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CD8+ T Cells Produce the Chemokine CXCL10 in Response to CD27/CD70 Costimulation To Promote Generation of the CD8+ Effector T Cell Pool

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Various cell types can produce the chemokine CXCL10 in response to IFN-γ stimulation. CXCL10 is generally viewed as a proinflammatory chemokine that promotes recruitment of CD8+ and Th1-type CD4+ effector T cells to infected or inflamed nonlymphoid tissues. We show that CXCL10 plays a role during CD8+ T cell priming in the mouse. Genome-wide expression profiling revealed the *Cxcl10* gene as a target of CD27/CD70 costimulation in newly activated CD8+ T cells. CD27/CD70 costimulation is known to promote activated T cell survival, but CXCL10 did not affect survival or proliferation of primed CD8+ T cells in vitro. Accordingly, CXCL10 could not fully rescue CD27 deficiency in mice infected with influenza virus. Rather, CXCL10 acted as chemoattractant for other activated CD8+ T cells. It signaled downstream of CD27 in a paracrine fashion to promote generation of the CD8+ effector T cell pool in the Ag-draining lymph nodes. Consistently, CD8+ T cells required expression of the CXCL10 receptor CXCR3 for their clonal expansion in a CD27/CD70-dependent peptide-immunization model. Our findings indicate that CXCL10, produced by primed CD8+ T cells in response to CD27/CD70 costimulation, signals to other primed CD8+ T cells in the lymph node microenvironment to facilitate their participation in the CD8+ effector T cell pool. *The Journal of Immunology*, **2013, 191: 000–000.**

The T cell response is characterized by the rapid proliferation of Ag-specific T cells, their differentiation into effector cells, and their migration to the site of infection. Throughout their life, T cells rely heavily on contacts and communication with cells in their microenvironment. In lymphoid organs, fibroblastic reticular cells offer steady-state survival signals (1), whereas dendritic cells (DCs) offer the essential signals for T cell priming (2). In infected tissues, various hematopoietic and nonhematopoietic cell types produce cytokines and chemokines that sustain, promote, and direct the T cell response. We highlight in this article that chemokines can optimize the T cell response by enabling cell–cell communication.

The chemokine CXCL10 was originally identified as an IFN-γ–inducible protein, produced by many cell types in inflamed or infected tissue, such as keratinocytes, macrophages, fibroblasts, and endothelial cells (3). Activated T cells can also produce CXCL10, both in mouse (4) and human (5). CXCL10 binds to one unique receptor, CXCR3, and acts as chemoattractant for various immune cells (6). CXCR3 is present on a small fraction of naive T cells, but it is rapidly upregulated upon T cell activation (7, 8) and expressed by most CD8+ effector T cells, as well as Th1-type CD4+ effector T cells (9, 10). CXCR3 is also found on NK cells (11), plasmacytoid DCs (12), and activated monocytes (13). Because of the preferential expression of CXCR3 on Th1 cells, rather than Th2 cells, and the IFN-γ–inducible nature of CXCL10, this chemokine pathway is typically associated with Th1-type immune responses.

Many studies report that local CXCL10 production serves to recruit effector T cells to infected or inflamed tissues. CXCL10 proved to be nonredundant with its close relatives CXCL9 and CXCL11 in promoting the accumulation of CD4+ and CD8+ effector T cells in infected tissues (14, 15). In a variety of disease models, the use of CXCL10-deficient or CXCL10-transgenic mice or the use of CXCL10-blocking Abs demonstrated that CXCL10 promoted the accumulation of CD4+ and CD8+ effector T cells in the infected, inflamed, or tumor tissues where it was produced (16–18).

However, certain studies report an effect of CXCL10 at the site of T cell priming. In CXCL10-deficient mice, the generation of CD8+ effector T cells in the spleen was impaired upon systemic virus infection (19). Likewise, generation of effector T cells in the lymph nodes upon OVA protein immunization was impaired in CXCL10-deficient mice (19). CXCL10 can influence T cell priming by enabling cell–cell communication: in lymph nodes, recently immigrated DCs produced CXCL10 to attract primed CD4+ T cells. The resulting cluster formation between the primed CD4+ T cells and the DCs promoted Th1-type effector differentiation (20). A recent report corroborated and further clarified the mechanism by

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The sequences presented in this article have been submitted to the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-1772.

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Abbreviations used in this article: aAPC, artificial APC; DC, dendritic cell; DLN, draining lymph node; IRES, internal ribosomal entry sequence; PI, propidium iodide; WT, wild-type.

which CXCL10 contributes to Th1 polarization during priming (21).

We report in this article that CXCL10 plays a role in $CD8^+$ T cell priming, under control of CD27/CD70 costimulation. CD27 is a TNFR family member that is expressed on naive and activated $CD4^+$ and $CD8^+$ T cells (22, 23). Expression of its ligand, CD70, is induced in DCs and lymphocytes upon immune activation. CD27/CD70 costimulation promotes primary and memory $CD8^+$ T cell responses and Th1-type $CD4^+$ T cell responses (24, 25). At the priming site, CD27/CD70 costimulation supports clonal expansion of $CD8^+$ T cells by antiapoptotic and prometabolic effects (25–27) and, thus, broadens the responder T cell repertoire (28). At the tissue effector site, CD27/CD70 costimulation improves the survival of effector $CD8^+$ T cells by autocrine IL-2 signaling (29). In $CD4^+$ T cells, CD27/CD70 costimulation supports clonal expansion, as well as Th1-type effector development and help for the memory $CD8^+$ T cell response (30, 31).

In an unbiased search for CD27-regulated transcripts in $CD8^+$ T cells, we identified the *Cxcl10* gene as one of the major CD27 target genes. In vitro studies, as well as in vivo studies with $CD8^+$ T cells genetically reconstituted with the *Cxcl10* gene, pointed out that the CXCL10 produced by $CD8^+$ T cells in response to CD27 costimulation acts on other $CD8^+$ T cells in the priming phase of the response. In influenza virus–infected mice, CXCL10 promoted the generation of an effector $CD8^+$ T cell population in the lung-draining lymph nodes (DLNs) in a paracrine fashion. Moreover, $CD8^+$ T cells required the CXCL10 receptor CXCR3 for their expansion in the Ag-DLNs in a peptide-immunization model that strongly relies on CD27/CD70 costimulation. Our findings suggest that the CXCL10 produced by $CD8^+$ T cells upon CD27 costimulation recruits other $CD8^+$ T cells to sites in the lymph node microenvironment that facilitate their participation in the $CD8^+$ effector T cell pool.

