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Distinct Populations of Innate CD8⁺ T Cells Revealed in a CXCR3 Reporter Mouse

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CXCR3, expressed mainly on activated T and NK cells, is implicated in host defenses against a variety of pathogens including bacterial, viral, and fungal infections including Leishmania major and HSV type 1 infections (2, 3). In most instances, CXCR3 mediates recruitment of T cells involved in Th1 type immunity to peripheral sites of infection. However, in some infectious and autoimmune diseases, CXCR3-expressing immune cells contribute to immunopathology (4, 5). Recent studies have also implicated CXCR3-expressing cells in animal models of autoimmune, neoplastic, and infectious diseases. However, investigation of the role of CXCR3 in infection in vivo or functional analysis of CXCR3-expressing cells ex vivo has been difficult because of the lack of appropriate tools for tracking and/or isolating CXCR3-expressing cells.

To study the role of CXCR3-expressing cells in vivo, we have generated and characterized a CXCR3 internal ribosome entry site bicistronic enhanced GFP reporter (CIBER) mouse using a bicistronic reporter system as described previously (7). CIBER mice are truly CXCR3 reporter mice, as all CXCR3-expressing cells in these mice also express enhanced GFP (EGFP). Using these mice, we have identified phenotypically and functionally distinct populations of innate CD8⁺ T cells based on CXCR3 expression. Innate CD8⁺ T cells, which are found in naive mice, develop via a different pathway from conventional CD8⁺ T cells (8–10). They phenotypically resemble bona fide memory CD8⁺ T cells and require IL-15 for their homeostatic maintenance (11). Several studies have shown that innate CD8⁺ T cells contribute to early host defenses against a variety of pathogens including Listeria monocytogenes, Salmonella typhimurium, and Mycobacterium tuberculosis (12). To our knowledge, our study is the first to reveal two distinct subsets of these nonconventional CD8⁺ T cells. CIBER...
mice should provide a valuable tool for researchers to study the role of CXCR3, as well as its regulation in vivo.

Materials and Methods

Mouse strains and infection protocol

All wild-type (WT) and knockout C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in a pathogen-free animal facility at The Ohio State University in accordance with National Institutes of Health and institutional guidelines. Mice were infected either with 2 × 10^5 S. typhimurium (aroA^- strain) orally, 2 × 10^5 Histoplasma yeast strain G217B intranasally, or 2 × 10^5 L. major promastigotes s.c. in their hind footpad. Mice were sacrificed at the indicated times postinfection, and single-cell suspensions from Peyrier’s patches, spleens, and lungs were prepared and analyzed by flow cytometry for the presence of GFP+ cells.

Intravital microscopy

Male CIBER mice were sensitized using the contact hypersensitivity (CHS) model with dinitrofluorobenzene (DNFB) as described previously (13), then challenged 6 d later on the skin surface over the cremaster muscle or on the ear dermis. Sixteen hours later, mice were anesthetized using isoflurane and trichloroethanol. Under anesthesia, the testicular cremaster muscle was surgically exposed using a dissecting microscope (2×; Nikon SMZ 645; Nikon, Tokyo, Japan) and mounted on a coherent cube. The cremaster muscle was bathed with Ringer’s lactate at 37°C, and leukocyte endothelial interaction was assessed in 5–10 venules using a Nikon Eclipse FN1 microscope (Nikon) with a 40×/0.80 W water immersed objective at a 2.0-mm working distance. Video images were captured and digitalized to 12-bit TIF images using MetaMorph software (version 7.1.2.0; MetaMorph, Downingtown, PA).

For CIBER mouse ear imaging, mouse was anesthetized and the inflamed ear dermis was visualized using a Zeiss LSM 700 confocal laser-scanning microscope. Fluorescent images were acquired as Z-stacks and three-dimensional images were generated using Zen software (Carl Zeiss MicroImaging, Jena, Germany).

