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Cytokine- and TCR-mediated regulation of T cell expression of Ly6C and Sca-1

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Abstract

Ly6C and Sca-1 (Ly6A/E) are Ly6 family GPI-anchored surface molecules that are differentially expressed by multiple immune populations. Ly6C expression has been used to distinguish short-lived effector $CD4^+$ T cells from memory precursor effector cells, whereas Sca-1 has been used in the identification of stem-like memory T (Tscm) cells. The present study examines the expression patterns of these molecules and establishes that, *in vitro*, IL-27, type I IFN and IFN-γ are potent inducers of Ly6C and Sca-1 in naïve mouse $CD4^+$ and $CD8^+$ T cells, while TGF-β limits their expression. The induction of Ly6C and Sca-1 by IL-27 and IFN-γ is dependent on STAT1, but not STAT3 or T-bet. In mouse splenocytes, at homeostasis, Ly6C and Sca-1 expression was not restricted to effector cells, but was also found at various levels on naïve and memory populations. However, in response to infection with *Toxoplasma gondii*, pathogen-specific T cells expressed high levels of these molecules and in this context, endogenous IL-27 and IFN-γ were required for the expression of Ly6C but not Sca-1. Together, these findings highlight the TCR-dependent and cytokine-mediated signals that modulate T cell expression of Ly6C and Sca-1 *in vitro* and *in vivo* during infection.

Keywords

IL-27; interferon-gamma; Sca-1; Ly6C; T cell effectors

Introduction

The T cell response to infection or immunization involves the generation of minimally differentiated memory cells as well as highly differentiated effector cells (1). Effector T cells

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produce the cytokines, granzymes, and other molecules necessary for immediate pathogen control, while central memory T cells are long-lived and can differentiate into effector cells upon rechallenge (1–3). The identification of surface molecules that distinguish effector and memory T cell populations has allowed researchers to track the expansion, evolution and contraction of the T cell response during infection and has provided insights into how these cells operate. For example, central memory cells upregulate CD44 and express CD62L, which keeps them localized to lymphoid organs. Effector cells are also $CD44^{hi}$ but lose expression of CD62L, allowing them to home to sites of inflammation. While these definitions have proven useful to define naïve, effector and memory T cells, additional markers, including KLRG1, CXCR3 and Ly6C have been used to further sub-divide these populations (4–8). In current models, a subset of highly-differentiated, short-lived effector cells (SLECs), which for $CD8^+$ T cells are often identified by expression of KLRG1 (8), are specialized to control acute infection. These cells produce high levels of cytokines and granzymes and survive poorly upon adoptive transfer to naïve hosts. Less-differentiated $CD8^+$ T cells, identified during acute toxoplasmosis as $CXCR3^+KLRG1^-$, exhibit the longevity, proliferative capacity and differentiation potential typical of memory cells (4).

Ly6C and Sca-1 (Ly6A/E) are members of a family of 21 Ly6-like proteins in mice, with 20 Ly6 family homologues in humans (9). Expression of Ly6C has been used to identify highly differentiated effector $CD4^+$ T cells (6, 10, 11). Among virus-specific effector $CD4^+$ T cells, $Ly6C^+$ cells produced more cytokines and effector molecules than $Ly6C^-$ cells (6, 10). Conversely, Sca-1, in combination with the IL-2R beta chain (CD122) and Bcl-2, has been used to identify mature $CD8^+$ T cells with stem-like properties, termed stem cell memory (Tscm) cells (12–14).

The finding that Ly6C is preferentially expressed by $CD4^+$ SLECs during acute infection suggests that identification of the factors that modulate Ly6C expression could yield insights into the signals that control the development of memory and effector populations. For example, sustained TCR signals have been found to skew T cells toward terminal differentiation and away from memory development (1, 15), while multiple cytokines influence the development of effector and memory populations (16). Relevant to this report, type I interferons, IFN-γ, and IL-27 have previously been implicated in promoting $Ly6C^+$ and $Sca\text{-}1^+$ populations in $CD4^+$, $CD8^+$ and regulatory T cells (17–21). However, previous studies have not been able to distinguish whether these stimuli induce Ly6C and Sca-1 expression or simply promote the outgrowth of $Ly6C^+$ or $Sca\text{-}1^+$ populations. Furthermore, extant reports have not examined how these cytokines intersect with TCR signaling to impact Ly6C and Sca-1 expression and what roles these signals play in modulating the expression of these molecules *in vivo*. In utilizing sorted $Ly6C^-Sca\text{-}1^-$ populations, the present study was able to show that TCR stimulation alone induces Sca-1 on $CD4^+$ and $CD8^+$ T cells, but is not sufficient to induce Ly6C on $CD4^+$ T cells. The cytokines IL-27, IFN-γ and type I IFN were found to broadly promote the expression of Ly6C and Sca-1, while TGF-β inhibited this expression. This cytokine-mediated induction of Ly6C and Sca-1 was largely STAT1-dependent and is not dependent on STAT3 or T-bet. A survey of naïve and antigen-experienced T cells in a range of differentiation states found that Ly6C expression is not restricted to effector cells and that Sca-1 expression is not limited to naïve/memory-like cells. Furthermore, *in vivo* studies during infection with *Toxoplasma gondii*

identified a role for endogenous IL-27 and IFN-γ in promoting effector cell expression of Ly6C. Together, these studies provide new insights into the signals that modulate the development of $Ly6C^+$ and $Sca\text{-}1^+$ T cell populations but indicate caution in the use of these molecules as markers of highly-differentiated effector cells (Ly6C) and minimally-differentiated stem cell memory cells (Sca-1).

Materials and Methods

Maintenance and care of experimental animals

Six week-old female C57BL/6 controls were purchased from Taconic. Mice deficient in Il27ra/WSX-1 (C57BL/6 background) were generated as described (22) and were originally provided by Amgen (Thousand Oaks, CA, USA). $STAT1^{-/-}$ mice (129S6/SvEv-Stat1tm1Rds) and 129S6 control mice were purchased from Taconic. Spleens from CD4-Cre x $STAT3^{-/-}$ mice were provided by Alejandro Villarino and John J. O'Shea (NIH). Mice were housed and bred in specific pathogen-free (SPF) facilities in the Department of Pathobiology at the University of Pennsylvania in accordance with institutional guidelines. The Me49 Strain of *T. gondii* was prepared from chronically infected CBA/ca mice and experimental animals were infected intraperitoneally with 20 cysts.