Materials and Methods

Mice

Wild-type (WT), OT-I (C57BL/6-Tg[TcraTcrb]1100Mjb/J) (The Jackson Laboratory), $Cd27^{-/-}$ (24), OT-I;$Cd27^{-/-}$, $Cxcr3^{-/-}$ (B6.129P2-$Cxcr3^{tm1Dgen}$/J) (The Jackson Laboratory), OT-I;$Cxcr3^{-/-}$, F5 (32), F5; $CD27^{-/-}$, and $Cd27^{-/-}$;CD11c-*Cd70*tg (26) mice on a C57BL/6 background were used for experiments at 7–12 wk of age. OT-I mice express a transgenic TCR with specificity for $OVA_{257–264}$ peptide in the context of $H\text{-}2K^b$, and F5 mice express a transgenic TCR with specificity for influenza virus $NP_{366–374}$ peptide in the context of $H\text{-}2D^b$. Mice were of the CD45.2 allotype, unless otherwise stated. To distinguish donor and recipient cell populations, OT-I;$Cxcr3^{-/-}$ mice were crossed with mice expressing GFP under the *Ubiquitin* promoter (C57BL/6-Tg[UBC-GFP]30Scha/J) (The Jackson Laboratory), or a CD45.1 barrier was used. Experiments were approved by the Experimental Animal Committee of The Netherlands Cancer Institute and performed in accordance with national guidelines.

T cell purification

For in vitro cultures, T cells were purified from spleens and lymph nodes, as described previously (30), or with the BD IMag mouse CD8 T lymphocyte enrichment kit – DM (BD; cat. no. 558471). Purity of the resulting T cell populations was checked by flow cytometry using anti-CD8 mAb, together with anti-TCR Vβ5.1/5.2 for OT-I T cells, and was 85–90%.

T cell stimulation in vitro

For the experiments depicted in Fig. 1, MEC.SigOVA cells were used as artificial APCs (aAPCs) for in vitro priming of OT-I T cells. These mouse fibroblasts express a minigene encoding the $OVA_{257–264}$ epitope, which is presented in the context of $H\text{-}2K^b$. The aAPCs were available in two versions: one that did express CD80 and one that did not (33). These lines were retrovirally transduced with a bicistronic internal ribosomal entry sequence (IRES)-GFP retroviral vector to express either mCD70 or empty vector together with GFP. Thus, the lines obtained expressed no CD80 or CD70, only CD80 or CD70, or both CD80 and CD70. These cell lines were sorted by flow cytometry to obtain lines with highly comparable levels of $H\text{-}2K^b$ expression. Recently sorted cell lines were used for OT-I stimulation to optimally reveal the effects of costimulation. The aAPCs were seeded at 10^5/well in 24-well plates and cultured overnight to form an adherent monolayer. The next day, wells were washed with medium to remove any nonadherent cells or cell debris. Purified WT or $Cd27^{-/-}$ OT-I T cells were added to the aAPCs at 0.5×10^6/well in culture medium. Next, plates were centrifuged at $900 \times g$ for 1 min, and cells were cultured for 2, 4, 8, or 14 h when used for microarray analysis. After culture and prior to mRNA isolation, OT-I T cells were further purified by MACS with the $CD8a^+$ T cell Isolation Kit II, according to the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of the resulting T cell populations was always >95% (Supplemental Fig. 1A). When stimulating for longer periods of time, the T cells were gently transferred to empty wells after 20 h, cultured further in the absence of APCs, and MACS purified. T cell numbers were determined using a CASY cell counter (Scharfe System). For the experiment depicted in Fig. 3, purified WT or $Cd27^{-/-}$ polyclonal $CD8^+$ T cells from nonimmunized mice were labeled with CFSE (0.5 μM), according the manufacturer's protocol (CellTrace; Invitrogen). Next, they were stimulated in vitro at 1×10^5 cells/well in 96-well flat-bottom plates, using 1 μg/ml coated anti-CD3 mAb 145.2C11 (BD; cat. no. 553057), in the presence or absence of 0.5 μg/ml agonistic soluble recombinant mouse CD70–Ig fusion protein (FcCD70) (27), 0.2 μg/ml rCXCL10 (PeproTech), or 20 μg/ml neutralizing rat anti-CXCL10 mAb (R&D Systems). Cell divisions and the absolute numbers of propidium iodide $(PI)^-$ (live) $CD8^+$ T cells were determined at the end of culture by cell counting and flow cytometry.

T cell stimulation in vivo

For gene-expression profiling, WT or $Cd27^{-/-}$ OT-I mice were immunized intranasally with 500 μg OVA protein and 1 μg cholera toxin (Sigma) in 50 μl HBSS (30). For the experiments depicted in Figs. 4–6, $Cd27^{-/-}$ F5 mice were infected intranasally with 25 hemagglutinin units of influenza virus strain A/NT/60/68, as described (24, 25, 34). For the experiments depicted in Fig. 7, $Cd27^{-/-}$; CD11c-*Cd70*tg mice were immunized s.c. in the left flank with 20 μg $OVA_{257–264}$ peptide in 100 μl PBS.

Flow cytometry

Cells were isolated from relevant organs, as described previously (27), and stained with fluorochrome-conjugated Abs (FITC, PE, PerCP/Cy5, PE/Cy7, Pacific Blue) and MHC tetramers. mAbs used were directed at CD8β (53-6.7), CD4 (L3T4 or GK1.5), CD27 (LG.3A10), anti-CD44 (IM7), anti-CD45.1 (A20), CD62L (MEL-14), CD69 (H1.2F3), CD70 (FR70), CD80 (16-10A1), Vβ5.1/5.2 (MR9-4), or Vβ11 (RR3-15). These mAbs were obtained from BD Biosciences or eBioscience or prepared as purified Ig from available hybridomas. The anti-CXCR3 mAb used for flow cytometry (CXCR3-173) was obtained from BioLegend, and the rabbit polyclonal anti-CXCR3 Ab was from Zymed. Allophycocyanin-labeled $H\text{-}2K^b/OVA_{257–264}$ and $H\text{-}2D^b/NP_{366–374}$ tetramers were prepared in-house by standard procedures. Cells were analyzed using a FACSCalibur (BD), FACSAria (BD; for CXCR3 phenotyping), or CyAn (Dako) flow cytometer. Data were analyzed with FlowJo analysis software (TreeStar). PI-stained dead cells were excluded from analysis. OT-I T cells used for ex vivo microarray, transduced aAPC lines, and retrovirally transduced T cells were sorted by flow cytometry (FACSAria; BD) and kept on ice immediately after sorting.

Gene-expression profiling

RNA extraction, amplification, and hybridization were performed as described (30). Microarrays spotted with the Operon v3 oligonucleotide library were obtained from the central microarray facility of The Netherlands Cancer Institute (http://microarrays.nki.nl/). Microarrays were scanned on an Agilent Technologies scanner, and data extraction was done using ImaGene 6.0 software (BioDiscovery, El Segundo, CA). Each experiment consisted of two microarrays to allow for dye reversal between the samples, thus reducing systemic errors due to oligonucleotide-specific dye preferences. The heat maps in Fig. 1A and Supplemental Fig. 2 were created using TIGR (The Institute for Genomic Research) MultiExperiment Viewer software, version 3.1. Genes were included in the analysis when differentially expressed with a p value < 0.00003 in at least two experimental settings. Hierarchy in this list was determined by the fold differential expression (M value) and the number of experimental settings in which the gene was differentially expressed. The p value is based on multiple parameters, including signal intensity (A value), ratio of representation (M value), and signal quality. All data are available at http://www.ebi.ac.uk/arrayexpress under accession numbers E-NCMF-38 and E-MTAB-1772.