In vitro T cell activation

Cells for in vitro T cell activation were obtained from excised lymph nodes and spleens of uninfected mice or from sorted populations using BD FACS Aria cell sorter (Becton-Dickinson Biosciences, San Jose, CA). Splenocytes were incubated with Boele’s solution at room temperature for 10 min to lyse RBCs, then washed with RPMI 1640 supplemented with 10% FBS. Enriched T cell populations were obtained by passing cell suspensions through nylon wool columns. Purified T cells or CD8^+ CD4^- CD2L^+ sorted cells were incubated at 0.5–2.5 × 10^6 cells/well with platebound anti-CD3 (3 μg/ml, clone 145–2C11) and anti-CD28 (4 μg/ml, clone 37.51) Abs (BioLegend, San Diego, CA) or with recombinant mouse cytokines (R&D Systems, Minneapolis, MN) for 48 h. After in vitro activation, cells were either rested in their conditioned media and analyzed by flow cytometry using PE-labeled anti-mouse CXCR3 Ab (BioLegend, San Diego, CA) or collected for real-time PCR analysis and ELISA of culture supernatants.

In vivo proliferation assays

Sorted CXCR3^+ and CXCR3^-/- innate CD8^+ T cells from WT, CIBER, or OT1-CIBER mice were stained with CellTrace Violet (Invitrogen, Carlsbad CA) according to the manufacturer’s instructions. Labeled cells were then injected i.v. into Thy1.1 recipient mice via the tail vein. The next day, recipient mice were injected with OVA, OVA/CAF, PBS/CAF, or PBS at the hind footpad. On day 3 postimmunization, draining popliteal lymph nodes were harvested and analyzed for proliferation by flow cytometry.

Migration assays

T cells from the spleens and lymph nodes of naive WT, CIBER, and cxcr3^-/- mice were isolated and activated in vitro with plate-bound anti-CD3 and -CD28 Abs for 48 h. Cells were then subsequently recultured in fresh media (in the absence of CD3/CD28 stimulation) for 48 h to induce CXCR3 expression in WT and CIBER mice T cells (14). For in vivo migration assays, CXCR3^+ T cells from WT and CIBER mice, as well as T cells from cxcr3^-/- mice, were labeled with cell tracking dyes Qdot 655 and Qdot 585, respectively, using the Qtracker Cell Labeling Kit (Invitrogen, Carlsbad, CA). Equal numbers were adoptively transferred to WT recipient mice. Twenty-four hours later, 10 μg CXCL10 in 50 μl was injected intratraehicularly into recipient mice. After another 24 h, mice were sacrificed and infiltrating cells in the lungs were isolated by bronchoalveolar lavage, then analyzed using BD FACS Canto flow cytometer (Becton-Dickinson Biosciences). Migration in DNFB-sensitized WT and CIBER mice was performed as described earlier for CIBER mouse ear imaging. Cells from mouse ears were obtained as described previously (15). In brief, mouse ear dorsal and ventral dermal sheets were separated and incubated dermal side down in culture media containing 2 mg/ml collagenase A and 0.5 mg/ml DNase I for 2 h at 37°C, then passed through a 70-μm strainer to obtain single-cell suspensions. Cells were stained with CD3 and CD8 Abs and analyzed by flow cytometry using a FACS Calibur flow cytometer (Becton-Dickinson Biosciences).

In vitro migration assays were performed as described previously (16). In brief, dilutions of chemokine in media were added to the bottom of 96-well chemotaxis plates (Corning). A total of 5 × 10^4 CXCR3^+ T cells was added to the top of the membrane and incubated at 37°C for 2 h. Cells in the bottom wells were subsequently counted using a microscope. Results were presented as chemotactic indices by dividing the number of cells in the bottom well in response to the chemokine by the average number of cells in the bottom well without the addition of chemokines.

Statistical analysis

Student unpaired t test was used to determine statistical significance of values obtained. The p values <0.01 were considered statistically significant.