Cell sorting and *in vitro* cell culture

Splenocytes from C57BL/6 mice were obtained by mechanically dissociating the spleen, filtering it through a 40 micron nylon strainer, and lysing red blood cells with ACK lysis buffer. T cells were enriched using a Mouse $CD3^+$ T Cell Enrichment Column (R&D Systems MTCC-25). Cells were then stained with Live/dead fixable Aqua dead cell stain (ThermoFisher L34957), anti-CD4 (GK1.5, Biolegend 100447), anti-CD8 (53–6.7, BD Biosciences 562283), anti-CD44 (IM7, eBioscience 0441-82), anti-CD62L (MEL-14, eBioscience 47-0621-82), anti-Ly6C (HK1.4, eBioscience 45-5932-82), and anti-Sca-1 (D7, eBiosceince 56-5981-82) antibodies and were sorted on a FACSAria II flow cytometer (BD Biosciences). Cells were plated in tissue culture-treated round-bottom 96-well plates, $1–2 \times 10^5$ per well in 200uL RPMI supplemented with 10% fetal bovine serum, 100U/mL penicillin, 100 U/mL streptomycin, 1 mM sodium pyruvate, 1x MEM non-essential amino acids (Gibco), 55 uM 2-Mercaptoethanol. The tissue culture plates were precoated with 1ug/mL anti-CD3 (145-2C11, BioXCell) for 3 hours at 37 degrees and excess anti-CD3 was rinsed off with PBS. Cells were stimulated in the presence of anti-CD28 (37.N.51.1, 1 ug/mL), IL-2 (Proleukin, 100U/mL), anti-IFN-γ (XMG1.2, BioXcell, 1ug/mL) (except when exogenous IFN-γ was tested) and anti-IL-4 (11B11, BioXcell, 1 ug/mL). Recombinant IL-27 (Amgen) was used at a concentration of 50 ng/mL, TGF-β (eBioscience) was used at 5ng/mL, and Universal type I IFN (PBL Assay Science) was used at a concentration of 2000 U/mL. IFN-γ (R&D Systems), IL-6 (eBioscience), IL-12 (eBioscience), TNF-α (eBioscience), IL-10 (eBioscience), and IL-7 (Peprotech) were used at 10 ng/mL. IL-15 (Peprotech) and IL-15Ra-Fc (R&D Systems) were incubated at 37 degrees for 30 minutes at a ratio of 2:9. The resulting IL-15 complexes were used at 55 ng/mL (10ng/mL IL-15, plus 45ng/mL IL-15Ra).

Flow cytometric Analysis

Cells were stained with the reagents used for cell sorting, described above, as well as antibodies specific for CD122 (5H4, BD Biosciences 554452), CD127 (SB/199 Biolegend 121105), CD69 (H1-2F3, eBioscience 12-0691-83), CD25 (PC61, BD Biosciences 553866), KLRG1 (2F1, eBioscience 25-5893-82), and CD49d (R1-2, Biolegend 103617). For analyses after infection, splenocytes were harvested as detailed above and peritoneal exudate cells were harvested by intraperitoneal lavage with 7 mL PBS. MHC-I monomers loaded with peptide (SVLAFRRL) from the *T. gondii* protein Tgd-057 were kindly provided by E. John Wherry (University of Pennsylvania) and tetramerized by incubation with streptavidin-conjugated PE or APC. Some experiments utilized PE- and APC-conjugated MHC-I tetramers loaded with the Tgd-057 peptide that were provided by the National Institutes of Health Tetramer Facility. PE- or APC-conjugated MHC-II tetramers loaded with the AS15 peptide AVEIHRPVPGTAPPS were also provided by the National Institutes of Health Tetramer Facility.

Cells were collected on an LSRFortessa (BD Biosciences) and analysis was performed with FlowJo (TreeStar). Cells were gated on lymphocytes (by forward scatter (FSC) and side scatter (SSC)), singlets (by FSC-W vs FSC-H and SSC-W vs SSC-H), and live cells (by exclusion of Aqua Dead Cell Stain). $CD4^+$ T cells were gated $CD4^+CD8^-FoxP3^-$ and $CD8^+$ T cells were gated $CD8^+CD4^-$.

Statistical Analysis

Statistical significance was determined using GraphPad Prism software, using Student's t test. P values less than 0.05 were considered significant.

Results

IL-27 promotes expression of Ly6C and Sca-1 on $CD4^+$ and $CD8^+$ T cells

Given previous studies that implicated IL-27 in the regulation of Ly6C on Treg cells (21) and Sca-1 on $CD8^+$ T cells *in vitro* and $CD4^+$ T cells *in vivo* (19, 20), initial experiments were performed to determine the relationship between IL-27 signaling and TCR stimulation in modulating expression of these molecules. Multiple experiments using bulk $CD4^+$ or $CD8^+$ splenocyte cultures showed that IL-27 in combination with TCR potently promotes T cell expression of Ly6C and Sca-1 (Supp Fig 1). Because subpopulations of splenic $CD4^+$ and $CD8^+$ T cells express Ly6C and/or Sca-1, these experiments were repeated with sort-purified $Ly6C^-Sca\text{-}1^-$ naïve ($CD44^{lo}CD62L^+$) T cells (>90% purity). Cells were then labeled with CFSE and cultured in the presence or absence of plate-bound anti-CD3 and soluble anti-CD28 stimulation (henceforth referred to as TCR stimulation) with IL-2, IL-27, and neutralizing anti-IFN-γ and anti-IL-4 antibodies (Fig 1A). After three days of culture in the absence of TCR stimulation or IL-27, the cells did not express Ly6C. Culture of $CD4^+$ or $CD8^+$ T cells with IL-27 alone induced modest Ly6C expression. TCR stimulation on its own induced robust proliferation (as seen by CFSE dilution), but did not induce expression of Ly6C on naïve $CD4^+$ T cells, and induced Ly6C on a small percentage of $CD8^+$ T cells. However, when naïve $CD4^+$ and $CD8^+$ T cells were provided TCR stimulation combined with IL-27, there was a synergistic effect on Ly6C expression, which was apparent even

during early divisions. It is notable that among $CD4^+$ T cells, TCR stimulation alone did not induce Ly6C expression, even in those cells that had proliferated. These results demonstrate that Ly6C is not a general activation marker on $CD4^+$ T cells, but in these experiments requires TCR activation in the presence of IL-27.

When the role of IL-27 and TCR stimulation in the regulation of Sca-1 was examined, each stimulus alone was sufficient to promote high expression of Sca-1 by $CD4^+$ and $CD8^+$ T cells (Fig 1B). However, TCR stimulation in the presence of IL-27 resulted in further induction of Sca-1 expression, which was upregulated in early divisions and maintained as cells divided. We previously reported that IL-2 downregulates the IL-27 receptor (23), raising the possibility that the exogenous IL-2 used in these cultures might limit the effect of IL-27. However, exogenous IL-2 did not limit expression of Ly6C and Sca-1 by $CD4^+$ T cells and enhanced their expression by $CD8^+$ T cells (Supp Fig 2). These results demonstrate that IL-27 has a major impact on Sca-1 and Ly6C expression, and that Sca-1 is more readily induced by these stimuli than Ly6C.

