defined by CFSE staining. Horizontal line indicates the median value for all animals within one experimental group. A representative experiment of four is shown. Results in *C* are from a different experiment with identical setup as in *A*, *B*, and *D*.

was reported (22). Our immunization protocol increased LN cellularity, as expected, but the number of cells recovered from the draining LNs was comparable in WT and ICOS-L KO mice over the entire observation period, as was the ratio of $CD4^+$ T cell to B cells (data not shown). Because the preparation of cells from single LN is accompanied by an undefined cell loss precluding the use of absolute cell numbers for quantification, all experiments were controlled by using the endogenous $CD4^+$ T cell population as the reference population.

To analyze the proliferation of Ag-specific T cells in vivo, naive OT-II T cells (<3.5% $CD62L^{low}$) were stained with CFSE and transferred into ICOS-L KO or WT recipients. The number of transgenic T cells 24 h after transfer (day 0) were identical in both groups, showing that ICOS does not influence the recruitment of T cells into lymphoid organs (Fig. 4*C*). After local immunization with OVA, the transgenic T cells became activated and almost uniformly up-regulated ICOS. Peak expression on days 2–3 was followed by a gradual decline in expression, with $ICOS^+$ T cells still detectable on day 14 (Fig. 4*A*). The cells expanded rapidly, with maximal cell numbers observed on days 4–5, when OT-II T cells represented around 14% of all $CD4^+$ T cells in the draining LN of WT recipients. In stark contrast hereto, when the same T cells were transferred into ICOS-L KO mice, the expansion was reduced to ~50% on days 5 and 10 (Fig. 4, *B* and *C*). An analysis of the CFSE signal revealed that a higher proportion of OT-II T cells in ICOS-L KO recipients was found in the division generations 0, 1, and 2. A substantially lesser proportion of OT-II T cells was found in the 7+ division generation (Fig. 4, *B* and *E*). Taking into account the overall reduced number of Ag-specific cells in ICOS-L KO recipients, this analysis finally means that the cell number in early generations is the same in both groups, whereas the division generation 7+ is dramatically reduced in ICOS-L KO recipients (Fig. 4*F*).

To investigate, whether an altered migration pattern was responsible for this difference, we analyzed peripheral blood and nondraining LNs at different time points beyond day 3, when the first transferred cells left the draining LN. Again, cells with a multiple division history (generation division 7+) were missing in the ICOS-L KO group (data not shown).

These data indicated that costimulation via ICOS substantially contributes to the expansion of Ag-specific T cells. Interestingly, no differences were found for the up-regulation of CD25, CD69, 4-1BB, CD28, CTLA-4, CD40L, and BTLA, when analyzed at different time points between days 1 and 6 (data not shown). However, the proportion of newly generated effector T cells with down-regulated CD62L was clearly lower on OT-II T cells transferred into ICOS-L KO recipients (Fig. 4, *B* and *D*). This finding reflected the decreased number of $CD62^{low}$ T cells in ICOS KO mice in the steady state (see Fig. 2).

ICOS costimulation contributes to the expansion of Ag-specific regulatory T cells

Local immunization with OVA plus adjuvant favored the expansion of $FoxP3^-$ effector T cells in the draining LN ($FoxP3^+$ T cells were below 3%), making this system unsuitable for the analysis of Ag-specific regulatory T cells (data not shown). We therefore switched to systemic immunization with endotoxin-free OVA, a protocol known to favor the generation of regulatory T cells (23). Twelve days after adoptive transfer and immunization with OVA, OT-II T cells were analyzed in the spleen. As in the local immunization protocol, we observed a clearly decreased number of Ag-specific T cells in ICOS-L KO recipients (Fig. 5*A*). In addition, we found a very specific reduction of T cells with an effector-memory phenotype