Quantitative real-time PCR

Expression of *Cxcl10* and *Hprt* mRNA was measured in the samples used for microarray analysis by real-time PCR (LightCycler 480 Real-Time PCR system; Roche). Fast SYBR Green Master Mix (Applied Biosystems) was used together with 10 ng cDNA template and 1 μM oligonucleotides. Levels of mRNA for the household gene *Hprt* were used for standardization. The oligonucleotides used to amplify the template DNA were *Cxcl10* fwd: 5′-CACACCCCGGTGCTGCGATG-3′, *Cxcl10* rev: 5′-AGCAGCTGATGTG-ACCACGGC-3′, *Hprt* fwd: 5′-CTGGTGAAAAGGACCTCTCG-3′, and *Hprt* rev: 5′-TGAAGTACTCATTATAGTCAAGGGCA-3′.

Western blotting

Supernatants of T cells activated in vitro were harvested at the indicated time points. Protein concentration was determined using the Bio-Rad protein assay. Equal amounts of total protein/sample were separated on Novex NuPAGE 8–14% Bis-Tris Gels (Invitrogen Life Technologies), and proteins were transferred to Protran nitrocellulose membrane (Schleicher & Schuell). The membrane was blocked with 5% powdered nonfat milk (Nutricia) in TBST. Next, the membrane was incubated with biotin-conjugated goat polyclonal antiserum directed against CXCL10 (R&D Systems) in TBST with 1% nonfat milk overnight at 4˚C, washed with TBST, and incubated with HRP-conjugated streptavidin for 2 h in TBST with 1% nonfat milk at 4˚C. After washing, HRP-conjugated streptavidin was detected using ECL (Amersham).

Transwell assays

T cells were purified from spleens of WT mice using a T lymphocyte enrichment set (BD) and were kept naive or were activated in vitro for 48 h with 2 μg/ml Con A (Omnilabo) in the presence of 1 ng/ml IL-7 (PeproTech). Transwells with a 5-μm pore size (Costar) were coated overnight with RetroNectin (Takara Bio, Otsu, Japan) at 50 μg/ml. Next, naive or activated T cells were added to the upper wells. IMDM or supernatants from T cells activated by aAPCs were added to the lower wells and supplemented with 0.2 μg/ml recombinant murine CXCL10 (PeproTech) or 20 μg/ml neutralizing rat anti-CXCL10 mAb (R&D Systems). T cells were allowed to migrate through the Transwell membrane for 3 h at 37˚C. The migrated cells were counted with a CASY cell counter (Scharfe System) and analyzed by flow cytometry.

Retroviral transduction

The mouse *Cxcl10* cDNA was obtained from the German Science Center for Genome Research and cloned into the pMX_{IRES}GFP vector that allows bicistronic expression of the gene of interest and GFP as the result of the presence of an IRES. Empty vectors pMX_{IRES}GFP or pMX_{IRES}YFP were used as controls. For virus production, retroviral constructs were transfected using FuGENE 6 (Roche) into Phoenix-ECO packaging cells, together with the pCL-Eco vector encoding the ecotropic retrovirus receptor. Medium that contained retrovirus was harvested from the Phoenix-ECO packaging cells 48 h later. For retroviral transduction, splenocytes from WT and *Cd27*$^{-/-}$ F5 mice were cultured with 2 μg/ml Con A and 1 ng/ml rIL-7 for 48 h. Next, they were resuspended at 2×10^6 cells/0.5 ml retrovirus-containing medium and placed in nontissue culture–treated 24-well plates (BD) coated with 50 μg/ml RetroNectin (Takara Bio). Plates were spun for 90 min at $450 \times g$, and cells were cultured for 20 h prior to their use for adoptive transfer.

Adoptive transfer

In the influenza virus–infection experiments, *Cxcl10*-transduced or empty vector–transduced splenocytes of WT and *Cd27*$^{-/-}$ F5 mice were incubated with allophycocyanin-conjugated anti-CD8 mAb in IMDM with FCS for 30 min on ice. Cells were washed and resuspended in IMDM with FCS and sorted by flow cytometry for GFP or YFP and CD8. The resulting purified transduced CD8$^+$ T cells were injected retro-orbitally at 5×10^3 cells/mouse in 100 μl HBSS. In the OVA protein or OVA peptide–immunization experiments, OT-I, OT-I;*Cd27*$^{-/-}$, and OT-I;*Cxcr3*$^{-/-}$;GFP cells were purified with the IMag mouse CD8 T Cell Enrichment Set (BD). OT-I T cells were injected retro-orbitally at 1×10^4 cells/genotype in 100 μl HBSS/mouse.

Statistical analysis

Statistical significance was determined using a two-tailed Student *t* test.

Results

Primed CD8$^+$ T cells produce CXCL10 in response to CD27/CD70 costimulation

We performed genome-wide mRNA expression profiling to identify the molecular mechanisms by which CD27/CD70 costimulation supports the CD8$^+$ T cell response. For this purpose, in vitro and in vivo experimental settings were used in which CD27/CD70 costimulation was the only variable. We used OT-I TCR transgenic T cells that recognize $OVA_{257–264}$ peptide in the context of H-2K^b as responder CD8$^+$ T cells. In vitro, purified OT-I T cells (Supplemental Fig. 1A) were activated by coculture with aAPCs in the form of mouse embryo fibroblasts that present $OVA_{257–264}$ peptide and did or did not express CD80 (33). In the two comparative settings, we stimulated WT OT-I cells with aAPCs that did or did not express CD70 or we stimulated WT and *Cd27*$^{-/-}$ OT-I T responder cells with aAPCs that expressed CD70 (Fig. 1A). This way, we selectively monitored the effect of CD27/CD70 costimulation in the presence or absence of CD28/CD80 costimulation. After 2, 4, 8, or 14 h of stimulation, the OT-I T cells were removed from the adherent aAPCs, purified further (Supplemental Fig. 1A), and used for RNA extraction. In vivo, WT or *Cd27*$^{-/-}$ OT-I T cells were activated by immunizing the mice intranasally with OVA protein. After 3, 4, or 8 d, the OT-I cells were purified from lung-DLNs, spleen, or lung by flow cytometric sorting, based on H-2K^b/$OVA_{257–264}$ tetramer and CD8 staining (Supplemental Fig. 1B). The mRNA isolated from the OT-I T cells at these time points was analyzed with microarrays that represent 72% of all known mouse genes (29, 30). Genes regulated by CD27/CD70 costimulation were selected on the stringent criteria of differential expression with a p value < 0.00003 in at least two experimental settings. A heat map was constructed that included in the hierarchy the fold differential expression and the number of experimental settings in which the gene was differentially expressed (Supplemental Fig. 2).