Results

Generation of CIBER mice and validation of GFP production by CXCR3-expressing cells

To examine the role of CXCR3-expressing cells during infection and inflammation in vivo, we generated and characterized a CXCR3 knock-in reporter (CIBER) mouse. A CXCR3 bicistronic reporter targeting vector was designed for integration by homologous recombination into the CXCR3 locus. A 13-kb mouse genomic fragment of the CXCR3 locus consisting of exons 1 and 2, a 5.8-kb flanking sequence and a 3.4-kb flanking sequence at the 5' and 3' ends of the coding region, respectively, was used to generate the targeting vector. The vector was modified by introducing an internal ribosome entry site element, EFGP, and a bovine growth hormone polyadenylation signal, followed by a loxP flanked neomycin cassette downstream of the translational stop codon and upstream of the endogenous polyadenylation signal in the 3' untranslated region of exon 2. A herpes simplex thymidine kinase cassette was cloned to one flank of the targeting construct to serve as a counter-selectable marker for targeted integration. The linearized targeting vector was electroporated into 129SVE embryonic stem cells. G418-resistant and ganciclovir-sensitive clones were screened by Southern blot for targeted integration into the CXCR3 locus (Fig. 1A, 1B). Mouse PCR genotyping revealed a 222-bp band for CIBER mice or a 448-bp band for WT mice, whereas heterozygous mice showed both bands (Fig. 1C). All mice were viable and healthy with no phenotypic defects. We have designated these mice as CIBER mice.

T cells that have been shown to express CXCR3 on activation by plate-bound anti-CD3 and anti-CD28 Abs, and subsequent re-expression in conditioned medium in the absence of external stimuli (14). To determine whether surface expression of CXCR3 on the cells from CIBER mice correlated with EFGP production, we activated T cells of WT, cxcr3^-/-, or CIBER mice using anti-CD3/anti-CD28 as described previously (17), and analyzed expression of EFGP in CXCR3^+ T cells stained using PE-conjugated anti-CXCR3 Ab. We found that in vitro-activated splenic T cells from CIBER mice that expressed CXCR3 on their surface also expressed EFGP (Fig. 1D). Levels of CXCR3 protein expression in CIBER mice were comparable with that of WT mice after in vitro T cell activation (Fig. 1D). Moreover, expression of CXCR3 in T cells was proportionate to EFGP production, and EFGP fluorescence was undetectable in the cells that did not express surface CXCR3. A similar expression pattern was observed in...
lymph node cells of CIBER mice. Taken together, these data show that CIBER mouse is a reliable reporter for CXCR3 expression.

Migration of CXCR3-expressing cells to inflamed tissues in vivo is not compromised in CIBER mice

Next, we examined whether CXCR3-expressing cells could be visualized in vivo during inflammation in real time in live mice. To do this, we elicited DNFB-mediated CHS in the skin over the groin of male CIBER mice and analyzed recruitment of GFP-expressing cells in the underlying cremasteric tissue by intravital microscopy (Fig. 2A). CXCR3-expressing (EGFP+) cells recruited into the inflamed tissue and tethered to the endothelial vessel wall of inflamed postcapillary venules in surgically exposed testicular cremaster muscle were readily visible (Fig. 2A). Similar results were obtained when CHS was induced in the ear dermis of CIBER mice (Fig. 2B). To further validate the use of CIBER mice in studying recruitment of CXCR3+ cells to different anatomical sites, we analyzed EGFP+ cells at infection sites of CIBER mice challenged intranasally with *Histoplasma capsulatum* or orally with *S. typhimurium*. EGFP+ cells were readily detectable in inflamed lung tissues of *Histoplasma*-infected, as well as in Peyer’s patches of *S. typhimurium*-infected, CIBER mice by flow cytometry (Fig.

