Retrogenic ICOS Expression Increases Differentiation of KLRG-1<sup>hi</sup>CD127<sup>lo</sup>CD8<sup>+</sup>T Cells during *Listeria* Infection and Diminishes Recall Responses

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Retrogenic ICOS Expression Increases Differentiation of KLRG-1\textsuperscript{hi}CD127\textsuperscript{lo}CD8\textsuperscript{+} T Cells during *Listeria* Infection and Diminishes Recall Responses

Danya Liu,*† Eileen M. Burd,‡ Craig M. Coopersmith,†§ and Mandy L. Ford*†

Following T cell encounter with Ag, multiple signals are integrated to collectively induce distinct differentiation programs within Ag-specific CD8\textsuperscript{+} T cell populations. Several factors contribute to these cell fate decisions, including the amount and duration of Ag, exposure to inflammatory cytokines, and degree of ligation of costimulating molecules. The ICOS is not expressed on resting T cells but is rapidly upregulated upon encounter with Ag. However, the impact of ICOS signaling on programmed differentiation is not well understood. In this study, we therefore sought to determine the role of ICOS signaling on CD8\textsuperscript{+} T cell programmed differentiation. Through the creation of novel ICOS retrogenic Ag-specific TCR-transgenic CD8\textsuperscript{+} T cells, we interrogated the phenotype, functionality, and recall potential of CD8\textsuperscript{+} T cells that receive early and sustained ICOS signaling during Ag exposure. Our results reveal that these ICOS signals critically impacted cell fate decisions of Ag-specific CD8\textsuperscript{+} T cells, resulting in increased frequencies of KLRG-1\textsuperscript{hi}CD127\textsuperscript{lo} cells, altered BLIMP-1, T-bet, and eomesodermin expression, and increased cytolytic capacity as compared with empty vector controls. Interestingly, however, ICOS retrogenic CD8\textsuperscript{+} T cells also preferentially homed to nonlymphoid organs and exhibited reduced multicytokine functionality and reduced ability to mount secondary recall responses upon challenge in vivo. In sum, our results suggest that an altered differentiation program is induced following early and sustained ICOS expression, resulting in the generation of more cytolytically potent, terminally differentiated effectors that possess limited capacity for recall response. *The Journal of Immunology*, 2016, 196: 000–000.

During the initiation of an Ag-specific CD8\textsuperscript{+} T cell response, the integration of multiple signals from the extracellular microenvironment collectively serves to induce distinct differentiation programs in Ag-specific T cells, such that the response is optimally adapted to respond appropriately to the insult. The factors that can influence this programmed differentiation include the amount and affinity of Ag, the type and duration of costimulation, and the level of inflammatory cytokines present (1–5). One aspect of programmed differentiation includes the cell fate decision to become either short-lived effector cells (SLECs), which exhibit potent cytolytic effector function during the peak of the response but are destined to undergo apoptosis during the contraction phase of the response, or long-lived memory precursor effector cells (MPECs), which may have less potent effector function but will go on to form the memory CD8\textsuperscript{+} T cell population that persists following clearance of Ag (5). Phenotypically, these differentiation programs can be identified on the basis of expression of KLRG-1 and CD127, which are reciprocally expressed on SLECs (KLRG-1\textsuperscript{lo}CD127\textsuperscript{hi}) and MPECs (KLRG-1\textsuperscript{hi}CD127\textsuperscript{lo}) (6, 7). Molecularly, the program is carried out by the expression of key transcription factors; in particular, high expression of T-bet and BLIMP-1 are thought to confer potent effector function but limited recall potential (8–15), whereas high expression of eomesodermin is thought to confer cytokine-secreting polyfunctionality as well as enhanced memory T cell recall potential (13, 14). Within a given T cell population, both SLEC and MPEC cell fates are induced following activation. The factors that dictate the induction of either an SLEC or MPEC differentiation program in a given T cell are thought to include the amount and duration of Ag exposure and the amount of inflammatory cytokine signaling, in particular IL-12 (also type 1 IFN) (5). However, the role of costimulatory signals in dictating SLEC versus MPEC cell fate decisions during CD8\textsuperscript{+} T cell differentiation is less well understood.

It is clear, however, that the type and duration of costimulatory signaling during T cell activation critically impacts the magnitude and quality of Ag-specific CD8\textsuperscript{+} T cell responses. One of the best-studied families of T cell costimulatory molecules is the CD28 family, and the programmed differentiation that ensues as a result of CD28 signals has been elucidated (16). CD28 signals lead to sustained IL-2 production and CD25 expression, promote cell division and survival, and enhance memory T cell development (17–20). The ICOS is a CD28 family member bearing some sequence homology to CD28 (21). In contrast to the constitutive expression of CD28 on T cells, ICOS is not expressed on resting CD4\textsuperscript{+} or CD8\textsuperscript{+} T cells but is induced upon encounter with Ag (22). It is also dynamically regulated such that during activation, there is a range of ICOS expression within a given population of Ag-specific CD8\textsuperscript{+} T cells. However, the impact of ICOS signaling on programmed differentiation is not well understood. It is thought that following upregulation and encounter of its ligand...
B7-H1 (ICOS ligand [ICOS-L]), ICOS delivers additional co-stimulatory signals to further enhance T cell activation and differentiation into cytokine-producing effector cells (22, 23).

Models of autoimmune immunity revealed that ICOS signaling is critical for T cell–mediated pathogenicity in experimental autoimmune encephalomyelitis and the development of type 1 diabetes (24) and that ICOS blockade could be efficacious in treating ongoing activated T cell responses and reversing autoimmunity during active disease (25, 26). Similarly, research in experimental transplant models has demonstrated that costimulation through ICOS is required for the development of both acute and chronic rejection (27, 28). In a recent study, ICOS antagonism synergized with CTLA-4–Ig to inhibit the effector function of donor-reactive memory T cells and prolong graft survival (29).