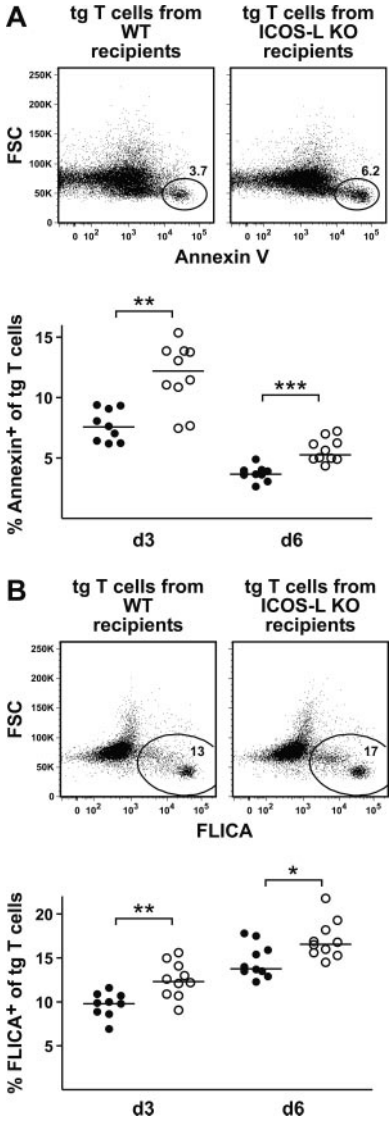

FIGURE 7. Role of ICOS for survival of Ag-specific T cells. OT-II TCR transgenic T cells were adoptively transferred into WT (●) or ICOS-L KO (○) recipients and immunized locally with OVA plus adjuvant. Popliteal LN were analyzed on days 3 and 6. Cells shown are gated on live ($DAPI^{low}$) Ag-specific Thy-1.1^+ $CD4^+$ T cells. *A*, Identification of early apoptotic cells by annexin V staining. Percentage of annexin-positive cells at different time points is also shown. **, $p < 0.01$; ***, $p < 0.001$. *B*, Alternative assay for apoptosis using a fluorochrome-labeled inhibitor of caspases (FLICA). Number in dot plot indicates percentage of cells gated in oval. Horizontal line indicates the median value for all animals within one experimental group. *, $p < 0.05$; **, $p < 0.01$. A representative experiment of six (*A*) or two (*B*) performed is shown.

($CD62L^{low}CD44^{high}$), and fewer cells with a multiple division history were found (Fig. 5*B* and data not shown). Most importantly, the proportion of $FoxP3^+$ Ag-specific T cells was significantly lower in the ICOS-L KO recipients when compared with the WT recipients (Fig. 5*C*). The reduced total number of transgenic T cells (approximately by a factor of 3) and the selective reduction in $FoxP3^+$ T cells within the transgenic population (approximately by a factor of 2) finally added up to a >6-fold difference in the number of Ag-specific regulatory T cells. This finding indicated that ICOS is also involved in the expansion of regulatory T cells.