**FIGURE 1.** Generation of CIBER mice and validation of GFP production by CXCR3-expressing cells. (A) Targeting vector for generation of CIBER mouse and Southern blot strategy for detection of targeted integration into the CXCR3 locus. (B) Southern blot screen of genomic DNA from embryonic stem cells electroporated with linearized targeting vector. (C) PCR genotyping of genomic DNA from mouse tails of WT, homozygous (CIBER), or heterozygous (CIBERhet) mice. (D) CXCR3 production by activated splenic T cells of WT, cxcr3−/−, CIBER, or CIBERhet mice. T cells were activated in vitro with plate-bound anti-CD3/CD28 Abs and subsequently recultured in conditioned media. CXCR3 expression is determined by PE-conjugated anti-CXCR3 Ab and fluorescent EGFP production. Experiment was repeated six times. b, *BamH1* site; E1, exon 1; E2, exon 2; p, probe; tk, thymidine kinase gene cassette.
These findings demonstrate that CXCR3+ cells in CIBER mice have the ability to migrate to inflamed tissues, and that these cells can be tracked in vivo during inflammation and infection.

Further, we determined whether there was any defect in the migration of CXCR3+ cells in CIBER mice as a result of insertion of the EGFP reporter gene into the CXCR3 locus. We therefore compared the migration of CXCR3+ cells in WT and CIBER mice using in vivo and in vitro models. CD8+ T cell recruitment to the ear dermis after DNFB mediated CHS induction of WT and CIBER mice was observed to be identical in both mice (Fig. 3A, 3B). In another in vivo model, CXCR3+ T cells from WT and CIBER mice, as well as T cells from cxcr3−/− mice, were labeled with different cellular tracking dyes and adoptively transferred to WT recipient mice. Recruitment to the lungs was analyzed after intratracheal administration of the CXCR3 ligand CXCL10. Flow cytometric analysis of lung infiltrates revealed similar numbers of adoptively transferred T cells from WT and CIBER mice, but not cxcr3−/− mice (Fig. 3C, 3D). To further demonstrate that migration of CXCR3-expressing cells in CIBER mice is not compromised, we compared migration of CXCR3+ T cells from WT and CIBER mice, as well as T cells from cxcr3−/− mice, with the CXCR3 ligands CXCL9, CXCL10, and CXCL11 in vitro. As with in vivo migration models, migration of CXCR3+ T cells to all CXCR3 ligands was similar in WT and CIBER mice (Fig. 3E–G). Furthermore, sorted T cells from WT and CIBER mice produced similar proliferative responses and cytokine levels after TCR or antigenic stimulation (Supplemental Fig. 1). Taken together, these findings demonstrate that CXCR3+ cells in CIBER mice have no functional or chemotactic defects.

Detection of CXCR3-expressing cells in naive CIBER mice

Previous reports have shown that CXCR3 is expressed on NK cells, NKT cells, and T cells in mice (18). Analysis of secondary lymphoid organs from naive CIBER mice, as well as anti-CXCR3 Ab staining of naive WT mice, confirmed expression of CXCR3 by these cell populations (Fig. 4A–C). In addition, we found expression of CXCR3 in a small population of B lymphocytes (Fig. 4D). CXCR3 expression on Ab-secreting cells of virally infected mice has been previously reported (19), but the significance of CXCR3 expression on B lymphocytes in naive, uninfected mice is
yet to be fully understood, although these cells are likely to be memory B cells. Furthermore, in CIBER mice infected with *L. major*, we observed a general increase in the expression of CXCR3 in lymphocytes (Supplemental Fig. 2).