In this study, we therefore sought to determine the role of increased and sustained ICOS expression on CD8+ T cell differentiation programs. Through the creation of ICOS retrogenic (ICOSrg) Ag-specific TCR-transgenic (tg) T cells, which constitutively express high levels of ICOS, we interrogated the phenotype, functionality, and recall potential of cells that receive high and sustained ICOS signaling during Ag exposure. Results indicated that ICOS signals critically impacted cell fate decisions of Ag-specific CD8+ T cells and imparted a differentiation program that rendered cells highly cytolytic but completely unable to sustain secondary recall responses upon heterologous rechallenge in vivo.

Materials and Methods

**Mice**

C57BL/6 (H-2b) mice were obtained from the National Cancer Institute (Frederick, MD). OT-I (30) and OT-II (31) tg mice, purchased from Taconic Farms (Germantown, NY), were bred to the Thy1.1 background at Emory University. mOVA mice (C57BL/6 background, H-2b) (32) were a gift from Dr. Marc Jenkins (University of Minnesota, Minneapolis, MN). All animals were maintained in accordance with Emory University Institutional Animal Care and Use Committee guidelines. All animals were housed in pathogen-free animal facilities at Emory University.

**Donor-reactive T cell adoptive transfers and Listeria infection**

For adoptive transfers of donor-reactive T cells, spleen and mesenteric lymph node (LN)–sorted cells isolated from Thy1.1–OT-I tg mice were processed and stained with mAbs for CD4 and CD8 (both from Invitrogen), Thy1.1, and Vε2 (BD Pharmingen) for flow cytometry analysis. Cells were reseeded in PBS and 10×10^6 Thy1.1–OT-I–infected tumor cells were injected i.v. Four hours after adoptive transfer, splenocytes were harvested and assessed for CD45.1 expression and CFSE labeling. For CD107a degranulation assays, cells were pulsed at 10 nmol OVA257–264 (SIINFEKL) (GenScript) where indicated, in the presence of 10 μg/ml streptomycin, 15 ml DMEM supplemented with 15% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mmol HEPES, 20 mg/ml murine IL-3, 50 ng/ml human IL-6, and 50 ng/ml murine stem cell factor (R&D Systems). The concentrated virus was transduced into the transduced BM cells. After 48 h incubation, BM cells were collected and washed. Subsequently irradiated (800 rad) wild-type (WT) B6 recipients were injected i.v. with 4×10^6 BM cells in PBS. Splenocytes from these BM chimeras were harvested 6–8 wk posttransplant and enriched by negative selection using a CD8a+ T cell isolation Kit II (Miltenyi Biotec). Purity of CD8a+ T cells was >80%. Cells were then stained with anti-CD8a Pac Orange, anti-Thy1.1 PerCP, and anti-ICOS APC, and CD8a-Thy1.1 ICOS+ T cells were purified by FACS sorting on a BD FACSaria (BD Biosciences). Postsort ICOS-OT-I T cell populations were >95% pure.

**Flow cytometry and intracellular cytokine staining**

Blood, spleens, graft-draining axillary, and brachial LNs, liver, and lung were isolated and stained for CD8 (both from Invitrogen) and Thy1.1 (BD Pharmingen). To isolate lymphocytes from liver, liver was perfused in situ via the portal vein, cells were made into a single-cell suspension, and Percoll gradient centrifugation was used as described (34). For isolation of lymphocytes from lung, murine lungs were perfused in situ with 3 ml PBS via the heart right ventricle, placed in digestion buffer (Eagle’s MEM plus 10% FCS), 1.5 mg/ml type IA collagenase (Sigma-Aldrich, St. Louis, MO), and 0.75 mg/ml type I hyaluronidase (Sigma-Aldrich), chopped into fragments of 0.5–2.0 mm3, and incubated at 37°C for 60 min as described (34). For phenotypic analysis, cells were also surface-stained with anti-ICOS, anti–KLRG-1, anti-CD127, anti-CD44, anti-CD62L, anti-Fas ligand (FasL), anti-CD103, and anti-CD69 (all Pharmingen). BLIMP-1, T-bet, comesemydamin, and granzyme B expression were measured intracellularly using an intracellular staining kit (BD Pharmingen). For 7-aminooxycinnamoyl D staining, cells were resuspended in 20 μl 7-aminooxycinnamoyl D/1×10^6 cells (BD Pharmingen) and analyzed by flow cytometry. Absolute numbers were calculated using TrueCount bead analysis according to the manufacturer’s instructions. Samples were analyzed on an LSRL II flow cytometer (BD Biosciences). Data was analyzed using FlowJo software (Tree Star, San Carlos, CA). For intracellular cytokine staining, splenocytes were stimulated with 10 nmol OVA257–264 (SIINFEKL) (GenScript) where indicated, in the presence of 10 μg/ml brefeldin A for 4 h. An intracellular staining kit was used to detect IL-2, TNF, and IFN-γ (all from BD Pharmingen), according to the manufacturer’s instructions.