In the comparative setting of WT versus *Cd27*$^{-/-}$ OT-I cells, the *Cd27* gene was consistently identified as the most important differentially expressed gene, confirming the validity of the approach (Supplemental Fig. 2). The *Il-2* gene was at the top of the hierarchy of CD27-regulated genes, as previously reported (29). At the indicated p value, this analysis revealed ~30 genes that were differentially expressed (Supplemental Fig. 2). To our surprise, the *Cxcl10* gene was number five on this hit list of potential CD27 target genes in primed CD8$^+$ T cells. CD27/CD70 costimulation induced *Cxcl10* mRNA expression both in vitro and in vivo (Fig. 1A). Quantitative real-time PCR confirmed the rapid upregulation of *Cxcl10* mRNA expression within 4 h after CD27 costimulation in both in vitro settings (Fig. 1B). CD27 costimulation also upregulated *Cxcl10* mRNA levels in a transient manner when polyclonal CD8$^+$ T cells were activated with anti-CD3 mAb (Supplemental Fig. 3A). This upregulation still occurred when protein synthesis was blocked, indicating that CD27 signaling directly promoted *Cxcl10* gene transcription and/or mRNA stability (Supplemental Fig. 3B).

To verify that CXCL10 was differentially expressed at the protein level, medium was harvested from cultures of WT and *Cd27*$^{-/-}$ OT-I cells that had been stimulated for 48, 72, or 96 h with CD70$^+$ aAPCs, and Western blotting was performed (Fig. 1C). The levels of CXCL10 protein were higher in the medium of WT OT-I T cells than that of *Cd27*$^{-/-}$ OT-I T cells at all time points tested, apparently due to the transient increase in *Cxcl10* mRNA levels as result of CD27 costimulation. CD27/CD70 costimulation is known to promote clonal expansion of T cells, but the numbers of live WT and *Cd27*$^{-/-}$ OT-I T cells were not significantly different at the 48- and 72-h time points (Fig. 1D), in-

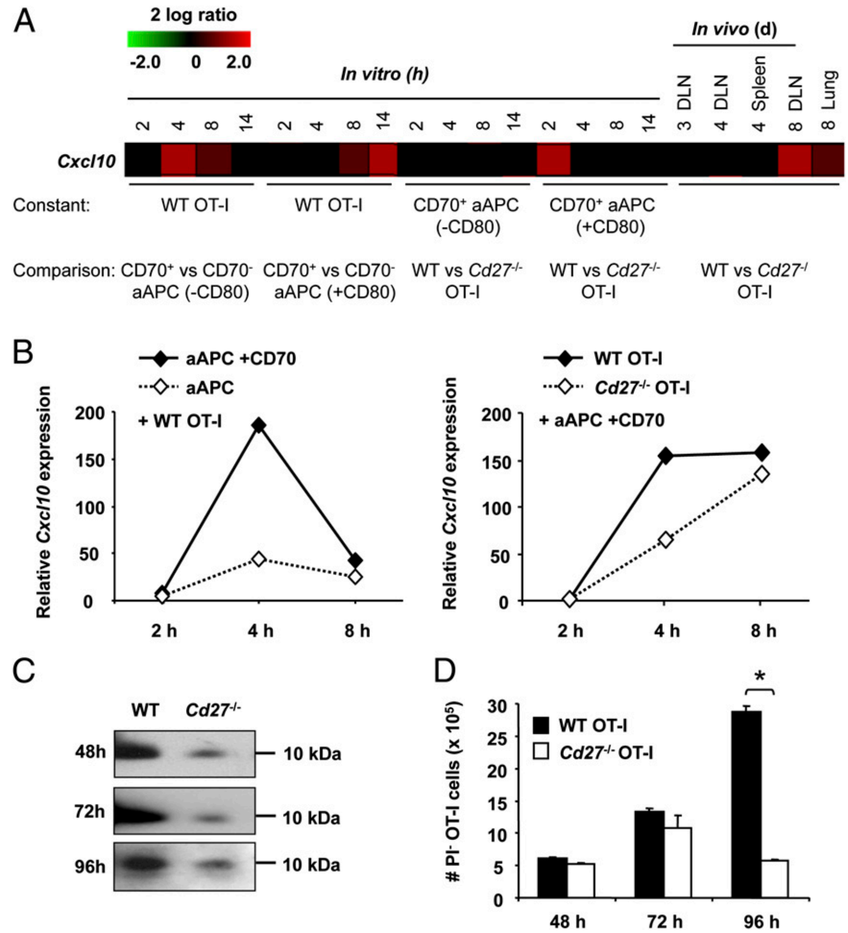

FIGURE 1. CD27/CD70 costimulation of CD8$^+$ T cells induces CXCL10 expression. (**A**) Differential expression of *Cxcl10* mRNA as a result of CD27 costimulation in the various experimental settings, as determined by microarray analysis. The data shown are part of the hit list that is depicted in Supplemental Fig. 2. (**B**). Validation of differential *Cxcl10* mRNA expression by quantitative real-time PCR in the indicated comparative settings. *Cxcl10* mRNA levels were related to those of the household gene *Hprt*. Data are means of two independent reactions. (**C**) Differential CXCL10 protein expression. WT and $Cd27^{-/-}$ OT-I T cells were stimulated for the indicated time periods with aAPCs that expressed CD80 and CD70. CXCL10 protein in the supernatants, as detected by Western blotting. (**D**) The absolute numbers of live OT-I cells/tissue culture well in the experiment shown in (C). Data are mean (+ SEM) of three samples and are representative of three independent experiments. $*p < 0.001$, Student t test.

dicating that WT OT-I T cells produced more CXCL10 on a per-cell basis. We conclude that CD27 costimulation of primed CD8$^+$ T cells increases CXCL10 protein expression by effects at the mRNA level. The protein synthesis–independent upregulation of *Cxcl10* mRNA by CD27 strongly suggests that CD27 signaling directly stimulates *Cxcl10* gene expression and/or mRNA stability.

CXCL10 produced upon CD27/CD70 costimulation attracts activated CD8$^+$ T cells

To examine which T cell subsets in priming organs might be responsive to CXCL10, we determined the expression of CXCR3, the unique receptor for CXCL10. In agreement with published results (35), a fraction of CD4$^+$ and CD8$^+$ T cells in spleen and inguinal lymph nodes of nonimmunized mice expressed CXCR3 (Fig. 2A, 2B). Most CXCR3$^+$ CD8$^+$ T cells had a central memory phenotype, whereas CXCR3$^+$ CD4$^+$ T cells predominantly had an effector (memory) phenotype (Fig. 2C, 2D). In addition, a proportion of naive phenotype CD4$^+$ and CD8$^+$ T cells expressed CXCR3 (Fig. 2C, 2D). In concordance with the predominant presence of CXCR3 on Ag-experienced cells, most splenic CD8$^+$ T cells acquired CXCR3 expression upon activation with Con A in vitro (Fig. 2E), before acquisition of a CD62L^{low} effector phenotype (data not shown). However, splenic CD4$^+$ T cells did not upregulate CXCR3 expression upon activation with Con A in vitro (Fig. 2E).