CXCR3 is generally an inducible receptor, although constitutive expression in innate lymphocytes including NK and NKT cells is well documented (18). Our analysis of CIBER mice revealed that, in addition to these subsets, a majority of CXCR3-expressing lymphocytes in the lymphoid organs of naive mice expressed the CD8 coreceptor (Fig. 4B). These CXCR3-expressing CD8+ T cells also expressed CD62L and CD44, suggesting similar characteristics with some central memory T cells (Fig. 4E). However, these populations were detected in uninfected naive mice, as well as in naive germ-free mice (Fig. 5A, 5B), and therefore could not be bona fide memory CD8+ T cells. Naturally occurring “memory phenotype” CD8+ T cells that originate from similar CD4+CD8+ thymocyte progenitors as with conventional naive CD8+ T cells but with a different lineage have been reported (8, 11). The phe-
CXCR3, suggestive of potential phenotypic differences between the CXCR3⁺ and CXCR3⁻ populations of innate CD8⁺ T cells (Fig. 5A).

**Role of STAT1, IFN-γ, and T-bet in CXCR3 expression by innate CD8⁺ T cells**

Given the constitutive nature of CXCR3 expression in a population of innate CD8⁺ T cells observed in naive CIBER mice, we determined whether T-bet, STAT-1, or IFN-γ were essential for the expression of CXCR3 on these cells. It is well established that expression of CXCR3 on activated T cells is tightly regulated by the transcription factor and master regulator of Th1 differentiation and function, T-bet (20). We have also previously shown that IFN-γ and STAT-1 are critical for the efficient induction of CXCR3 in CD4⁺, but not CD8⁺, T cells on activation (17). To determine whether STAT1 and/or T-bet are required for CXCR3 expression on innate CD8⁺ T cells, we analyzed innate CD8⁺ T cells isolated from the spleens and lymph nodes of naive T-bet⁻/⁻, stat1⁻/⁻, and ifn-γ⁻/⁻ mice for cellular CXCR3 expression by flow cytometry. CXCR3⁺ innate CD8⁺ T cells were present in all groups of mice (Fig. 5C, 5D). However, the proportion of CXCR3⁺ innate CD8⁺ T cells was slightly reduced in T-bet⁻/⁻ mice compared with WT mice (Fig. 5C, 5D). Taken together, our results show that T-bet might contribute to the expression of CXCR3 in innate CD8⁺ T cells. However, T-bet does not seem to be required for the presence of this population.

**CXCR3⁺ and CXCR3⁻ innate CD8⁺ T populations possess distinct phenotypic characteristics**

Although antigenic diversity within the innate CD8⁺ T cell repertoire has been described, phenotypic and functional classifications of this population are poorly characterized. As noted earlier, we observed that not all innate CD8⁺ T cells in naive mice express CXCR3. This led us to examine whether phenotypic differences exist between CXCR3⁺ and CXCR3⁻ populations of innate CD8⁺ T cells. We therefore isolated CXCR3⁺ and CXCR3⁻ innate CD8⁺, as well as naive CD8⁺ T cells, from uninfected CIBER mice, then analyzed their gene expression profiles. We observed that CXCR3⁺ innate CD8⁺ T cells display higher levels of T-bet and IFN-γ mRNA compared with CXCR3⁻ innate CD8⁺, suggesting a greater potential for this population to produce IFN-γ (Fig. 6A). Differences in the expression of genes involved in T cell development and function were also observed between both populations of innate CD8⁺ T cells, including higher levels of Spi-6, Spi-2α, GATA-3, and IL-10, and lower levels of Id-2, Notch, PD-1, and CTLA-4 expressed by CXCR3⁺ innate CD8⁺ T cells (Fig. 6A).

To further characterize phenotypic differences between CXCR3⁺ and CXCR3⁻ innate CD8⁺ T cells, we analyzed the expression of cell-surface markers by both populations. CXCR3⁺ innate CD8⁺ T cells expressed significantly greater levels of Ly6C than CXCR3⁻ innate CD8⁺ T cells (Fig. 6B). We also observed significant differences in the cell-surface expression of IL-2Rα, a receptor for IL-2 and IL-15, which was consistently higher in CXCR3⁺ innate CD8⁺ T cells (Fig. 6B). This finding suggests that subsets of innate CD8⁺ T cells have varied responses to IL-2 and IL-15. Taken together, our results demonstrate that CXCR3⁺ and CXCR3⁻ innate CD8⁺ T cells possess distinct phenotypic characteristics.