Expression of Ly6C and Sca-1 is modulated by multiple cytokines

Given the impact of IL-27 on Ly6C and Sca-1, studies were performed to assess whether other cytokines (IFN-γ, type I IFN, IL-6, IL-12, IL-4, IL-10, IL-7, IL-15, and TNF-α) modulate expression of these molecules. $Ly6C^-Sca\text{-}1^-$ naïve ($CD62L^+CD44^{lo}$) T cells were sort purified as described above and were given TCR stimulation in the presence of the different cytokines for 72 hours before being assayed for expression of Ly6C or Sca-1. Across multiple experiments, IFN-γ, type I IFN, and IL-12 induced expression of Ly6C on naïve $Ly6C^-$ $CD4^+$ T cells, though not as robustly as IL-27 (Fig 2A). For naïve $CD8^+$ T cells, both IFN-γ and type I IFN induced expression of Ly6C but type I IFN was consistently the strongest inducer of Ly6C in these experiments (Fig 2B). Stimulation of naïve $CD4^+$ or $CD8^+$ T cells with TCR alone induced high expression of Sca-1, but this was not further upregulated by cytokines (data not shown). IL-4, IL-10, IL-7, IL-15 and TNF-α did not modulate expression of Sca-1 or Ly6C on $CD4^+$ or $CD8^+$ T cells (Fig 2, bar charts). Although there is variation in the impact of individual cytokines on different T cell populations, when taken together, these data identify two groups of related cytokines (the interferons and the IL-6 family members) that utilize similar signaling pathways that promote T cell expression of Ly6C and Sca-1.

Requirements for IL-27-mediated upregulation of Ly6C and Sca-1

There are several common elements to the signaling pathways used by the cytokines that most robustly induced expression of Ly6C and Sca-1, specifically the ability to activate STAT1 and to upregulate expression of T-bet (24–26). We previously reported that Ly6C expression is T-bet-dependent *in vivo* during toxoplasmosis (27), but culture of $T\text{-}bet^{-/-}$ T cells demonstrated that T-bet is not required for IL-27-mediated induction of Ly6C and Sca-1 *in vitro* (Supp Fig 3A, B). These findings agree with an earlier study that found that stimulation with anti-CD3/28 antibodies overcomes the need for T-bet in the induction of Ly6C (28). A single experiment using T cells from $STAT3^{fl/fl}$ x CD4-Cre mice suggested that STAT3 is not required for IL-27-mediated expression of Ly6C or Sca-1 on $CD4^+$ or $CD8^+$ T cells (Supp Fig 3C, D). In contrast, when naïve $CD8^+$ $Ly6C^-Sca\text{-}1^-$ T cells from

STAT1$^{-/-}$ mice were given TCR stimulation in the presence of IL-27 or IFN-γ, the induction of Ly6C was found to be almost entirely STAT1-dependent (Fig 3A), as was the induction of Sca-1 (Fig 3B). However, the type I IFN-mediated induction of Ly6C and Sca-1 was not STAT1-dependent in this system (Fig 3A, B). Similar results were seen for CD4$^+$ T cells (Fig 3C, D). These results demonstrate a key role for STAT1 in IL-27 and IFN-γ-mediated induction of Ly6C and Sca-1.

TGF-β antagonizes Ly6C and Sca-1 expression

While multiple cytokines promote expression of Ly6C and Sca-1, this screening process revealed that TGF-β was a potent inhibitor of the expression of these molecules. The addition of TGF-β reduced IL-27-mediated expression of Ly6C on CD4$^+$ T cells (Fig 4A, C) and CD8$^+$ T cells (Fig 4B, C), which is similar to results from a previous study that used P14 cells in the context of LCMV infection (29). Addition of TGF-β also limited proliferation of CD4$^+$ and CD8$^+$ T cells, as illustrated by the reduced dilution of CFSE (Fig 4A, B, D, E). This was true in the presence and absence of IL-27 and is consistent with the ability of TGF-β to limit the proliferation and differentiation of naïve T cells into terminally-differentiated effector cells (30). Addition of TGF-β also reduced TCR-mediated induction of Sca-1 on CD4$^+$ T cells (Fig 4D, F) and on CD8$^+$ T cells (Fig 4E, F), but did not affect the expression of Sca-1 in the presence of IL-27. The relatively modest effect that TGF-β has on IL-27-mediated expression of Sca-1 is consistent with the data in earlier figures that Sca-1 is more robustly expressed than Ly6C, and also indicates that the inhibitory effects of TGF-β is most closely associated with reduced TCR signaling.

Analysis of Ly6C and Sca-1 expression on diverse T cell populations

Ly6C has been used to identify terminally-differentiated effector CD4$^+$ T cells during infection (6, 10, 11), but its expression during homeostasis is not well described. To determine which T cell populations express Ly6C under homeostatic conditions, a survey of uninfected SPF mice was conducted. The expression of high levels of CD44 was used to identify antigen-experienced cells (31) and CD62L was used to identify cells that home to lymph nodes, which are primarily naïve and central memory cells (32). In this setting, 20% of naïve (CD62L$^+$CD44^{lo}) and 10% of memory (CD62L$^+$CD44^{hi}) CD4$^+$ T cells expressed Ly6C and only 10% of CD4$^+$ T cells with an effector phenotype (CD62L$^-$CD44^{hi}) were Ly6C$^+$ (Fig 5A). For CD8$^+$ T cells, 20% of naïve (CD44^{lo}CD62L$^+$) CD8$^+$ T cells expressed Ly6C, while approximately 30% of CD44^{hi}CD62L$^-$ CD8s and 90% of CD44^{hi}CD62L$^+$ cells expressed Ly6C. Thus, Ly6C expression was concentrated on the antigen-experienced cells, but further subsetting based on T cell expression of CD25, KLRG1, CD127, CD69 or CD49d indicated that Ly6C expression is not restricted to a particular effector/memory T cell population (data not shown). However, it is notable that in this survey, the majority of CD122$^+$ CD8$^+$ T cells expressed Ly6C, regardless of their expression of CD62L and CD44 (Supp Fig 4). CD122 is the beta subunit of the IL-2 and IL-15 receptors and along with CD44 and Ly6C, is upregulated on T cells undergoing homeostatic proliferation (2, 33, 34).