**In vivo cytokinesis assay**

As previously published (35), CD45.1-congenic splenocyte target cells were labeled with high (1 μmol) or intermediate (100 nmol) concentrations of CFSE. The CFSE-lll target cells were pulsed at 10 nmol OVA257–264 peptide; CFSE-lll target cells were incubated without peptide. A total of 10^6 target cells in a 50:50 mixture of unloaded and peptide-loaded target cells were adoptively transferred i.v. into each LM-OVA–infected recipient. Four days after adoptive transfer, splenocytes were harvested and assessed for CD45.1 expression and CFSE labeling. For CD107a degranulation assay, splenocyte suspensions were incubated in R10 media at 37°C in a 96-well plate (4×10^6 cells/well) for 5 h with mAbs and anti–CD107a–FITC in the presence or absence of 10 nmol OVA257–264 peptide as previously described (36). After incubation, surface staining with anti-Thy1.1 and anti-CD8a was performed. Degranulation was measured as the frequency of CD8a-Thy1.1+ cells that were CD107a+.

**Anti-ICOS mAb treatment**

Where indicated, animals were injected with 250 μg/dose of an anti-ICOS mAb (clone 1709; BioXCell, West Lebanon, NJ) diluted in 500 μl sterile PBS on days 0, 2, 4, and 6 postinfection.

**Skin transplantation**

Full thickness tail and ear skins were transplanted onto dorsal thorax of recipient mice and secured with adhesive bandages as previously described (37). Where indicated, animals were treated with 100 μg anti-CD28

(15338–100, Invitrogen). Cells were transiently transfected with 10 μg DNA (ICOS plasmid DNA or empty vector control). After 48 h incubation, the culture supernatant was harvested, and virus was concentrated per manufacturer’s instructions (RV-201, Cell Bios)
domain Ab on days 0, 2, 4, and 6 (38). Mice were monitored for graft survival, and rejection was defined as <10% viable donor tissue remaining.

Statistical analysis
Survival data were plotted on Kaplan–Meier curves, and log-rank tests were performed. For analysis of T cell responses, nonparametric Mann–Whitney U tests were performed. Results were considered significant if \( p < 0.05 \). All analyses were done using GraphPad Prism software (GraphPad Software). In all legends and figures, \( *p < 0.05, **p < 0.01, ***p < 0.001 \).

Results

Differential expansion and contraction in ICOShi versus ICOSrg Ag-specific CD8+ T cell populations in vivo

We sought to determine the impact of ICOS expression on programmed T cell differentiation. Initial experiments demonstrated that following in vivo antigenic stimulation, Thy1.1+ CD8+ OT-I T cells specific for SIINFEKL divide and upregulate ICOS (Fig. 1A). Data from CFSE analysis revealed that highly divided (CFSElo) CD8+ OVA-specific T cells exhibited increased ICOS expression (mean fluorescence intensity [MFI]) relative to less divided (CFSEhi) CD8+ OVA-specific T cells (Fig. 1B). Importantly, we observed that although Ag-specific ICOShi cells significantly outnumbered Ag-specific ICOSlo cells at day 10 (the peak of the response) (Fig. 1C, 1D), by day 14, there was no significant difference in the number of ICOShi versus ICOSlo Ag-specific CD8+ T cells (Fig. 1D). The relative enrichment for ICOSlo cells compared with ICOShi cells at day 14 (Fig. 1E) suggests one of two possibilities: 1) ICOSlo cells underwent a greater fold contraction from day 10 to day 14 relative to ICOShi cells; or 2) ICOS was downregulated such that ICOSlo cells converted into ICOShi cells by day 14. In order to differentiate between these two possibilities, we created TCR Ig T cells that expressed ICOS via a constitutively active retroviral promoter, such that they could not modulate the expression of ICOS following activation. We hypothesized that high expression of ICOS may result in a T cell differentiation program that is more SLEC-like, resulting in increased contraction within the population of Ag-specific CD8+ T cells that express the highest levels of ICOS.

Retrogenic expression of ICOS on Ag-specific CD8+ T cells results in enhanced expansion and accumulation at day 7 postinfection

In order to determine the impact of early and sustained ICOS expression during T cell activation and programmed differentiation, we used a retrogenic approach to constitutively express ICOS within the Ag-specific CD8+ T cell population. Briefly, CD45.2+ Thy1.1+ OT-I BM was transduced with a construct that expresses ICOS under a constitutively active promoter. The construct also contained an IRES-GFP to facilitate tracking the cells. Approximately 10% of Thy1.1 OT-I BM cells expressed either GFP alone (pMY control vector-transduced cells) or both GFP and ICOS (for ICOS vector-transduced cells) on day 3 following transduction (not shown). BM cells were then adoptively transferred into irradiated CD45.2+Thy1.2+ animals. At 8–10 wk post–in vivo BM transfer, Thy1.1+ pMY and ICOSrg cells OT-I T cells were detectable in the blood at comparable frequencies (Fig. 2A, left panel). Mature Thy1.1+ OT-I T cells from spleen and LN of pMY (GFP+ICOS-) or ICOSrg (GFP+ICOS+) chimeric animals were FACS sorted (Fig. 2A, right panel) and adoptively transferred (10^5/recipient) into naive B6 hosts. Animals were then infected with LM-OVA. Prior to adoptive transfer, we confirmed that expression of ICOS was significantly higher on ICOSrg as compared with pMY cells (Fig. 2B). Expression of CD62L and CD44 on ICOSrg OT-I versus pMY empty vector control T cells were not different (Fig. 2C), demonstrating that there was no difference in the naive versus memory status of ICOSrg OT-I T cells prior to secondary transfer and suggesting that the expression of ICOS during T cell development did not dramatically alter baseline activation status of these cells. Furthermore, to ascertain the degree of surface ICOS expressed by the retrogenic T cells as compared with those cells that upregulate ICOS as a consequence of activation, we compared the MFI of ICOS expressed on ICOSrg OT-I T cells (at day 10 postinfection) to the MFI of ICOS expressed on WT OT-I T cells at day 10 postinfection. As shown in Fig. 2D, ICOS expression was similar between groups. These data suggest that the level of ICOS expression on ICOSrg OT-I T cells falls within the physiologic range.