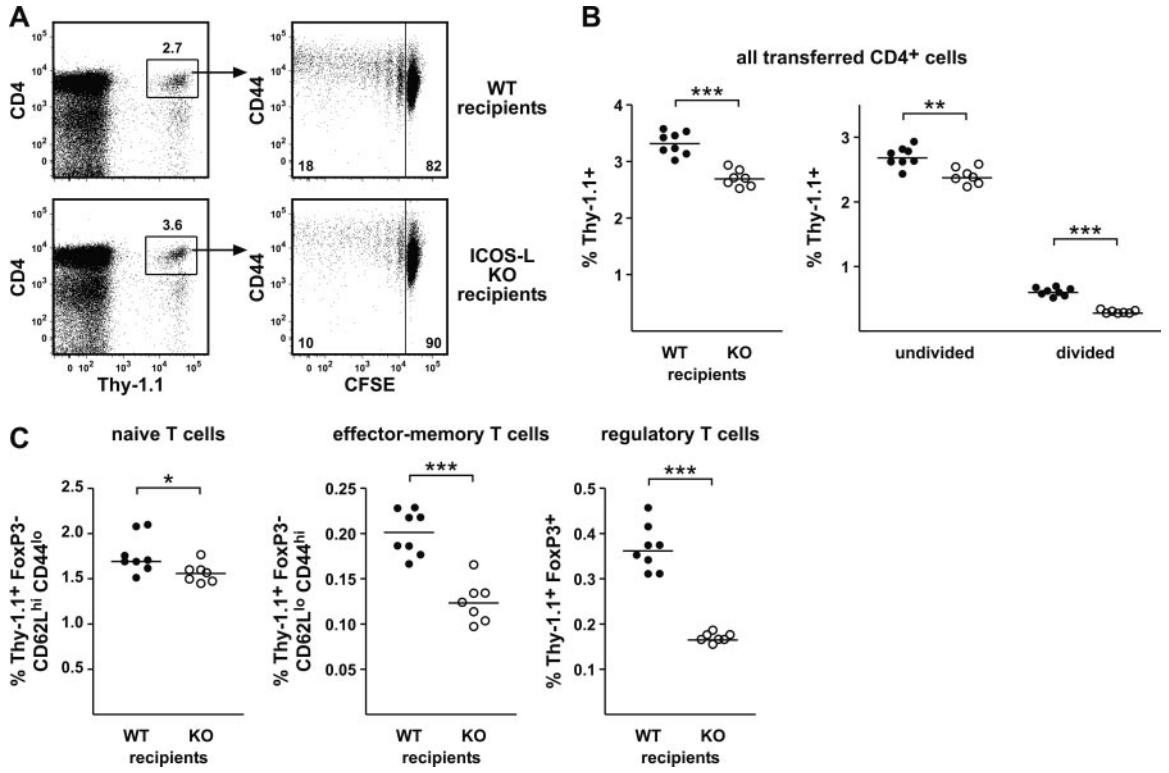

FIGURE 8. Homeostatic proliferation of adoptively transferred T cells. LN cells (25×10^6) from B6.PL mice (Thy-1.1$^+$) were labeled with CFSE and transferred into WT or ICOS-L KO mice (Thy-1.2$^+$). Cells from peripheral LNs of recipient mice were analyzed by flow cytometry on day 20. Residual Thy-1.1$^+$ T cells were divided into T cell subpopulations using the same gating strategy as shown in Fig. 1. *A*, Representative staining shows the CFSE dilution on all transferred T cells. Number in dot plots indicates percentage of cells in the indicated gate. Frequency of transferred cells in relation to the recipients' CD4$^+$ T cells is shown for all transferred CD4$^+$ T cells (*B*) and for the various subpopulations (*C*). Horizontal line indicates the median value for all animals within one experimental group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. A representative experiment of four (*B*) or two (*C*) is shown.

ICOS does not influence cell cycle progression

Many of our findings left room for the following two mutually nonexclusive interpretations: 1) ICOS costimulation sustains proliferation at later cycles of cell division, and 2) ICOS is important for long-term survival of T cells. To further distinguish between these possibilities, we combined CFSE staining with short-term BrdU incorporation to assess the ongoing proliferation rate at any defined time point.

On days 1.5 and 3, up to 35% of all OT-II T cells incorporated BrdU during a 2 h pulse, whereas on days 5 and 8 only a small number of T cells still synthesized DNA (Fig. 6*A*). In all experiments, the BrdU incorporation rate was consistently somewhat smaller in the ICOS-L KO group than in the WT group, but not to a significant degree (Fig. 6*B*). The proliferation rate in later cell division generations was not selectively diminished in the ICOS-L KO group (Fig. 6*D*). This result excludes the possibility that lack of ICOS costimulation results in cell cycle arrest after a certain number of cell divisions. In addition, the mean fluorescence intensity of the BrdU signal was identical in both experimental groups, indicating that the rate of DNA synthesis in cells in the S phase was independent of ICOS signaling (Fig. 6*C*).

Lack of ICOS increases the apoptosis rate of T cells

Taken together, these findings point to a role of ICOS in the survival of activated T cells. We addressed this question directly using DAPI and annexin V staining to detect T cells in early apoptosis. Importantly, the fraction of early apoptotic cells was significantly higher in OT-II cells without ICOS costimulation (Fig. 7*A*). A more detailed analysis relating annexin V staining to the number of cell divisions showed that this significant difference was true for all cell generations (data not shown). Same results were obtained using alternative apoptosis assays with fluorescent-labeled caspase inhibitors (FLICA) or intracellular staining for active caspase-3 (Fig. 7*B* and data not shown).