**CXCR3⁺ innate CD8⁺ T cells proliferate more robustly and produce greater levels of IFN-γ and granzyme B on stimulation**

Innate CD8⁺ T cells generally display a more activated surface phenotype and respond more rapidly to TCR stimulation than conventional naive CD8⁺ T cells (8). Furthermore, they have the

![FIGURE 4. Identification of CXCR3-expressing cells in naive CIBER mice. (A-D) Representative dot plots showing CXCR3 expression in NK cells (A), CD8⁺ T cells (B), CD4⁺ T cells (C), and B cells (D) on lymph nodes of naive CIBER and WT mice. (E) Expression of CD44 and CD62L in CXCR3⁺ and CXCR3⁻ CD8⁺ T cells in lymph nodes of naive CIBER mice. All experiments were performed at least three times.](http://www.jimmunol.org/)
ability to produce IFN-γ in response to cytokine stimulation without TCR engagement (10). As such, they play a significant role in early innate responses against bacterial infection before the onset of adaptive immunity (12). Because we observed phenotypic and transcriptional differences between CXCR3+ and CXCR3- innate CD8+ T cells, we determined whether functional differences exist in both populations. Given that CXCR3 expression is associated with activated T cells, we hypothesized that CXCR3+ innate CD8+ T cells would display a more activated and proliferative phenotype than those not expressing CXCR3. To test this hypothesis, single-cell suspensions from spleen and lymph nodes of naive CIBER mice were stimulated with IL-12/IL-18, IL-12/IL-15, IL-12/IL-2, or PMA/ionomycin, then analyzed for surface CD8+, CD3+ lymphocytes. (A) Total numbers of CXCR3+ innate CD8+ T cells in blood and lymphoid organs of WT and germ-free mice. Numbers correspond to the percentage of CXCR3+ or CXCR3- innate CD8+ T cell populations. (B) Total numbers of CXCR3+ innate CD8+ T cells in blood and lymphoid organs of WT and germ-free mice. (C) Representative flow cytometric plots of CXCR3+ innate CD8+ T cell populations in the spleens of WT, stat1-/-, T-bet-/-, ifn-γ-/-, and cxcr3-/- mice. Numbers correspond to the percentage of CXCR3+ innate CD8+ T cell populations. Cells are gated on CD8+, CD3+ lymphocytes. (D) Total number of CXCR3+ innate CD8+ T cells in the spleens of WT, stat1-/-, T-bet-/-, ifn-γ-/-, and cxcr3-/- mice. *p < 0.05.

To confirm this functional difference in the proliferative response of these cells to antigenic stimulation in vivo, we sorted CXCR3+ and CXCR3- innate CD8+ T cells from OT1-CIBER mice, labeled them with CellTrace Violet Cell Proliferation kit, and then adoptively transferred them to WT Thy1.1 recipients. Proliferative responses of transferred cells in the draining lymph nodes were analyzed after injection of recipient mice with OVA. Consistent with our in vitro results, CXCR3+ innate CD8+ T cells proliferated more robustly than CXCR3- innate CD8+ T cells (Fig. 8E). Proliferative responses and cytokine production of sorted CXCR3+ or CXCR3- innate CD8+ T cell populations to in vitro stimulation from WT mice were similar to those observed in CIBER mice (Supplemental Fig. 3). Taken together, our results suggest that CXCR3+, but not CXCR3-, innate CD8+ T cells could be involved in the generation of a more rapid effector response after antigenic or cytokine stimulation.
IL-2 and IL-15 enhance the effector functions of CXCR3-expressing innate CD8+ T cells