Animals containing pMY-OT-I or ICOSrg-OT-I were then infected with LM-OVA, and peripheral blood was assessed for Ag-specific T cell expansion over time. Results indicated that ICOSrg Thy1.1+CD8+ T cells initially expanded to a greater extent than control pMY-OT-I (Fig. 2E, 2F), such that by day 7, there was a significantly increased population of cells in the ICOSrg mice (Fig. 2E). However, this increase in frequencies of ICOSrg Thy1.1+ CD8+ T cells was not sustained, such that there was no difference in the observed frequencies of ICOSrg Thy1.1+ CD8+ cells as compared with pMY-OT-I in these animals on days 10, 14, and 20 postinfection. In addition, there was no difference in bacterial loads measured in the spleen at day 3 postinfection (Supplemental Fig. 1A).

Retrogenic ICOS overexpression on Ag-specific CD8+ T cells results in increased KLRG-1hiCD127lo phenotype and enhanced cytolytic function

In order to determine whether ICOS overexpression altered the programmed differentiation of the Ag-specific CD8+ T cell response, we assessed phenotypic markers normally associated with SLECs versus MPECs. Results revealed that the frequencies of ICOSrg Thy1.1+ CD8+ cells expressing the SLEC-associated marker KLRG-1 were increased at the peak of the response (day 10 postinfection; Fig. 3A), whereas the frequencies of ICOSrg Thy1.1+ CD8+ cells expressing the MPEC-associated marker CD127 were decreased at day 10 postinfection relative to pMY-transduced OT-I controls (Fig. 3B). These data demonstrate the early and sustained ICOS expression contribute to Ag-specific CD8+ T cell differentiation and increase the proportion of SLEC-like cells.

Given these observed changes in KLRG-1 and CD127 expression on ICOSrg cells following *Listeria* infection, we sought to characterize the tissue distribution of pMY and ICOSrg cells in this system. Results demonstrated that although the frequencies and number of Thy1.1+ cells in isolated from these two groups were identical in the spleen and blood (Fig. 3C–E), there were significantly more ICOSrg cells in the liver (Fig. 3C, 3F) and a trend toward more ICOSrg OT-I in the lung (Fig. 3C, 3G). These data further corroborate the idea that ICOSrg cells are more effector memory-like.

Retrogenic ICOS expression on Ag-specific CD8+ T cells results in increased cytolytic capacity in vivo but reduced multicytokine-producing functionality

We next sought to determine whether this increased effector-like phenotype of the ICOSrg CD8+ T cells was associated with any change in functionality. In order to accomplish this, we performed an in vivo CTL assay in which naive B6 mice were adoptively transferred with either ICOSrg Thy1.1+ OT-I T cells or pMY Thy1.1+ OT-I controls and were infected with LM-OVA. On day 10 postinfection, 5 × 10^6 CD45.1+CFSEhi SIINFEKL-pulsed target cells were transferred into the recipients along with 5 × 10^6 CD45.1+CFSElo unpulsed control targets (Fig. 4A). Four hours
later, mice were sacrificed, and the relative proportion of peptide-pulsed versus unpulsed CD45.1+ CFSE-labeled targets was determined, and the percent specific lysis was calculated as described in Materials and Methods. Results revealed that mice containing ICOSrg OT-I T cells exhibited significantly higher percent specific lysis than mice containing pMY OT-I controls (Fig. 4B, 4C). Importantly, the frequency and absolute number of Thy1.1+ CD8+ OT-I T cells in the spleens of pMY versus ICOSrg recipients were similar at this time point (Fig. 3E), suggesting that the cells possessed increased cytolytic capacity on a per-cell basis; interestingly, however, in vitro measures of cytolytic function including granzyme B expression and CD107a degranulation assay failed to demonstrate any difference between pMY and ICOSrg OT-I T cells isolated from these animals (Supplemental Fig. 1B–D). Taken together, these data indicate that ICOSrg CD8+ Ag-specific T cells exhibit both phenotypic and functional characteristics of more highly activated, potent effectors in vivo during the course of an immune response.

Given the above results demonstrating enhanced in vivo cytolytic function of ICOSrg CD8+ T cells, we next interrogated their ability to produce the inflammatory cytokines TNF, IFN-γ, and IL-2. Cells isolated from the spleens of day 10 LM-OVA–infected recipients of 10^6 ICOSrg or pMY control OT-Is were restimulated ex vivo with SIINFEKL Ag for 4 h in the presence of brefeldin A and then stained intracellularly for the presence of cytokines. Interestingly, results revealed that ICOSrg OT-I T cells were compromised in their ability to make cytokines, in that the frequencies of cells making IFN-γ were reduced (Fig. 4D–F). Strikingly, however, the frequencies of both IFN-γ+TNF+ (Fig. 4D, 4G) and IFN-γ+IL-2+ (Fig. 4E, 4H) multicytokine producers were even more diminished in the Thy1.1+CD8+ ICOSrg population as compared with the pMY controls. Live/dead staining performed on these samples did not detect any difference in the frequency of apoptotic Thy1.1+CD8+ OT-I T cells between the groups (Supplemental Fig. 1E). Taken together, these data demonstrate that although ICOSrg CD8+ T cells are more potent cytolytic effectors, their
ability to differentiate into high-quality multicytokine producers is diminished.