When a similar survey was performed to assess the expression of Sca-1 on naïve, effector, and memory T cells in SPF mice, 20% of naïve (CD44^{lo}CD62L$^+$) CD4$^+$ T cells expressed Sca-1, while 80% of CD44^{hi}CD62L$^-$ effector CD4$^+$ T cells were Sca-1$^+$ (Fig 5B). Similarly,

minimal Sca-1 expression was seen in $CD44^{lo}CD62L^{+}$ $CD8^{+}$ T cells, while the highest Sca-1 expression by $CD8^{+}$ T cells (15%) was seen in the $CD44^{hi}CD62L^{-}$ population (Fig 5B). These findings indicate that Sca-1 was enriched on $CD44^{hi}$ $CD4^{+}$ and $CD8^{+}$ T cells, but in this survey was not exclusively expressed by any particular effector/memory population examined.

Ly6C and Sca-1 are upregulated on pathogen-specific T cells during toxoplasmosis

The above survey utilized established surface markers to distinguish antigen-experienced and naïve cells, but in this setting, it is difficult to determine how expression of Ly6C or Sca-1 correlates with previous antigen exposure. To examine an effector population with a well-defined history, mice were infected with *T. gondii* and the expression of Ly6C and Sca-1 on parasite-specific T cells during acute toxoplasmosis was examined. Mice were infected intraperitoneally with *T. gondii* and spleens and peritoneal exudate cells (PECs) were harvested 10 days post-infection. Toxoplasma-specific T cells were identified by staining with parasite-specific MHCI or MHCII tetramers in combination with high expression of LFA-1 (35). At day 10 post infection, the majority of parasite-specific $CD4^{+}$ and $CD8^{+}$ T cells in the peritoneum expressed Ly6C, demonstrating that toxoplasmosis promotes Ly6C expression by parasite-specific T cells (Fig 6A).

A recent study (4) proposed that during toxoplasmosis, minimally-differentiated memory $CD8^{+}$ T cells that are $CXCR3^{+}KLRG1^{-}$ give rise to an intermediate $CXCR3^{+}KLRG1^{+}$ population that in turn downregulates CXCR3 when it differentiates into terminally-differentiated effector cells. Consistent with this previous report, at day 10 of infection, 20% of splenic parasite-specific $CD8^{+}$ T cells were $CXCR3^{+}KLRG1^{-}$, 60% were $CXCR3^{+}KLRG1^{+}$ and less than 10% were $CXCR3^{-}KLRG1^{+}$. This analysis was extended to $CD4^{+}$ T cells, in which 20% were $CXCR3^{+}KLRG1^{-}$, 30% were $CXCR3^{+}KLRG1^{+}$ and 30% were $CXCR3^{-}KLRG1^{+}$ (Fig 6B). 70% of parasite-specific $CD4^{+}$ T cells expressed Ly6C (Fig 6A). When the cells were subsetted by expression of CXCR3 and KLRG1, Ly6C was expressed by 60% of $CXCR3^{+}KLRG1^{-}$, 75% of $CXCR3^{+}KLRG1^{+}$, and 70% of $CXCR3^{+}KLRG1^{-}$ parasite-specific $CD4^{+}$ T cells. Similar results were seen for $CD8^{+}$ T cells, as Ly6C was expressed by 90% of CXCR3+$KLRG1^{-}$, 95% of CXCR3+$KLRG1^{+}$, and 85% of $CXCR3^{-}KLRG1^{+}$ parasite-specific $CD8^{+}$ T cells. Therefore, Ly6C expression on these individual subsets was not exclusive to the $KLRG1^{+}CXCR3^{-}$ population for $CD4^{+}$ or $CD8^{+}$ T cells, demonstrating that Ly6C and KLRG1 are not interchangeable markers of differentiation (Fig 6C).

When a similar analysis was performed for Sca-1, 85% of parasite-specific $CD4^{+}$ and $CD8^{+}$ T cells expressed Sca-1 (Fig 6D). When these cells were subsetted by their expression of KLRG1 and CXCR3, approximately 90% of the $CXCR3^{+}KLRG1^{-}$ and $CXCR3^{+}KLRG1^{+}$ populations expressed Sca-1 in both $CD4^{+}$ and $CD8^{+}$ T cells. 70% of $CXCR3^{-}KLRG1^{+}$ $CD4^{+}$ T cells and 60% of $CXCR3^{-}KLRG1^{+}$ $CD8^{+}$ T cells expressed Ly6C, indicating that Sca-1 is present at a lower frequency on more highly differentiated cells. Nonetheless, Sca-1 is more widely expressed during toxoplasmosis than KLRG1 or CXCR3, and does not enable the ready differentiation of distinct antigen-experienced T cell populations in this experimental system.

IL-27 and IFN-γ promote T cell expression of Ly6C during toxoplasmosis

IL-27 and IFN-γ are key cytokines during toxoplasmosis (36, 37) and are two of the strongest inducers of Ly6C and Sca-1 *in vitro*. To determine the contribution of IL-27 and IFN-γ signaling to the expression of Ly6C and Sca-1 during infection, WT and IL-27 receptor (*Il27ra*)-deficient mice were infected with *T. gondii*. Mice were also treated with an isotype antibody or a neutralizing anti-IFN-γ antibody on day 3 and 6 of infection. When peritoneal tetramer-positive T cells were examined at day 9 of infection, the percentage of parasite-specific $CD4^+$ T cells expressing Ly6C was substantially lower in *Il27ra*-deficient mice than in WT mice. Ly6C levels were significantly reduced in $CD8^+$ T cells as well, providing evidence that IL-27 promotes the $Ly6C^+$ population in this system (Fig 7A, B). Additionally, neutralizing IFN-γ resulted in less Ly6C expression by $CD4^+$ T cells but in *Il27ra*-deficient mice did not result in a complete ablation of Ly6C expression. In contrast, the absence of the IL-27 receptor did not limit the expression of Sca-1 by parasite-specific T cells. Surprisingly, the blockade of IFN-γ increased Sca-1 expression in both WT and *Il27ra* $^{-/-}$ mice, possibly because the absence of IFN-γ leads to a marked increase in parasite replication and antigen load that might lead to increased T cell activation. Together, these studies establish that IL-27 and IFN-γ are involved in the regulation of Ly6C expression during toxoplasmosis, but in this setting they were not required for maximal Sca-1 expression.