Our observation that surface expression of IL-2Rβ (a coreceptor for IL-2 and IL-15) is greater among CXCR3+ innate CD8+ T cells led us to examine whether both populations differ in their response to in vitro stimulation by IL-2 or IL-15 alone. Numerous studies have demonstrated a requirement for IL-15 in the homeostatic maintenance and functions of innate CD8+ T cells (8–11), and these cells have been shown to proliferate in vitro to IL-2 alone (21, 22). However, the ability of IL-2 or IL-15 alone to induce the production of effector molecules by innate CD8+ T cells independent of Ag has not been fully explored. In view of the observed variation in response to mixed cytokine stimulation, we stimulated splenocytes and lymph node cells with IL-2, IL-12, IL-15, or IL-18 alone, and analyzed intracellular IFN-γ production by flow cytometry. We observed that CXCR3+ innate CD8+ T cells preferentially responded to IL-2 or IL-15 stimulation by producing greater amounts of intracellular IFN-γ than CXCR3− innate CD8+ T cells (Fig. 9A).

Because whole-spleen and lymph-node cell preparations also contain other immune cells that could influence responsiveness of innate CD8+ T cells to cytokines and affect CXCR3 expression, we isolated CXCR3+ and CXCR3− innate CD8+ T cells from naive CIBER mice by FACS sorting and subjected them to similar cytokine stimulation conditions. Consistent with our previous data, we observed a much higher induction of IFN-γ and granzyme B mRNAs in CXCR3+ innate CD8+ T cells after stimulation with IL-2 and IL-15, but not IL-12 and IL-18 (Fig. 9C). Furthermore, CXCR3+ innate CD8+ T cells secreted greater amounts of IFN-γ (Fig. 9B), and showed greater proliferation (Fig. 9D), than cells not expressing CXCR3 after stimulation with IL-2 or IL-15, but not IL-12 or IL-18. However, we did not observe any significant differences in the expression of c-myc, and bcl-2 transcripts were lower in CXCR3+ innate CD8+ T cells than in CXCR3− innate CD8+ and naive CD8+ T cells after IL-2 and IL-15 stimulation (data not shown). Taken together, these findings confirm the functional differences that exist between CXCR3+ and CXCR3− innate CD8+ T cells.

Discussion

Bicistronic reporter mouse models that characterize immune-related gene-expressing cells are valuable tools that greatly enhance our understanding of gene regulation, function, and cellular expression in vivo. This system has been successfully used to examine the roles of IL-4, IL-10, and FoxP3, and their contribution to the immune response (7, 23, 24). Our data demonstrate that CIBER mouse is an ideal reporter system for CXCR3 expression, because EGFP production correlates with CXCR3 expression.
levels. Unlike cytokines that could potentially be stored within the cell before release, CXCR3 is primarily regulated at the transcriptional level (25). Furthermore, unlike other chemokine receptors, CXCR3 is not recycled back to the cell surface after constitutive or ligand-induced internalization but is rapidly degraded. As such, replenishment of cell-surface CXCR3 is dependent on de novo synthesis of CXCR3 protein and subsequent transport through the Golgi (25). This makes CXCR3 mRNA transcript levels a suitable indicator for cell-surface CXCR3 expression and function, which could be induced by binding with anti-CXCR3 Abs.

In this study, using CIBER mice, we identified CXCR3+ and CXCR3− populations of innate CD8+ T cells that are transcriptionally and phenotypically distinct. CXCR3+ innate CD8+ T cells expressed higher transcript levels of Spi-6 and Spi-2a and lower levels of PD-1 than CXCR3− innate CD8+ T cells. The serine protease inhibitors Spi-6 and Spi-2a have been shown to block the activity of granzyme B and cathepsin B and favor the longevity of memory T cells (26, 27). Downregulation of the inhibitory receptor, PD-1, is crucial to the formation of functional memory CD8+ T cells after acute infection (28). The pattern of gene ex-
pression displayed by CXCR3+ innate CD8+ T cells appears to be similar to those of bona fide central memory CD8+ T cells, favoring long-term persistence and preventing apoptosis (29). Therefore, the roles played by CXCR3+ innate CD8+ T cells during the innate phase of an immune response might very well be comparable with the functions of central memory CD8+ T cells during adaptive immunity to recall infections.