ICOS KO T cells do not lack expression of key survival factors

Although little is known about factors regulating the survival of CD4$^+$ T cells, some key factors have been identified recently (21, 24). We analyzed activated Ag-specific T cells in our adoptive transfer system, as well as T cells from nonimmunized ICOS KO mice for the expression of IL-7R, OX-40, intracellular Bcl-2, and Bcl-x_L, but did not identify any differences (data not shown). Another key survival factor, especially for regulatory T cells, is IL-2 (25). Several publications on ICOS and ICOS-L KO mice reported a reduction or even complete lack of IL-2 production by T cells after stimulation in vitro (5, 10). This finding prompted us to measure IL-2 in vivo. We transferred unseparated OT-II T cells (including memory T cells) into ICOS-L KO or WT recipients, reisolated these cells 6 h after immunization, and directly measured IL-2 production (without in vitro restimulation) using a cytokine capture assay (26). In both groups ~38% of Ag-specific T cells produced IL-2 (data not shown). Therefore, different from the reported findings in vitro, lack of ICOS costimulation in vivo did not affect IL-2 production.

ICOS costimulation positively influences homeostatic proliferation and survival of T cells

The adoptive transfer system with Ag-specific T cells demonstrated the role of ICOS for controlling the pool size of activated T cells. To learn more about the function of ICOS in a steady-state situation without Ag, we switched to another transfer system with polyclonal T cells. Total LN cells from B6.PL mice, a C57BL/6 congenic strain, which can be distinguished by the 1.1 variant of Thy-1, were labeled with CFSE and transferred into WT or ICOS-L KO mice. Analysis of peripheral LNs on day 20 revealed that a homeostatic proliferation has taken place in both recipient groups, but the overall number of surviving T cells was significantly higher in WT recipients (Fig. 8, *A* and *B*). As in the OVA-specific model, the proportion of divided cells was lower without ICOS costimulation, and there were fewer cells with a history of multiple divisions (Fig. 8, *A* and *B*). Also the absolute number of undivided cells was reduced (Fig. 8*B*). Analysis of different T cell subsets revealed that the reduction of transferred cells was most pronounced within the effector-memory phenotype and regulatory T cell subset (Fig. 8*C*). This situation exactly resembles the one found in the ICOS KO mice (Fig. 2).

Taken together, the data demonstrate the positive contribution of ICOS to T cell survival not only in an ongoing Ag-specific response, but also in the steady state.

Discussion

Originally, ICOS KO mice were reported to have a rather normal phenotype in the steady state, showing impaired T cell effector functions only in an ongoing immune response (5–7). Our detailed analysis of T cell subsets now revealed that ICOS KO mice have up to 4-fold lower numbers of effector-memory T cells already in the steady state. Also the number of regulatory T cells is severely reduced. Whereas the generation of regulatory T cells in CD28 KO mice is already defective in the thymus (27, 28), we did not observe any deficiency in thymic $FoxP^+$ T cells in ICOS KO mice, despite the high expression of ICOS on CD4 single positive thymocytes (29). Therefore, ICOS seems to have major impact on the homeostasis of regulatory T cell in the periphery.

Early in vitro studies suggested that ICOS costimulation has broad effects on T cell activation by up-regulating cell surface activation molecules (e.g., CD69, CD25, CD40L), by enhancing cytokine synthesis, proliferation, and cell survival (2, 3, 6, 7, 13). However, some subsequent reports using cells from ICOS or ICOS-L KO mice for in vitro assays did not find any effects on proliferation (6, 8) and T cell survival (8). One study described effects of ICOS costimulation on the expansion of Ag-specific T cells, but could not attribute this effect to altered cell division or any other mechanism (16). Many of these older studies were limited in certain technical aspects as they could not control for untoward effects of "blocking" Abs (e.g., depletion, partial costimulation, Fc-mediated effects) or could not follow up Ag-specific T cells in vivo. Without the tracing of Ag-specific cells, the experimental information is somewhat restricted because a population reduced to 50%, but fully activated, cannot be easily distinguished from a population activated to 50%.

Using an adoptive transfer system with Ag-specific T cells, we now could more precisely define the contribution of ICOS to T cell biology in vivo. In our system, WT OT-II TCR transgenic T cells were transferred into ICOS-L KO vs WT recipients, which is advantageous because of an identical starting T cell population. However, the reduced number of regulatory T cells in the ICOS-L KO recipient mice could bias the activation/expansion of the transferred T cells. As control for this potential bias, we additionally transferred OT-II ICOS KO and OT-II WT T cells into normal C57BL/6 recipients as an alternative setup. In all experiments both strategies led to comparable results (data not shown).