Discussion

Studies to understand the functions of Ly6 molecules have been performed since the 1970s, but questions still remain about their functions and the factors that influence their expression(38). The association of Ly6C expression with short-lived effector T cells has been reported in multiple experimental systems (6, 10) while the combination of Sca-1, CD122 and Bcl-2 can be used to identify $CD8^+$ stem cell memory (Tscm) cells (12–14). A previous study showed that treatment of mice with IL-27 promotes the development of a memory precursor population of tumor antigen-specific $CD8^+$ T cells, characterized by high expression of Bcl-6, SOCS3, and Sca-1 (19). However, the expression patterns of Ly6C and Sca-1 have not been compared and there is a limited appreciation of how cytokine and TCR-mediated signals are integrated to promote their expression. The *in vitro* experiments performed here utilized a defined population of naïve $Ly6C^-$ $CD4^+$ and $CD8^+$ T cells to address any concerns that the stimuli used here might preferentially expand a $Ly6C^+$ population. This approach showed quite modest effects of IL-27, the interferons, or TCR alone on Ly6C expression, but the combination of cytokine plus TCR synergistically promoted the expression of Ly6C by a subset of activated $CD4^+$ and $CD8^+$ T cells. That this activity was STAT1-dependent correlated well with the range of cytokines that could modulate Ly6C, while cytokines that predominantly utilize STAT3, STAT4 or STAT6 had minimal effects. TGF-β was the only signal identified that suppressed the induction of Ly6C and Sca-1, which correlates with its ability to suppress T cell activation and proliferation (39, 40).

Since Ly6C and Sca-1 have been used as markers to identify T cells at different stages of differentiation, it was notable that the expression of these two molecules was upregulated by

the same cytokine signals, in the same cells. Sca-1 was potently induced by either TCR stimulation or cytokine signaling and consequently appeared to be more widely expressed than Ly6C. Indeed, a survey to determine if either of these molecules could be associated with different effector or memory populations found that it was difficult to link them to memory-like or terminally-differentiated effector cells based on differential expression of KLRG1 and CXCR3. The ability of these cytokines to induce Ly6C and most notably Sca-1 in the absence of TCR stimulation suggest the need for caution in using these molecules alone to identify Ag-experienced populations.

Infection with *T. gondii* is dominated by the generation of parasite-specific $CD4^+$ and $CD8^+$ T cells that produce IFN-γ, but this is also a system in which endogenous IL-27 is required to limit the inflammatory response (37). While the loss of either cytokine signal during infection reduces Ly6C expression, it was relevant to note that in the setting of IL-27R-deficiency, there are markedly elevated levels of IFN-γ (37), but IFN-γ blockade did not result in a further reduction in the numbers of parasite-specific effectors that expressed Ly6C. The observation that IL-27 was more important in driving Ly6C in $CD4^+$ T cells than in $CD8^+$ T cells during toxoplasmosis is consistent with the larger effect of interferons in promoting Ly6C in $CD8^+$ T cells seen *in vitro*. Nevertheless, the *in vivo* studies presented here indicate that IL-27 and IFN-γ are not redundant in promoting Ly6C expression during toxoplasmosis.

While the section above focuses on the regulation of Ly6C, the *in vitro* and *in vivo* studies identified common pathways that influence expression of Ly6C and Sca-1, but also highlighted some notable differences. Again, the ability to utilize a defined, naïve, $Sca\text{-}1^-$ starting population helped establish the profound impact of TCR stimulation alone or in combination with cytokines on Sca-1 expression. As seen for Ly6C, not every cytokine was a potent inducer of Sca-1 and those that activated STAT1 seemed dominant, although the use of STAT1-deficient cells does indicate the presence of additional pathways that are involved in this process. Potential STAT1-independent pathways relevant to the ability of IL-27 and type I IFNs include p38 MAPK and ERK1/2 (24, 25). However, unlike Ly6C, Sca-1 expression was not attenuated by the loss of IL-27 or IFN-γ in *in vivo* studies. This contrasts with an earlier report using a model of colitis, in which *in vivo* administration of an AAV vector encoding IL-27 was associated with reduced inflammation and increased expression of Sca-1 by $CD4^+$ T cells (20). This contradiction likely reflects the complexity in trying to distinguish a role for endogenous IL-27 or IFN-γ in a systemic infection, in which many signals including TCR and other cytokines readily promote Sca-1 expression, versus a dominant signal provided by overexpression of IL-27.

There have been few studies that have directly addressed the function of Ly6C and Sca-1 in the immune response. Ly6C has been proposed to be involved in T cell homing to secondary lymphoid organs, possibly through an association with LFA-1 (41–43), and ImageStream analysis of *T. gondii*-specific effectors shows co-localization of Ly6C and CD11a (a subunit of LFA-1) on the surface of these cells (27). IL-27 has been proposed to modulate T cell homing through upregulation of ICAM-1 and selectin ligands on conventional $CD4^+$ T cells (21, 44, 45), as well as CXCR3 on Tregs (21). Thus, upregulation of Ly6C may be an additional mechanism by which IL-27 and/or interferons modulate T cell trafficking. There

is also a literature that suggests a regulatory role for Ly6C and Sca-1 in limiting the T cell response. A mutation in the promoter of Ly6C reduces its expression in NOD, NZB/W and ST mice, which are strains that spontaneously develop autoimmune diseases (46). Moreover, in the context of TCR stimulation, antibodies that crosslink Ly6C or Sca-1 on the surface of T cells limit their ability to produce IL-2 and proliferate (47–49). Furthermore, in mice genetically engineered to lack Sca-1 expression, T cells exhibit enhanced proliferation in response to TCR stimulation (50). Additional evidence for a regulatory function of Sca-1 is the finding that transgenic overexpression of Sca-1 limits T cell proliferation (48, 49, 51) and suppresses lymphoproliferation and autoimmunity in *lpr/lpr* mice (51). Together, these findings suggest that Ly6C and/or Sca-1 may have a role in limiting T cell responses. A suppressive function for Ly6C and Sca-1 would complement reports that IL-27 promotes inhibitory pathways including IL-10 and LAG-3 (52–58), and that IL-27 and IFN-γ promote expression of PD-L1 (58–60). Additional studies are needed to determine whether Ly6C and Sca-1 primarily function to promote T cell activation and migration, or if any of the shared immune-regulatory effects of IL-27 and the IFNs are mediated through the induction of Ly6C and/or Sca-1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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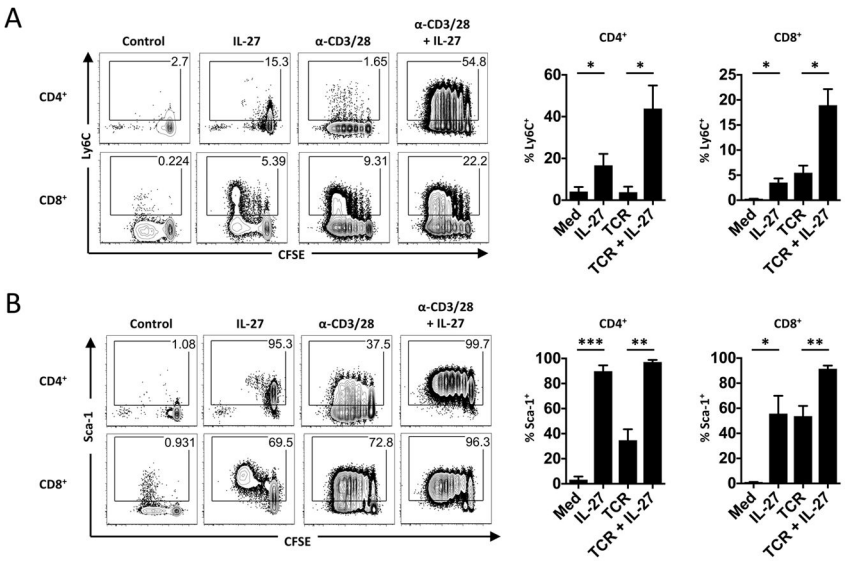