Blockade of ICOS signaling during Listeria infection results in fewer KLRG-1+ cells and delayed contraction of the Ag-specific CD8+ T cell response

Given the above results suggesting that constitutive ICOS signaling resulted in the generation of more KLRG-1+ short-term effector-like cells, we next queried whether blockade of ICOS signals during the elicitation of an anti-Listeria T cell response would result in the opposite effect. To address this, naive B6 animals were adoptively transferred with 10^6 WT OT-I T cells and infected with LM-OVA 2 d later (Fig. 5A). Immediately prior to infection and on days 2, 4, and 6 thereafter, mice were injected with an anti-ICOS blocking Ab as described in Materials and Methods. Results indicated that on day 7 postinfection, the magnitude of the OT-I T cell response in animals...
treated with anti-ICOS was not different compared to untreated controls, both in terms of frequency and absolute number (Fig. 5B–D). However, OT-I T cells isolated from anti–ICOS-treated mice exhibited a trend toward decreased frequencies of KLRG-1hi cells in the peripheral blood, and a statistically significant reduction in the absolute number of KLRG-1hi Thy1.1+ CD8+ T cells (Fig. 5E–G). By day 10 postinfection, there were significantly more Thy1.1+ OT-I T cells in the anti–ICOS-treated animals relative to untreated controls, both in terms of frequencies (Fig. 5H) and absolute numbers (not shown). Taken together, these data suggested that blockade of ICOS signals during the primary CD8+ T cell response to *Listeria-OVA* resulted in diminished contraction of the response between day 7

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** ICOSrg overexpression on Ag-specific CD8+ T cells results in increased KLRG-1hiCD127lo phenotype and increased presence in non-lymphoid tissues. Thy1.1+ OT-I T cells from spleen and LN of pMY (GFP+ICOS) or ICOSrg (GFP+ICOS+) 8–10-wk chimeric animals were FACS sorted and adoptively transferred (10^4/recipient) into naive B6 hosts. Animals were then infected with LM-OVA. Thy1.1+CD8+ pMY or ICOSrg cells in peripheral blood were stained with anti–KLRG-1 (A) and anti-CD127 (B) and analyzed by flow cytometry. Summary data are shown from three independent experiments with a total of nine mice per group. (C) Animals described above were sacrificed on day 10 posttransplant, and CD8+Thy1.1+ pMY or ICOSrg OT-I T cells were assessed in the blood, spleen, liver, and lung. (D–G) Summary data of frequencies and absolute numbers of the CD8+Thy1.1+ pMY or ICOSrg OT-I T cells described in (C) (n = 5 mice/group). *p < 0.05, **p < 0.01, ***p < 0.0001.
FIGURE 4. ICOSrg expression on Ag-specific CD8+ T cells results in increased cytolytic capacity in vivo but reduced multicytokine-producing functionality. (A) Naive B6 mice were adoptively transferred with either ICOSrg Thy1.1+ OT-I cells or pMY Thy1.1+ OT-I controls and infected with LM-OVA. On day 10 postinfection, $5 \times 10^6$ CD45.1+ CFSEint SIINFEKL-pulsed target cells were transferred into the recipients along with $5 \times 10^6$ CD45.1+ CFSEhi unpulsed control targets. Four hours later, mice were sacrificed, and the relative proportion of peptide pulsed versus unpulsed CD45.1+ CFSE-labeled targets was determined (representative animals shown in B), and the percent specific lysis was calculated as described in Materials and Methods. (C) Summary data of cytolysis experiments from two independent experiments with a total of five mice per group. (D) Day 10 splenocytes of the animals described above were restimulated ex vivo with SIINFEKL Ag for 4 h in the presence of brefeldin A and then stained intracellularly for the presence of cytokines. Unstimulated cells that were not exposed to peptide served as negative controls. (D and E) Plots are gated on CD8+Thy1.1+ cells and depict IFN-γ+ single producers (F), IFN-γ+TNF+ double producers (G), and IFN-γ+IL-2+ double producers (H). Representative animals from pMY and ICOSrg animals are shown. (F) Summary data depicting frequencies of total IFN-γ+Thy1.1+ CD8+ cells in both groups. (G) Summary data depicting frequencies of IFN-γ+TNF+Thy1.1+ CD8+ cells in both groups. (H) Summary data depicting frequencies of IFN-γ+IL-2+Thy1.1+ CD8+ cells in both groups. (D–H) Data shown are cumulative from three independent experiments with a total of 14-29 animals/group. *p < 0.05, **p < 0.01, ***p < 0.0001.
Impact of ICOS blockade on Ag-specific CD8+ T cell cytolytic and cytokine effector function

To further corroborate our findings from the retrogenic model, we interrogated OT-I T cell acquisition of cytolytic and cytokine function in the setting of ICOS blockade. LM-OVA–infected recipients of OT-I T cells were treated with anti-ICOS mAb as described above, and 10 d later, CFSE-labeled CD45.1+ OVA-pulsed target cells and unpulsed controls were transferred into animals as described in Materials and Methods. As shown in Fig. 6A, anti-ICOS-treated animals exhibited reduced cytolytic function on a per-cell basis as compared with untreated controls. Interestingly, Ag-specific Thy1.1+ CD8+ T cells isolated at this time point (day 10) exhibited reduced expression of FasL, suggesting that the
reduced cytolytic function observed in anti–ICOS-treated animals may be a result of decreased engagement of the Fas/FasL death pathway. Furthermore, consistent with our findings that retrogenic ICOS expression resulted in diminished multicytokine effectors (Fig. 4D–H), OT-I T cells isolated from anti–ICOS-treated animals exhibited increased frequencies of IFN-γ/IL-2+ multicytokine producers on day 10 postinfection (Fig. 6D, 6E).