In vitro Transwell assays were performed to assess T cell migration in response to CXCL10 or potentially other factors produced in response to CD27 costimulation. Polyclonal CD8$^+$ T cells isolated from the spleens of nonimmunized mice migrated toward recombinant murine CXCL10 after Con A activation, and this was completely blocked by a neutralizing anti-CXCL10 mAb, thus validating the assay (Supplemental Fig. 3C). Splenic CD4$^+$ T cells did not detectably respond to CXCL10, either before or after Con A activation (Supplemental Fig. 3C), in agreement with lack of CXCR3 upregulation (Fig. 2E). Next, we tested the effect of soluble factors, produced by OT-I T cells in response to CD27/CD70 costimulation, on the migration of activated polyclonal CD8$^+$ T cells. For this purpose, we used culture medium from WT or $Cd27^{-/-}$ OT-I T cells that had been activated for 48 h by CD70-expressing aAPCs. Medium from WT OT-I T cells attracted more activated CD8$^+$ T cells than did medium from $Cd27^{-/-}$ OT-I T cells (Fig. 2F), in agreement with the larger amount of CXCL10 present (Fig. 1C). Addition of CXCL10-neutralizing mAb to the medium of WT OT-I T cells strongly inhibited the migration of polyclonal CD8$^+$ T cells (Fig. 2F). By mAb-mediated blocking of CXCL10, the migratory response toward WT OT-I T cell medium became comparable to the migratory response toward $Cd27^{-/-}$ OT-I T cell medium (Fig. 2D). Therefore, we conclude that CXCL10 is the only chemoattractant for activated CD8$^+$ T cells that is produced by CD8$^+$ T cells in response to CD27/CD70 costimulation.

CXCL10 does not affect survival or clonal expansion of primed CD8$^+$ T cells in vitro

CD27/CD70 costimulation is known to promote the survival of primed CD8$^+$ T cells. In lymph nodes, CD27/CD70 costimulation induces expression of Bcl-x_L and the Pim-1 kinase that provide antiapoptotic and prometabolic signals to CD8$^+$ T cells during clonal expansion (27). It was suggested that CXCL10 can promote T cell proliferation (36). For this reason, we tested the potential

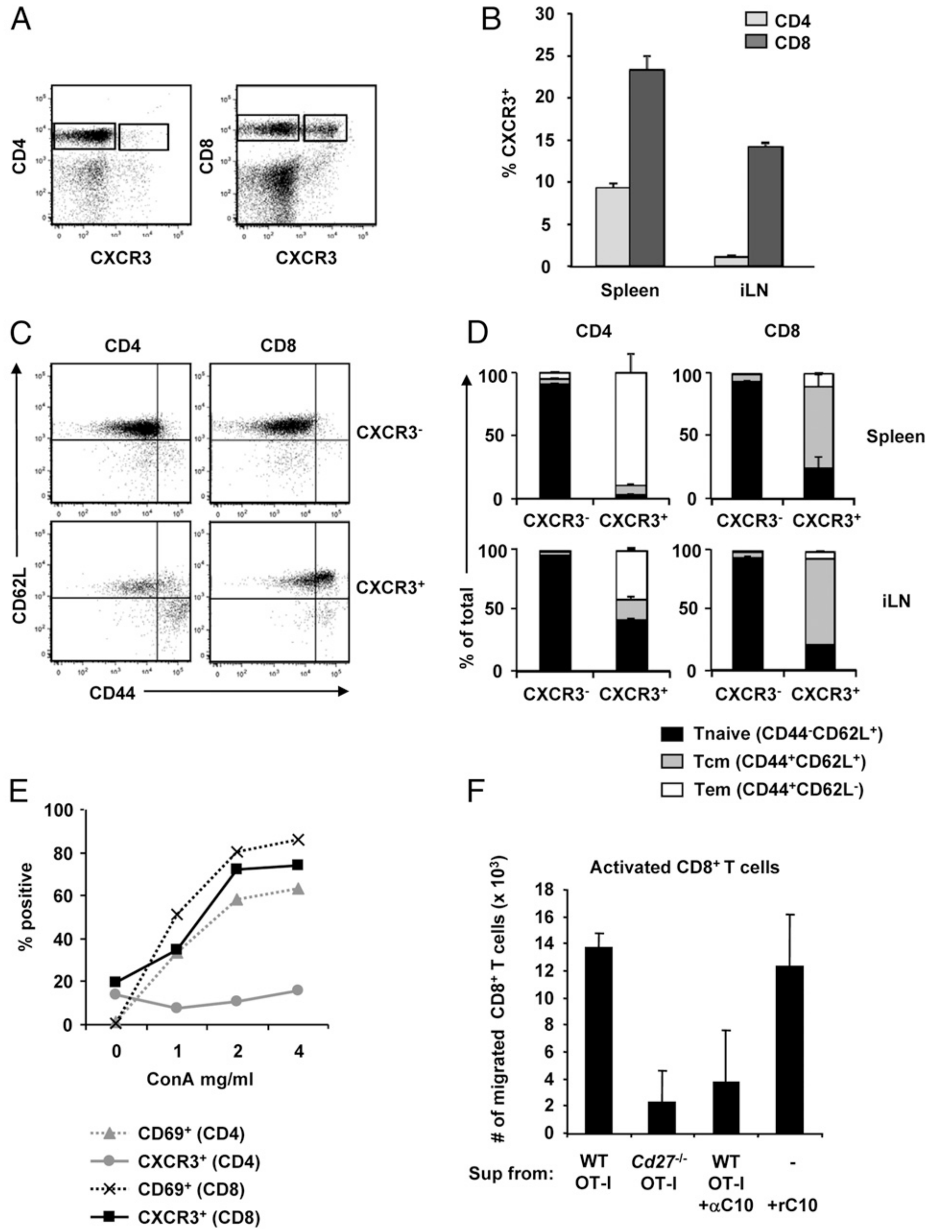

FIGURE 2. CXCL10 produced in response to CD27/CD70 costimulation attracts activated CD8$^+$ T cells. (**A**–**D**) Detection of CXCR3$^+$ cells among CD4$^+$ and CD8$^+$ T cells in spleen and inguinal lymph nodes (iLN) of nonimmunized, 8-wk-old female mice (n = 3), as determined by flow cytometry. Cells were stained with the following combination of Abs—CXCR3-PE, CD8a–PerCp–Cy5.5, CD4–allophycocyanin–H7, CD62L-FITC, and CD44–PE–Cy7—and gated for live cells using PI exclusion. (A) Representative flow cytometric analysis of CXCR3 and CD4 or CD8 staining on cells from iLN. (B) The percentage of CXCR3$^+$ cells among total CD4$^+$ and CD8$^+$ T cells in spleen and the iLN. (C) Representative flow cytometric analysis of CD44 and CD62L staining on gated CXCR3$^-$ or CXCR3$^+$ CD4$^+$ and CD8$^+$ T cells from the iLN. (D) The fractions of naive (CD44$^-$CD62L$^+$), central memory (Tcm; CD44$^+$ CD62L$^+$), and effector (memory) (Tem; CD44$^+$CD62L$^-$) phenotype cells among total CXCR3$^-$ or CXCR3$^+$ CD4$^+$ and CD8$^+$ T cells in spleen and iLN. Data in (B) and (D) are means + SD. (**E**) Effect of Con A activation on CD69 and CXCR3 expression on CD4$^+$ and CD8$^+$ T cells. Purified T cells were activated in vitro with Con A for 2 d. Fluorescence intensity of CD69 and CXCR3 on gated CD4$^+$ and CD8$^+$ T cells was determined, and the percentage of positive cells is indicated. Data are representative of two independent experiments. (**F**) Transwell assay using as chemoattractant in the lower chamber the supernatant (Sup) from WT or *Cd27*$^{-/-}$ OT-I T cells that had been activated with CD70$^+$ aAPCs for 48 h. Sup was tested alone or together with neutralizing anti-CXCL10 mAb (αC10). Control medium (-) with rCXCL10 (rC10) was used as positive control. Con A–activated splenic CD8$^+$ T cells from WT mice were added to the upper chamber as responder cells. The numbers of CD8$^+$ T cells that migrated through the membrane to the lower chamber are shown. Data are the means of two independent assays with triplicate samples (+ SD).