Figure 1. IL-27 induces Ly6C and Sca-1 on CD4$^+$ and CD8$^+$ T cells

Naive Ly6C$^-$Sca-1$^-$ CD4$^+$ or CD8$^+$ T cells were sorted and stained with CFSE before a 3 day culture in the presence or absence of TCR stimulation and/or IL-27. **A–B.** Induction of Ly6C (A) or Sca-1 (B) on naive CD4$^+$ or CD8$^+$ T cells. *Left*, representative flow cytometry plots. *Right*, bar charts summarize the results of four experiments. Error bars indicate SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

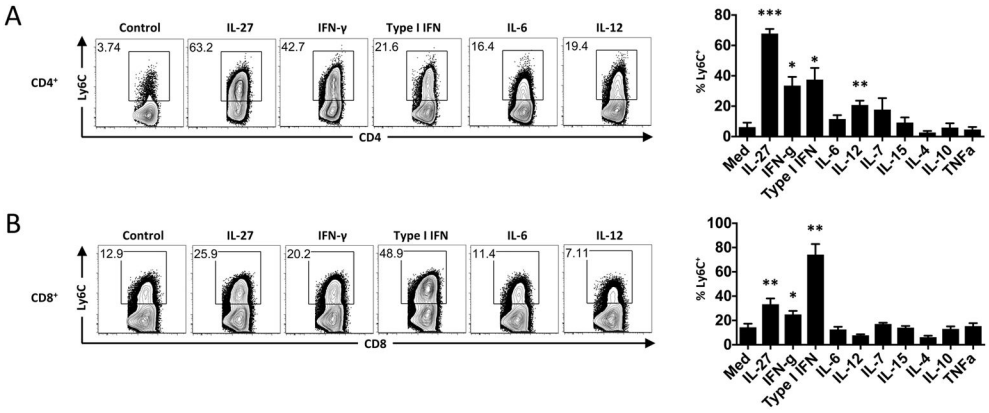

Figure 2. Ly6C expression is modulated by multiple cytokines *in vitro*

Naive Ly6C^{-}Sca-1^{-} CD4^{+} or CD8^{+} T cells were sorted and stained with CFSE before a 3 day culture in the presence of TCR stimulation and Type I IFN, IL-6, IL-12, IL-4, IL-10, IL-7, or IL-15. **A–B.** Expression of Ly6C by CD4^{+} (A) and CD8^{+} (B) T cells. **C**. Expression of Sca-1 by CD4^{+} T cells by a panel of cytokines. *Left*, representative plots for select cytokines. *Right,* bar charts summarize the results of four experiments. Error bars indicate SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

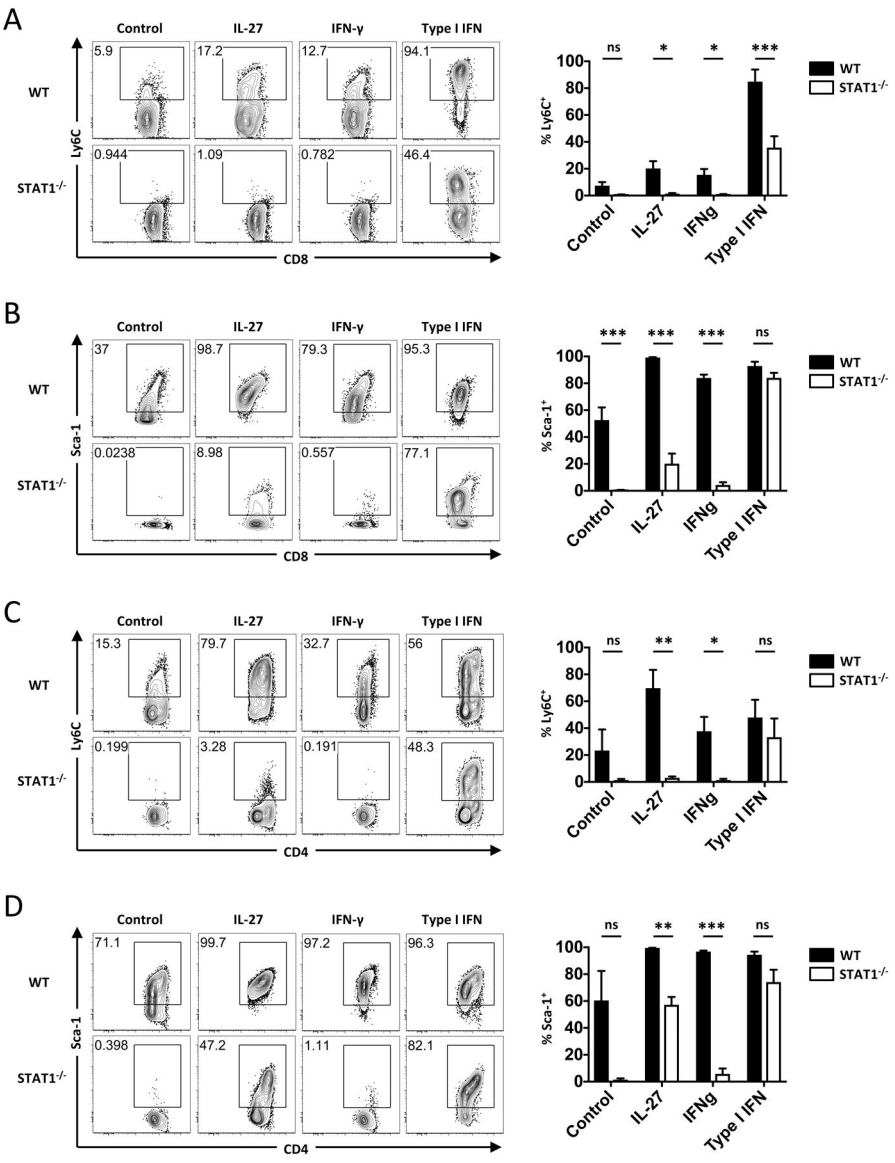

Figure 3. Signaling pathways involved in cytokine-mediated Ly6C and Sca-1 expression *in vitro*