Our data suggested that retrogenic ICOS expression resulted in increased effector responses within tissues such as lung and liver (Fig. 3C–G). To further understand the role ICOS signaling in Ag-specific CD8+ T cell responses within peripheral tissues, we interrogated the impact of ICOS blockade on the development of CD69+CD103+ tissue-resident effector-memory T cells following *Listeria* infection. As depicted in Fig. 6F and 6G (left panel), a significantly reduced frequency of CD69+CD103+ cells was observed within the Ag-specific CD8+ Thy1.1+ T cell population in the lungs of anti–ICOS-treated animals as compared with untreated controls. Similarly, whereas CD103+ CD8+ T cells were very rare in the liver, data revealed a significantly reduced frequency of CD69+ cells within the Ag-specific CD8+ Thy1.1+ T cell population in the livers of anti–ICOS-treated animals as compared with untreated controls (Fig. 6G, right panel). Taken

**FIGURE 6.** ICOS blockade impacts Ag-specific CD8+ T cell cytolytic and cytokine effector function. Thy1.1+ OT-I T cells from spleen and LN of WT mice were adoptively transferred (10^5/recipient) into naive B6 hosts. Animals were then infected with LM-OVA. Where indicated, animals were treated with anti-ICOS Ab (clone 17G9; 250 μg/dose) on days 0, 2, 4, and 6 postinfection. (A) On day 10 postinfection, 5 × 10^6 CD45.1+CFSEint SIINFEKL-pulsed target cells were transferred into the recipients along with 5 × 10^6 CD45.1+CFSEhi unpulsed control targets. Four hours later, mice were sacrificed, the relative proportion of peptide-pulsed versus unpulsed CD45.1+ CFSE-labeled targets was determined, and the percent specific lysis was calculated as described in Materials and Methods. Summary data of cytolysis experiments from two independent experiments with a total of five mice per group. (B and C) Day 10 splenocytes of the animals described above were stained ex vivo for FasL. Representative flow cytometry staining (B) and summary data (C) depicting frequencies of FasL+ cells of Thy1.1+CD8+ T cells in untreated versus anti–ICOS-treated animals. (n = 4 to 5 animals/group). (D and E) Day 10 splenocytes of the animals described above were restimulated ex vivo with SIINFEKL Ag for 4 h in the presence of brefeldin A and then stained intracellularly for the presence of cytokines. Unstimulated cells that were not exposed to peptide served as negative controls. (D) Representative flow cytometry plots are gated on CD8+Thy1.1+ cells and depict IFN-γ+IL-2+TNF+ double producers. (E) Summary data depicting frequencies of IFN-γ+IL-2+Thy1.1+ CD8+ cells in both groups (n = 5/group). (F and G) Analysis of CD103 and CD69 expression on Ag-specific CD8+ Thy1.1+ T cells isolated from lungs and liver of untreated or anti–ICOS-treated animals on day 10 postinfection. Data depict representative flow cytometry plots of CD8+Thy1.1+ T cells isolated from the lung (F) and summary data of n = 5 animals/group from the lung (G, left panel) or liver (G, right panel). *p < 0.05, **p < 0.01.
together, these data suggest that blockade of ICOS signals may result in diminished generation of CD69<sup>+</sup>CD103<sup>+</sup> tissue-resident effector memory T cells following Listeria infection.

**ICOSrg CD8<sup>+</sup> T cells exhibit impaired recall responses following challenge**

Our data thus far has suggested that ICOS expression on Ag-specific CD8<sup>+</sup> T cells confers a potent effector phenotype characterized by enhanced early expansion and cytolytic effector function but compromised ability to produce critical T cell cytokines, including IL-2. We therefore sought to determine the impact of increased ICOS expression on Ag-specific CD8<sup>+</sup> T cell recall responses. To accomplish this, we used a skin allograft model in which LM-OVA-infected recipients of either ICOSrg OT-I or control pMY-OT-I were heterologously rechallenged on day 20 postinfection with skin derived from mice that constitutively express membrane-bound OVA under the β-actin promoter (mOVA mice) (Fig. 7A). At day 5 postchallenge, mice were sacrificed, and graft-draining LN cells were harvested for analysis. As observed in our earlier studies, there was no difference in either the frequency or absolute number of ICOSrg CD8<sup>+</sup>Thy1.1<sup>+</sup> OT-I T cells as compared with pMY-OT-I controls in ungrafted (no rechallenge) recipients (Fig. 7B). In the recipients that received a skin graft rechallenge, mice containing control pMY-OT-I exhibited significant recall response to the graft challenge with an ~7-fold increase in the absolute number of graft-specific Thy1.1<sup>+</sup> OT-I T cells. In contrast, however, animals containing ICOSrg CD8<sup>+</sup> memory T cells failed to mount a recall response upon heterologous rechallenge, in that there was no observed increase in either the frequency or absolute number of ICOS rg OT-I T cells in the draining LN following skin transplantation (Fig. 7B, 7C). To determine whether this effect was restricted to heterologous skin transplant rechallenge, we assessed recall responses to secondary rechallenge with a high dose of Listeria-OVA. Animals that had received pMY or ICOSrg OT-I T cells and been infected with LM-OVA 20 d earlier were rechallenged with a high dose (10<sup>6</sup> CFU) of LM-OVA, and OT-I T cell responses were assessed 5 d later in the blood. As shown in Fig. 7D, recipients of pMY-OT-I T cells exhibited strong Thy1.1<sup>+</sup> CD8<sup>+</sup> T cell responses in the blood, whereas Thy1.1<sup>+</sup>CD8<sup>+</sup> T cells were barely detectable in recipients of ICOSrg OT-I T cell responses. Comparison of absolute numbers of OT-I T cells in the blood of these animals revealed a significant, two-log reduction in the magnitude of the ICOSrg versus pMY OT-I secondary recall response (Fig. 7E).