We observed significant differences in the surface expression of Ly6C between CXCR3+ and CXCR3− populations of innate CD8+ T cells. Ly6C is involved in signal transduction and cytokine production during T cell activation and has recently been shown to play a role in the trafficking of central memory T cells (30). Its function in CXCR3+ innate CD8+ T cells is not clear, but it is possible that Ly6C plays similar functional and migratory roles. Indeed, migratory differences are further suggested by our observation that CCR9, a gut homing chemokine receptor, is downregulated in the CXCR3+ innate CD8+ T cells compared with innate CD8+ T cells that do not express CXCR3 (Fig. 6A).

CXCR3+ innate CD8+ T cells showed higher cell-surface expression of IL-2Rβ. IL-2 and IL-15 signal through IL-2Rβ...
FIGURE 9. IL-2 and IL-15 enhance the effector functions of CXCR3-expressing innate CD8+ T cells. (A) Intracellular IFN-γ produced by lymph node cells and splenocytes obtained from naive CIBER mice and treated with individual cytokines in vitro. Innate CD8 T cell populations are gated on CD3+ CD8+ CD44+ CD62L+. (B) IFN-γ levels of sorted CXCR3+ and CXCR3− innate CD8+ T cells stimulated in vitro with individual cytokines determined by cytokine ELISA. (C) Real-time PCR analysis of IFN-γ and granzyme B gene expression from sorted CXCR3+ and CXCR3− innate CD8+ T cells, and naive CD8+ T cells after individual cytokine stimulation. Data are presented as fold induction over unstimulated cells. (D) Cellular proliferation of sorted CXCR3+ and CXCR3− innate CD8+ T cells and naive CD8+ T cells stimulated in vitro with individual cytokines. Proliferation is determined using CellTrace Violet proliferation kit. Numbers represent division index. Error bars represent SEM. All experiments were performed at least three times. *p < 0.01.
(CD122), a common cytokine receptor γ subunit (CD132) and a cytokine-specific α-chain (31). Memory T cells express IL-2Rβ, and the expression of this receptor on innate CD8+ T cells, as well as the dependence of this population on IL-15 for homeostatic maintenance, has been reported (11). However, we observe differential expression of IL-2Rβ among innate CD8+ T cells correlating with the expression of CXCR3 on these cells. Our results suggest that CXCR3+ innate CD8+ T cells are more responsive than CXCR3− innate CD8+ T cells to IL-2– and IL-15–mediated signaling, resulting in increased cell proliferation, activation, and induction of effector genes. Signal transduction through IL-2Rβ involves the Jak1, Jak3, STAT3, and STAT5 (31). PI3K/AKT and Ras/Raf/MAPK pathways are also stimulated, leading to the induction of c-myc, bcl-2, and c-fos/c-jun, which promote cell survival, proliferation, and cytotoxic activity of CD8+ memory T cells and NK cells (32, 33). However, we did not observe increased levels of c-myc and bcl-2 transcripts in CXCR3+ innate CD8+ T cells, it is possible that other signaling pathways are induced in CXCR3+ innate CD8+ T cells after stimulation with these cytokines.

In conclusion, we demonstrate, using our newly generated CIBER mice, the existence of distinct populations of innate CD8+ T cells that differ in activation status, transcriptional profile, and surface marker expression based on the expression of the chemokine receptor CXCR3, which suggests distinct functional and phenotypic characteristics. Targeting this population of innate CD8+ T cells may provide an early source of IFN-γ during intracellular bacterial infections before the onset of adaptive immunity. We believe that CIBER mouse will further enable assessment of the contributions made by various CXCR3-expressing cell populations to the overall immune response in a variety of infectious, autoimmune, and neoplastic diseases.

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