WT or STAT1$^{-/-}$ splenocytes were enriched for T cells by negative-selection bead enrichment before a 3 day culture in the presence or absence of TCR stimulation and/or IL-27, IFN-γ or Type I IFN. **A, B.** Expression of Ly6C (A) or Sca-1 (B) by WT and STAT1$^{-/-}$ CD8^{+} T cells. **C, D.** Expression of Ly6C (C) or Sca-1 (D) by WT and STAT1$^{-/-}$ CD4^{+} T cells. *Left*, representative plots. *Right,* bar charts summarize the results of three experiments. Error bars indicate SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

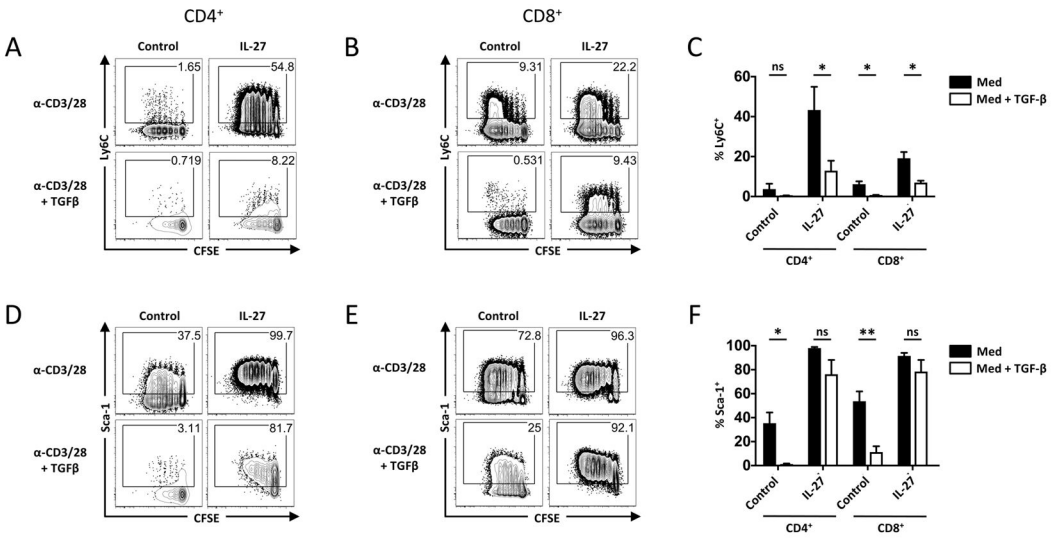

Figure 4. TGFβ inhibits induction of Ly6C and Sca-1

Naive Ly6C^{-}Sca-1^{-} CD4^{+} or CD8^{+} T cells were sorted and stained with CFSE before a 3 day culture with TCR stimulation in the presence or absence of IL-27 and/or TGFγ. **A, B.** Expression of Ly6C by CD4^{+} (A) and CD8^{+} (B) T cells after culture. **C, D**. Expression of Sca-1 by CD4^{+} (C) and CD8^{+} (D) T cells after culture. *Left*, representative plots. *Right,* bar charts summarize the results of four experiments. Error bars indicate SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