We hypothesized that altered programmed differentiation in the presence of high ICOS expression was underlying the observed diminished recall responses. We therefore interrogated the expression of BLIMP-1, T-bet, and eomesodermin, T cell transcription factors known to be expressed in memory T cells and that help determine potency of recall responses. We observed that ICOSrg CD8<sup>+</sup>Thy1.1<sup>+</sup> OVA-specific effectors (day 10 postinfection) expressed reduced levels of BLIMP-1 (Fig. 7F), T-bet (Fig. 7G), and eomesodermin (Fig. 7H) as compared with pMY-OT-I controls. Taken together, these data suggest that high expression of ICOS during a primary response integrate to decrease expression of memory T cell-associated transcription factors and thus diminish recall potential. We next queried the impact of this altered differentiation and recall potential on the ability of these cells to carry out graft rejection. Naïve recipients of pMY or ICOSrg OT-I T cells were infected with Listeria-OVA. On day 30 postinfection, animals received an OVA-expressing skin graft and were treated with anti-CD28 domain Ab immunosuppression as described in Materials and Methods. As shown in Fig. 7I, we found that recipients containing ICOSrg memory OT-I T cells experienced accelerated rejection as compared with those possessing WT pMY memory OT-I T cells (p = 0.0394). These data indicate that although ICOSrg memory T cells possess poorer recall potential in the draining node, they possess a superior ability to mediate rejection of peripheral tissues.

**Discussion**

In this study, we demonstrated that early and sustained ICOS expression is instructive for enhanced early expansion and differentiation into KLRRG-1<sup>hi</sup>, CD127<sup>lo</sup> short-lived effector cells, followed by more exaggerated contraction and failure to mount recall responses upon secondary rechallenge. The data are corroborated by blocking studies in which the absence of ICOS signaling during T cell activation resulted in reduced differentiation into KLRRG-1<sup>hi</sup>, SLEC and diminished contraction of the Ag-specific CD8<sup>+</sup> T cell response. These studies highlight a key role for ICOS expression in programmed differentiation and confirm and extend previous studies on the role of ICOS in the development and maintenance of T cell memory. For example, it is known that both ICOS<sup>−/−</sup> and ICOS-L<sup>−/−</sup> mice exhibit reduced frequencies of CD4<sup>+</sup> T effector memory cells as the mice age (39). Intriguingly, this deficit was confined to the T effector memory compartment and was not observed within the ICOS<sup>−/−</sup> or ICOS-L<sup>−/−</sup> T central memory population (39). Furthermore, antigenic stimulation of ICOS<sup>−/−</sup> cells results in less downregulation of CD62L and CCR7 as compared with WT cells (40). Together with these findings, our data suggest that ICOS expression is critical for effector/effector memory differentiation following infection. These conclusions are also supported by data from a published study with human patients with a rare genetic disorder in ICOS signaling (41). Nine patients have been identified worldwide possessing an identical mutation in the Icos gene, and all exhibit immunologic abnormalities ranging from immune deficiency to autoimmunity.

Our data also demonstrate enhanced cytolysis but reduced multifunctional cytokine secreting cells within ICOSrg CD8<sup>+</sup> T cell populations and conversely reduced cytolytic function and increased multifunctional cytokine-secreting cells in OT-I T cells in the setting of ICOS blockade. The observation that ICOSrg CD8<sup>+</sup> T cells, which have higher propensity to differentiate into KLRRG-1<sup>hi</sup>CD127<sup>lo</sup> SLEC-like cells and are found in higher numbers in nonlymphoid tissues such as liver and lung, also have reduced multicytokine-producing ability is for the most part consistent with many published reports on the general functional capacities of SLECs versus MPECs (5, 42, 43). For instance, it is known that KLRRG-1<sup>hi</sup> Ag-specific CD8<sup>+</sup> T cells have diminished capacity to produce IL-2 relative to Ag-specific KLRRG-1<sup>hi</sup> CD8<sup>+</sup> T cells (5, 44, 45). However, most studies show that KLRRG-1<sup>hi</sup> and KLRRG-1<sup>lo</sup> Ag-specific CD8<sup>+</sup> T cells are equivalent in their abilities to induce cytolysis and produce IFN-γ (5, 44, 45). Thus, we conclude that although some aspects of the loss of cytokine-producing functionality observed in the population of CD8<sup>+</sup> ICOSrg cells could be entirely dependent on their increased frequency of KLRRG-1<sup>hi</sup> cells, other aspects of this loss of cytokine functionality are likely independent of the SLEC/MPEC paradigm and represent a distinct aspect of ICOS-mediated T cell differentiation. This impact of ICOS on multicytokine-producing ability has also previously been described in the literature, in that populations of influenza virus–specific CD4<sup>+</sup> T cells possessing higher IFN-γ/TNF-IL-2 multicytokine-producing functionality also exhibited reduced ICOS expression (46).