impact of CXCL10 on clonal expansion and survival of primed CD8$^+$ T cells in vitro. WT and *Cd27*$^{-/-}$ polyclonal CD8$^+$ T cells were activated by low-level TCR/CD3 triggering with anti-CD3 mAb and costimulated via CD27 with a soluble recombinant CD70 protein (FcCD70) (27, 29). Cell division was read out by dilution of CFSE, and cell survival was assessed by uptake of PI. In these cultures, CD27 costimulation with FcCD70 greatly increased the yield of live WT CD8$^+$ T cells at day 3 of culture (Fig. 3A). FcCD70 did not have any effect on *Cd27*$^{-/-}$ CD8$^+$ T cells, demonstrating that it acted by engaging CD27 (Fig. 3A). Recombinant CXCL10 or neutralizing anti-CXCL10 mAb did not influence the yield of live WT or *Cd27*$^{-/-}$ CD8$^+$ T cells, either in the presence or in the absence of FcCD70 at day 3 of the response (Fig. 3A) or at day 4 (data not shown).

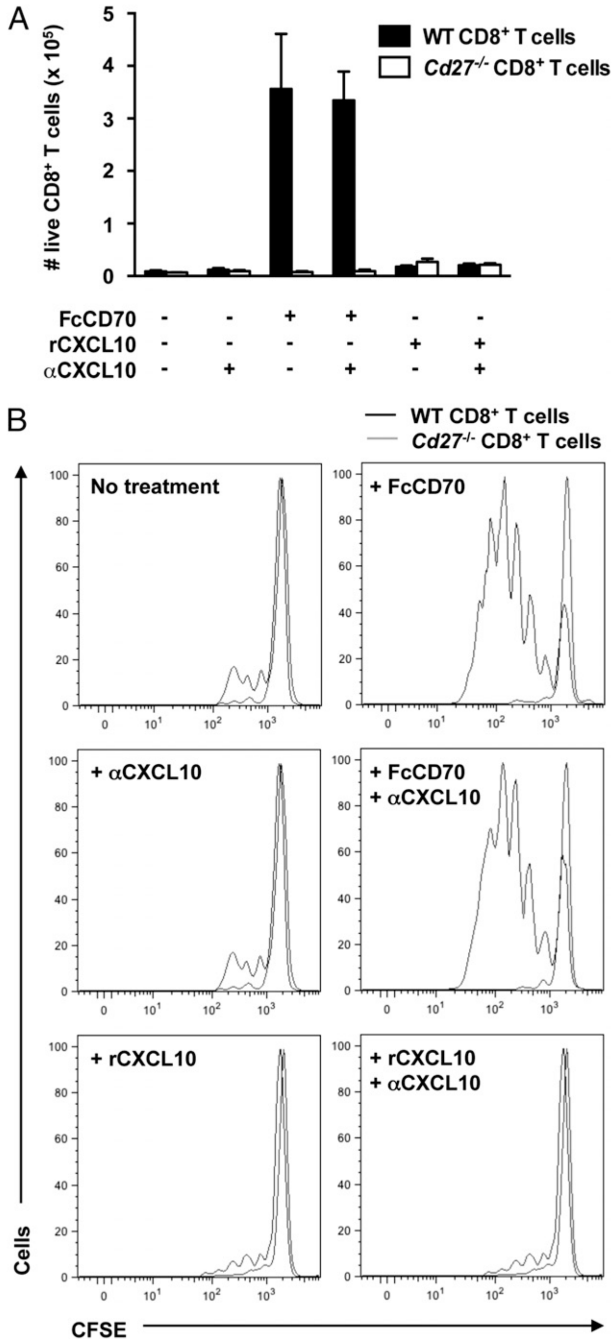

FIGURE 3. CXCL10 does not affect clonal expansion or survival of primed CD8$^+$ T cells in vitro. Purified WT or *Cd27*$^{-/-}$ polyclonal CD8$^+$ T cells were stimulated in vitro with 1 μg/ml coated anti-CD3 mAb 145.2C11, in combination with CD27-agonistic recombinant mouse CD70–Ig fusion protein (FcCD70), rCXCL10, or neutralizing anti-(α)CXCL10 mAb. (**A**) Absolute numbers of live CD8$^+$ T cells present after 72 h of culture. Data are the mean + SD of triplicate samples in one experiment with pooled T cells from two age- and sex-matched mice/genotype. (**B**) Representative flow cytometric plots indicating CFSE dilution in the anti-CD3–stimulated T cells from the assay in (A) at 72 h. Cells received no additional treatment, rCXCL10, αCXCL10, and/or FcCD70. Triplicates showed highly similar results. Data in (A) and (B) are representative of three independent experiments.

Under conditions of low TCR/CD3 input, CD27/CD70 costimulation is known to promote cell cycle entry of murine CD8$^+$ T cells, most likely as the result of survival signaling (27). This was also revealed in the current assay by CFSE dilution. With TCR/CD3 stimulation alone, only a very small proportion of cells had divided at day 3, whereas in the presence of CD27 costimulation, the great majority of cells had entered division and completed multiple cycles (Fig. 3B, no treatment versus FcCD70). Addition of CXCL10 had no effect on CD8$^+$ T cell cycling, nor did the addition of neutralizing anti-CXCL10 mAb, either in the absence or in the presence of CD27 costimulation (Fig. 3B). Therefore, we conclude that, in vitro, survival and clonal expansion of CD8$^+$ T cells, driven by TCR/CD3 and CD27 signaling, does not rely on CXCL10.

Reconstitution of the Cxcl10 *gene in Cd27*$^{-/-}$ *CD8*$^+$ *T cells improves in vivo priming*

Next, we determined how CXCL10 contributed to CD27 function in vivo. For this purpose, we used influenza virus infection, a model that we used previously to delineate the contribution of CD27/CD70 costimulation to the CD8$^+$ T cell response (24, 25, 34). As responders, we used CD8$^+$ T cells of the F5 TCR transgenic mouse strain that recognize the immunodominant $NP_{366–375}$ epitope in the context of H-2D^b. Our aim was to determine to what extent reconstitution of *Cxcl10* gene expression could rescue the defects that *Cd27*$^{-/-}$ CD8$^+$ T cells display in this model system. WT and *Cd27*$^{-/-}$ F5 cells were transduced to express the *Cxcl10* gene or empty control vector in an IRES-GFP configuration that enabled flow cytometric purification of transduced F5 T cells. These cells were adoptively transferred into *Cd27*$^{-/-}$ recipient mice that were subsequently infected with influenza virus (Fig. 4A).