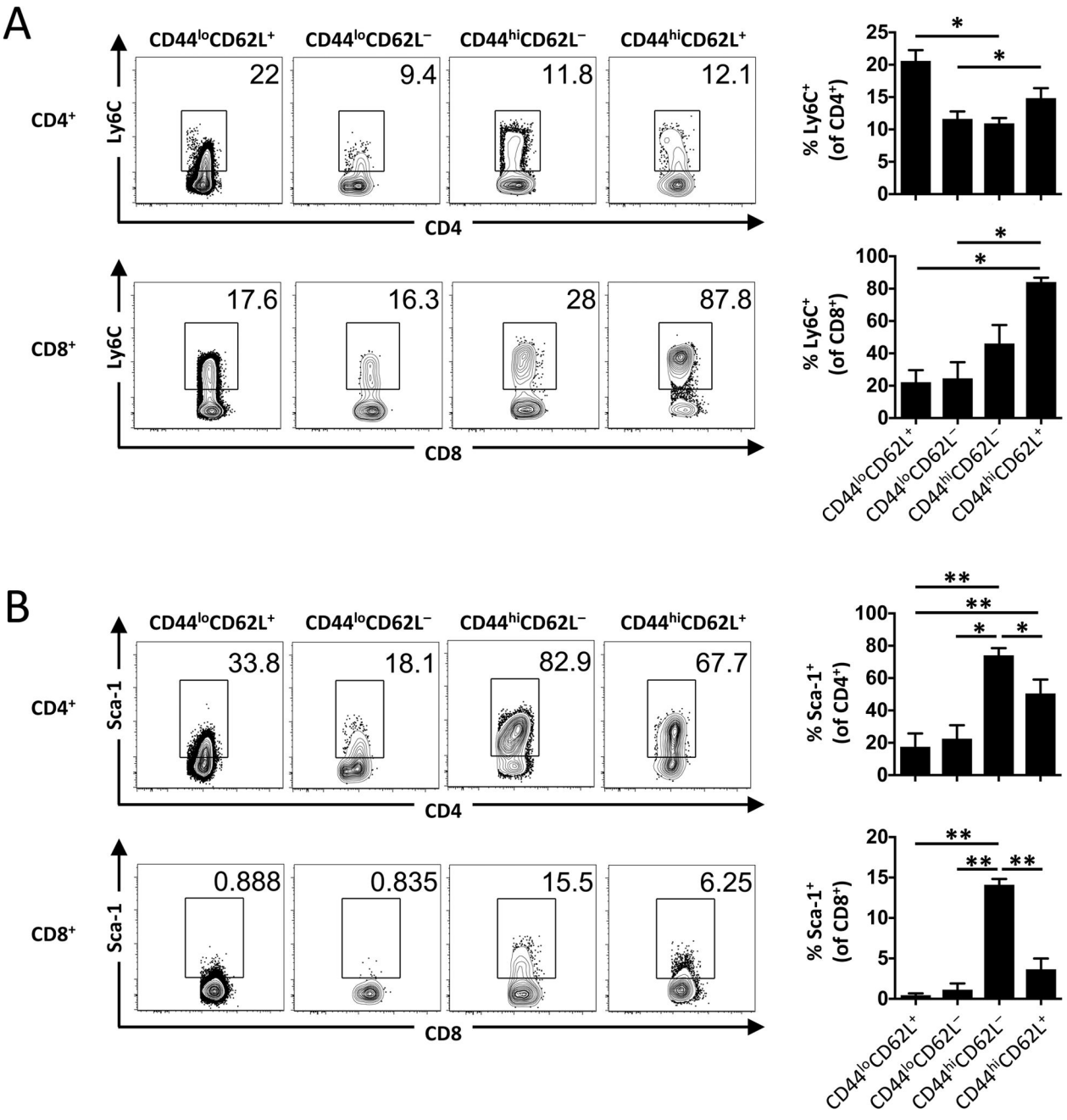

Figure 5. Expression of Ly6C and Sca-1 by naïve, effector and memory populations

Splenocytes from WT C57BL/6 mice were harvested and analyzed for expression of Ly6C and Sca-1. **A.** Expression of Ly6C by $CD4^+$ (top) and $CD8^+$ (bottom) T cell populations. **B.** Expression of Sca-1 by $CD4^+$ (top) and $CD8^+$ (bottom) T cell populations. *Left*, representative plots. *Right,* bar charts summarize the results of three experiments. Error bars indicate SEM. * $p < 0.05$, ** $p < 0.01$

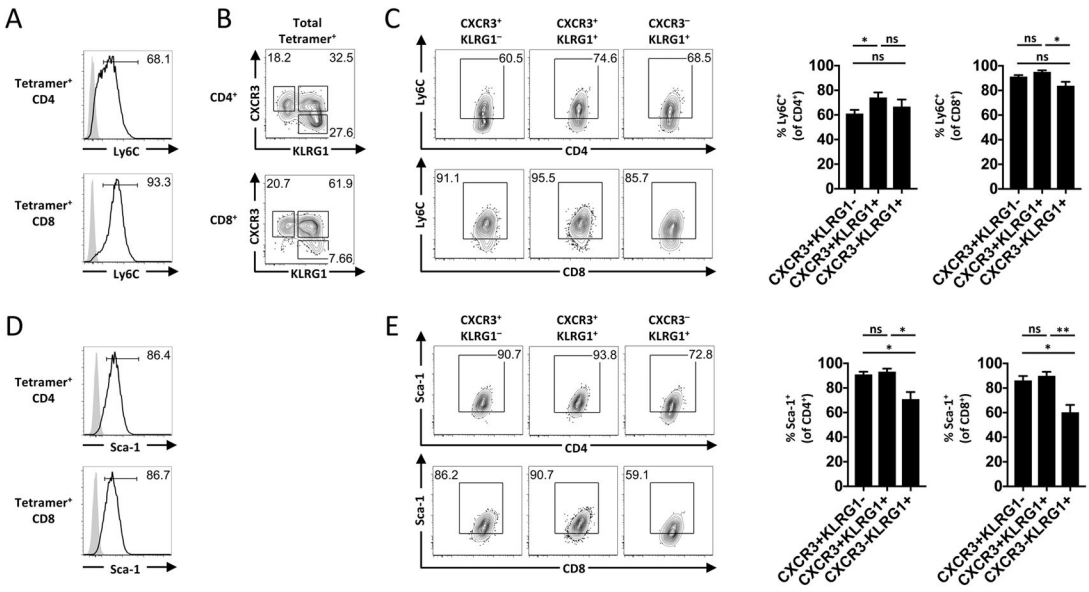

Figure 6. Toxoplasmosis upregulates Ly6C and Sca-1 in T cells

WT C57BL/6 mice were infected with 20 *T. gondii* cysts intraperitoneally. Splenocytes were harvested and analyzed at day 9 of infection. *T. gondii*-specific T cells were identified by MHCI and MHCII tetramers. **A, D.** Expression of Ly6C (A) or Sca-1 (D) by $CD4^+$ and $CD8^+$ T cells. **B**. Expression of CXCR3 and KLRG1 by *T. gondii*-specific $CD4^+$ and $CD8^+$ T cells. **C, E.** Expression of Ly6C (C) or Sca-1 (E) by different populations of T cells, distinguished by expression of CXCR3 and KLRG1. *Left*, representative plots. *Right,* bar charts summarize results from one of two experiments, $n = 3–4$ mice per experiment. Error bars indicate SEM. * $p < 0.05$, ** $p < 0.01$

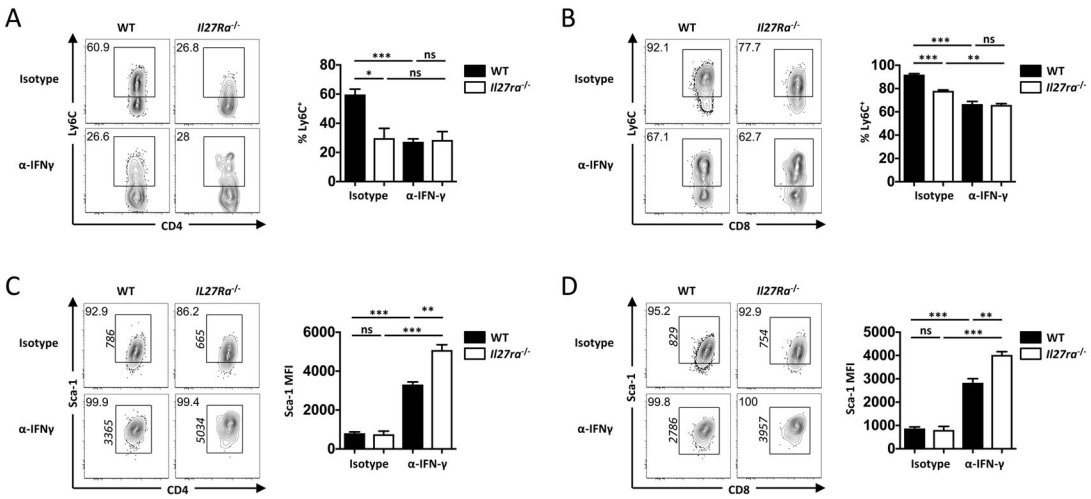

Figure 7. IL-27 and IFN-γ promote expression of Ly6C *in vivo* during toxoplasmosis

WT or *Il27ra*$^{-/-}$ mice were infected with 20 *T. gondii* cysts intraperitoneally and a subset of mice were treated with anti-IFN-γ antibody on day 3 and 6 of infection. Peritoneal cells were harvested 9 days after infection and *T. gondii*-specific cells were identified using MHCI and MHCII tetramers. Ly6C expression by tetramer^{+} CD4^{+} (**A**) or CD8^{+} (**B**) T cells was examined. Sca-1 expression by tetramer^{+} CD4^{+} (**C**) or CD8^{+} (**D**) T cells was also examined. *Left*, representative plots with MFI indicated by vertical italic numbers. *Right,* bar charts show representative results from one of two experiments, n = 2–4 mice per group, per experiment. Error bars indicate SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$