Taken together with these results, our study demonstrates a role for ICOS signaling in driving T cells toward a less multifunctional, more terminally differentiated effector phenotype.
FIGURE 7. ICOSrg CD8+ T cells exhibit impaired recall responses following challenge. Thy1.1+ OT-I T cells from spleen and LN of pMY (GFP+ICOS+) or ICOSrg (GFP+ICOS-) 8–10-wk chimeric animals were FACS sorted and adoptively transferred (10^4/recipient) into naive B6 hosts. Animals were then infected with LM-OVA. (A) On day 20 postinfection, animals were heterologously rechallenged with skin derived from mice that constitutively express membrane-bound OVA under the β-actin promoter (mOVA mice). (B) Mice were sacrificed at day 5 posttransplant, and graft-draining LN cells were harvested for analysis. Representative data shown are gated on CD8+ T cells. (C) Summary data of the absolute numbers of CD8+Thy1.1+ pMY versus ICOSrg cells from two independent experiments with a total of 7–10 mice/group. (D and E) Recipients of either pMY or ICOSrg OT-I T cells were infected with LM-OVA and then reinfected with a higher dose (10^6 CFU) on day 20 postinfection. (D) Representative flow cytometry plots showing frequencies of Thy1.1+ of CD8+ T cells in the peripheral blood at day 5 post–secondary rechallenge with high-dose LM-OVA. (E) Summary data of n = 5 animals/group. CD8+ Thy1.1+ T cell effectors isolated on day 7 or 10 post–primary Listeria-OVA infection were fixed, permeabilized, and stained intracellularly for the transcription factors BLIMP-1 (F), eomesodermin (Eomes) (G), and T-bet (H). Cumulative data from two independent experiments with a total of 10–14 animals per group are depicted. (I) Mice containing memory pMY (n = 6) or ICOSrg (n = 7) T cells were challenged with an OVA-expressing skin graft as described above and treated with 100 μg anti-CD28 domain Ab on days 0, 2, 4, and 6. Mice were monitored for graft rejection. *p < 0.05, **p < 0.01.
Overall, our study suggests that ICOS functions on Ag-specific CD8+ T cells to increase early expansion and cytolytic function, but somewhat paradoxically results in reduced long-term memory and recall potential. These results may have clinical implications in that ICOS-blocking reagents being developed for the treatment of both autoimmunity and transplantation have met with somewhat limited success (47). Indeed, ICOS blockade studies presented in this manuscript demonstrated an increased proportion of KLRG-1lo MPEC-like cells and increased multifunctional cytokine producers in the setting of ICOS blockade. Thus, in light of the findings put forth in this manuscript, it is interesting to speculate that perhaps the observed limited efficacy of these reagents was due in part to the generation of more multifunctional CD8+ T cell responses with enhanced recall potential in the setting of reduced ICOS signaling. Conversely, these results may also have implications for protective immunity and vaccine design, in that blockade of ICOS signals during the induction of Ag-specific CD8+ T cell responses may result in more long-lived, multifunctional cellular immunity. One important caveat to note, however, is that these findings pertain to Ag-specific CD8+ T cells, and the critical role of ICOS on CD4+ T follicular helper cells for the provision of help during the generation of Ab responses is likely indispensable (48).

Our data revealed an increase in the frequency of Ag-specific CD8+ T cells in ICOSrg cells as compared with pMY controls at day 7 post-Listeria infection; however, this initially augmented response failed to persist by day 10. Conversely, blockade of ICOS signals resulted in increased persistence of OT-I T cells at days 10. Together, these data suggest that strong or preserved ICOS signals may promote early T cell contraction, whereas the absence of ICOS signaling favors T cell survival. Previous studies examining the impact of ICOS deficiency on the expansion and accumulation of CD8+ effector T cells have shown dichotomous results, with some studies demonstrating decreased CD8+ T cell proliferation in ICOS−/− animals (49) and other studies concluding that accumulation of activated CD8+ T cells is not altered in ICOS−/− animals (50). Interestingly, we also assessed the expansion of ICOSrg CD8+ T cells in response to a skin transplant as opposed to pathogen infection and found that under these conditions, ICOSrg OT-I T cells did not exhibit enhanced accumulation and an altered KLRG-1/CD127 profile (data not shown). Taken together, these results suggest that the role of ICOS in CD8+ T cell expansion and differentiation may be dependent on the precise stimulation conditions present. It is interesting to speculate that one factor impacting whether ICOS functions to significantly enhance CD8+ T cell accumulation during an immune response may be expression patterns of ICOS-L. Unlike CD80/CD86, which are primarily restricted to APC, ICOS-L can be expressed on parenchymal cells (51, 52) and is upregulated in the presence of inflammatory cytokines such as TNF (52). Thus, different types of inflammatory conditions such as those present during transplantation versus pathogen infection might result in differential availability of ICOS-L; this possibility warrants further investigation.

One of the most striking aspects of ICOSrg expression in our study was a reduced ability of ICOSrg memory T cells to mount recall responses when subjected to rechallenge, via either a second LM-OVA infection or heterologous challenge with an OVA-expressing skin graft. This finding is consistent with the decreased proportion of KLRG-1loCD127hi memory precursor cells in these animals; however, it is important to note that we did not observe a difference in the number of Ag-specific pMY versus ICOSrg CD8+ T cells present at the time of rechallenge. Thus, although ICOS overexpression did not result in a quantitative difference in the generation of memory, it did result in the altered expression of transcription factor cemeosdermin and BLIMP-1, both transcription factors known to be important for memory T cell differentiation (8–14). Reduced expression of cemeosdermin in these cells is consistent with several published reports that demonstrated the critical role of this molecule in the generation and maintenance of CD8+ memory T cells. Interestingly, previous reports on BLIMP-1 and T-bet in CD8+ T cells have demonstrated the increased expression of these molecules in KLRG-1loCD127hi SLECs (10, 12), whereas our data showed a decrease in BLIMP-1 and T-bet on ICOSrg cells relative to pMY controls. This possible discrepancy may be reconciled by a recent report that demonstrated a critical role for BLIMP-1 during recall responses in influenza virus–infected animals (9), but it is also possible that intense ICOS signaling induces the differentiation of cells that are distinct from classical SLECs. We conclude that a distinct profile of transcription factor expression is induced following early and sustained ICOS expression, which results in the generation of more cytolytically potent, terminally differentiated effectors that possess limited capacity for recall response.

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Disclosures

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References