We found that ICOS costimulation does not affect the general activation status of the T cells as defined by the expression of several cell surface markers. Instead, we could show that ICOS regulates the survival of activated effector T cells as well as resting (nondividing) memory T cells. As a result, ICOS very significantly influences the availability of Ag-experienced T cells in later phases of the immune response. Compared with in vitro cultures the percentage of annexin V-positive $DAPI^-$ early apoptotic cells observed in vivo was rather small, but one should take into account that in vivo these cells are rapidly cleared by phagocytic cells. Therefore, the 2-fold difference in the apoptosis rate between WT and ICOS-L KO mice observed at a given time point can easily explain the different pool size of effector T cells. In that respect, ICOS biology shows some resemblance to the effects of OX-40, which has recently been shown to promote long-term T cell survival by up-regulating Bcl-2 and $Bcl\text{-}x_L$ (30).

Whether ICOS actively regulates T cell proliferation is difficult to judge. The BrdU incorporation rate in ICOS-L KO mice was only slightly reduced, but the cumulative effect of this observed difference may become biologically relevant after several rounds of cell division. At the same time, the intensity of the BrdU signal was identical in both groups. This may indicate that ICOS affects to a certain degree the probability of a cell to enter the cell cycle, but not the rate of DNA synthesis in the S phase. Alternatively, the small difference in BrdU incorporating cells can be attributed to the death of cells during the BrdU pulse. Taken together, the possible contribution of ICOS to cell proliferation seems to be at best small compared with the potent effect of CD28 costimulation on cell cycle progression (31).

The observed contribution of ICOS to the expansion of effector and memory T cells is in line with earlier data obtained in a greater number of disease models. These studies demonstrated an important proinflammatory role for ICOS in the late effector phase (13–15), or in the secondary, T memory-dependent B cell response (6–9). A reduced pool of memory T cells will be less effective in providing help to B cells for recall Ags (32). This mechanism could contribute to the almost complete absence of memory B cells in ICOS-deficient patients (17).

So far, most studies pointed to a biological role of ICOS as a positive costimulator for effector T cells. However, some more recent results could not easily be reconciled with such a functional paradigm. In models of inhalational or mucosal tolerance, the blockade or absence of ICOS led to an exacerbation of the disease (33–35). Further, in a mouse diabetes model that relies on a sensitive balance of effector and regulatory T cells, blockade of ICOS aggravated the condition and appeared to be related to a reduced number of $CD4^+CD25^+$ regulatory T cells (36).

The present study finds that ICOS not only influences the pool size of effector and memory T cells, but the pool size of $FoxP3^+$ regulatory T cells. These results may help to resolve the seeming discrepancies of ICOS biology in vivo. In mouse airway inflammation models, which are induced by Ag plus adjuvant, effector T cells producing IL-4, IL-5, and IL-13 are crucial in the initiation and maintenance of the disease process. Under these conditions ICOS will increase the pool size of proinflammatory cells and thus contribute to the degree of inflammation. Other airway inflammation models based on antigenic challenge without adjuvants have been shown to induce a high number of regulatory T cells (33, 34). Under these conditions, the absence of ICOS can be expected to lead to a reduced number of Ag-specific $FoxP3^+$ regulatory T cells and thus to a breakdown of tolerance with resulting inflammation.

Based on a number of studies, we have earlier on proposed a concept according to which ICOS is not hard-wired to certain T cell differentiation pathways such as Th1 or Th2, but acts as an agonist in the "effector" phase of a T cell (12, 19). Our present in vivo experimental system demonstrated that ICOS has a similar role on such functionally divergent cells as effector T cells and FoxP3$^+$ regulatory T cells. In view of these findings, we would like to extend the agonist molecule concept to all T cells expressing high levels of ICOS on the cell surface. These T cells may be proinflammatory effector cells, FoxP3$^+$ regulatory effector cells, Th1 or Th2 responder cells, or Th17 cells (37). In all cases, ICOS can be expected to support T cell expansion and survival. Consequently, the absence of ICOS function in a given animal model will lead to a phenotype reflecting a deficiency of the dominating effector T cell type.

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Disclosures

The authors have no financial conflict of interest.

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