At days 7 and 8 postinfection, mediastinal-DLNs and spleen of recipient mice were analyzed for the number of F5 T cells, using GFP and CD8 as identifiers. At both days 7 and 8, *Cd27*$^{-/-}$ F5 T cells with empty vector showed a reduced accumulation compared with WT F5 donor cells with empty vector in DLNs (Fig. 4B), as well as to some extent in the spleen (Fig. 4C). This was expected because CD27/CD70 costimulation promotes clonal expansion and survival of primed CD8$^+$ T cells, particularly in the DLNs in this model system (25, 27). In the case of WT F5 T cells, *Cxcl10* gene transduction did not alter the response in DLNs or spleen (Fig. 4B, 4C). However, in case of *Cd27*$^{-/-}$ F5 T cells, *Cxcl10* gene transduction significantly improved accumulation in the DLNs at day 8 postinfection (Fig. 4B), but the response was still deficient compared with WT cells. In the spleen, the response of *Cd27*$^{-/-}$ F5 T cells was not significantly affected by *Cxcl10* gene transduction (Fig. 4C). Western blotting validated the expression of CXCL10 protein from the retroviral construct (Fig. 4D).

In conclusion, *Cxcl10* gene transduction did not affect the response of WT CD8$^+$ cells, indicating that it specifically complemented defects of *Cd27*$^{-/-}$ CD8$^+$ T cells. As expected from the fact that CD27 acts also via Bcl-x_L and Pim-1 during CD8$^+$ T cell priming (27), *Cxcl10* gene transduction could not fully rescue the defects in clonal expansion and accumulation of *Cd27*$^{-/-}$ responder CD8$^+$ T cells. However, it did improve their clonal expansion in the DLNs.

CXCL10 acts in a paracrine fashion to promote the generation of CD8$^+$ *effector T cells*

In the same experiment, we also determined how *Cxcl10* gene transduction in donor F5 T cells affected the T cell response of the *Cd27*$^{-/-}$ recipient mice. This was done by enumerating CD8$^+$ and CD4$^+$ T cells that had acquired a CD62L^{low} effector phenotype. At day 8 postinfection, generation of CD8$^+$ CD62L^{low} effector T cells in the DLN was significantly lower in mice that had received *Cd27*$^{-/-}$ F5 donor T cells with empty vector compared with mice

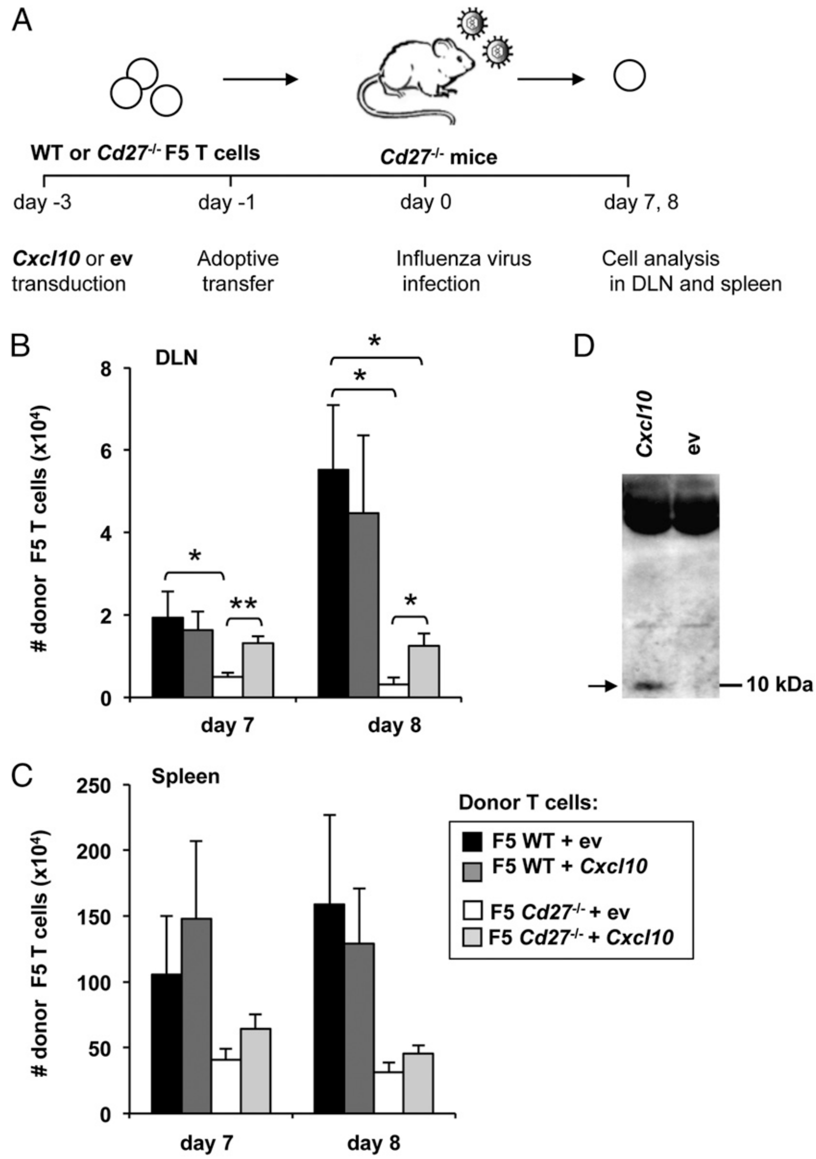

FIGURE 4. Reconstitution of the *Cxcl10* gene in $Cd27^{-/-}$ $CD8^+$ T cells improves in vivo priming. Effect of *Cxcl10* gene transduction on the response of F5 T cells to influenza virus. (**A**) Experimental scheme. WT and $Cd27^{-/-}$ influenza virus–specific F5 T cells were retrovirally transduced with a vector encoding CXCL10 and GFP in an IRES configuration or with empty vector (ev) encoding GFP only. Two days later, transduced F5 T cells were sorted for CD8 and GFP expression and adoptively transferred (5000 cells/mouse) into $Cd27^{-/-}$ recipients, which were infected with influenza virus on the following day. At day 7 or 8 postinfection, cells were harvested from DLNs and spleen, enumerated, and analyzed by flow cytometry. Absolute numbers of GFP^+ $CD8^+$ F5 T cells of the indicated genotypes recovered from DLNs (**B**) or spleen (**C**) at days 7 and 8. Data are mean + SEM ($n = 4$ mice/group). The experiment is representative of three independent experiments. (**D**) Verification of CXCL10 protein expression by transduced $Cd27^{-/-}$ F5 cells before adoptive transfer. Western blotting with anti-CXCL10 mAb was performed on supernatant of F5 T cells transduced with empty vector (ev) or *Cxcl10*-IRES-GFP vector. *$p < 0.05$, Student t test.

that had received WT F5 donor T cells with empty vector (Fig. 5A). A similar phenotype was observed for $CD4^+$ effector T cell generation (Fig. 5B). This result indicated that CD27 deficiency of the F5 donor T cells impacted on the $CD8^+$ and $CD4^+$ T cell responses of the ($Cd27^{-/-}$) recipient mice. When $Cd27^{-/-}$ F5 donor T cells had been transduced to express the *Cxcl10* gene, they improved the $CD8^+$ T cell response of the recipients to the level observed in mice that had received WT F5 donor T cells (Fig. 5A). *Cxcl10* reconstitution into $Cd27^{-/-}$ F5 T cells also significantly improved the $CD4^+$ T cell response (Fig. 5B). *Cxcl10* gene transduction into WT F5 T cells did not enhance $CD8^+$ or $CD4^+$ T cell responses of the recipients (Fig. 5A). This means that *Cxcl10* gene transduction specifically complemented the deficiency of $Cd27^{-/-}$ F5 T cells. F5 donor T cells expressing the *Cxcl10* gene did not affect the responses of $CD8^+$ or $CD4^+$ T cells in the spleen (Fig. 5C, 5D). We conclude that CXCL10 produced by virus-specific $CD8^+$ T cells upon CD27 costimulation acts in a paracrine fashion to promote the response of other $CD8^+$ T cells and $CD4^+$ T cells in the Ag-DLNs.

To further examine the paracrine effect of CXCL10, the responses of $Cd27^{-/-}$ F5 T cells that expressed either the *Cxcl10* gene or empty vector were compared side-by-side in the same recipient mice. For this purpose, $Cd27^{-/-}$ F5 T cells were transduced with a *Cxcl10*-IRES-GFP vector or an empty IRES-YFP vector, mixed in a 1:1 ratio, and adoptively transferred into $Cd27^{-/-}$ recipient mice. As a control, $Cd27^{-/-}$ F5 T cells were transduced with an empty IRES-YFP vector or with an empty IRES-GFP vector, mixed in a 1:1 ratio, and adoptively transferred into a different group of $Cd27^{-/-}$ recipient mice. Mice were subsequently infected with influenza virus, and the absolute numbers of GFP- and YFP-expressing F5 T cells were determined in DLNs and spleen of the recipient mice (Fig. 6A). In the DLNs at day 6, the F5 T cell response was significantly higher in mice that received *Cxcl10*-transduced F5 cells than in mice that received only empty vector–transduced F5 cells (Fig. 6B, *left panel*). This indicated that CXCL10 produced by the transduced F5 cells promoted the F5 T cell response. Importantly, $Cd27^{-/-}$ F5 T cells with empty vector accumulated to similar levels as did the *Cxcl10*-transduced F5 cells when they were present in the same recipients (Fig. 6B, *left panel*). This indicated that CXCL10 produced by F5 T cells acted in a paracrine fashion to promote the accumulation of F5 T cells that did not express CXCL10. There was no difference between the groups in the spleen (Fig. 6B, *right panel*), confirming that CXCL10 selectively promoted the accumulation of primed $CD8^+$ T cells in DLNs but not in the spleen.

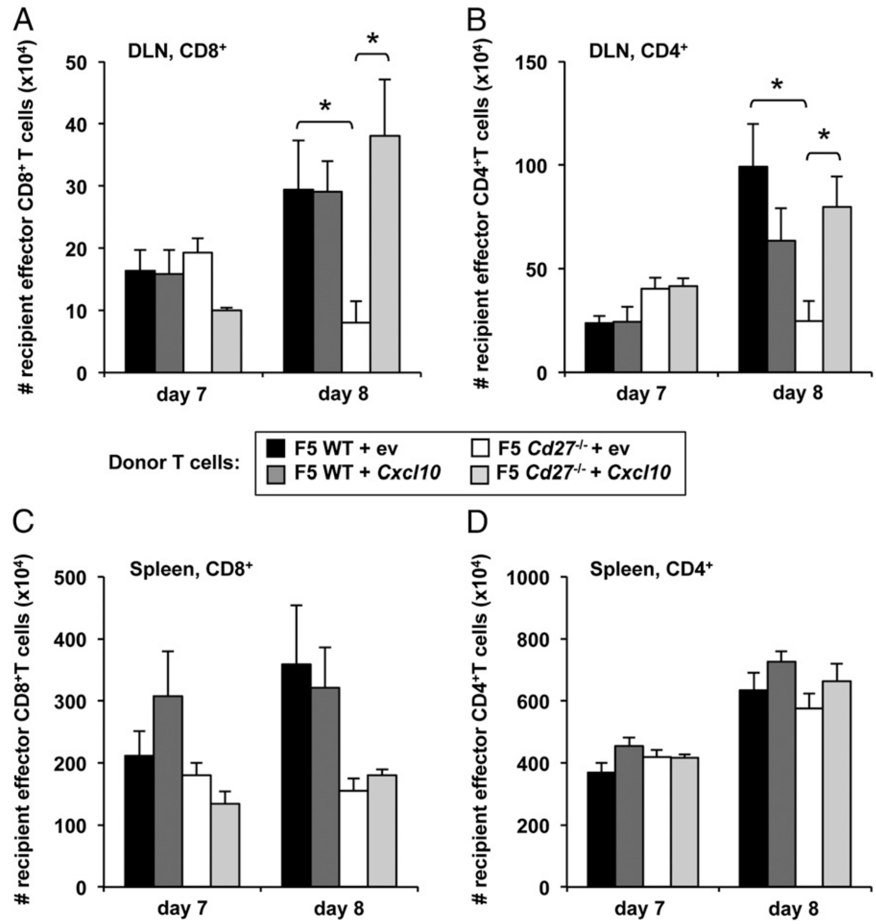

FIGURE 5. CXCL10 produced by $CD8^+$ donor T cells promotes the response of *Cd27*$^{-/-}$ $CD8^+$ T cells in the recipients. In the experiment outlined in Fig. 4, we also monitored the response of $CD8^+$ and $CD4^+$ T cells in the *Cd27*$^{-/-}$ recipients. (**A** and **C**) $CD8^+$ T cell responses. Absolute numbers of recipient $CD62L^-$ effector $CD8^+$ T cells formed in DLN (A) or spleen (**B**) of *Cd27*$^{-/-}$ mice that had received donor F5 T cells of the indicated genotypes. (B and **D**) $CD4^+$ T cell responses. Absolute numbers of recipient $CD62L^-$ effector $CD4^+$ T cells formed in DLNs (C) or spleen (D) of *Cd27*$^{-/-}$ mice that had received donor F5 T cells of the indicated genotypes. Data are mean + SEM of four mice/group. The experiment is representative of three independent experiments.

To examine the paracrine effect of CXCL10 on the $CD8^+$ T cell response of the recipient mice, the absolute numbers of recipient $CD62L^{low}$ effector $CD8^+$ T cells were determined at days 6 and 8 postinfection. At day 8, the number of recipient $CD8^+$ effector T cells in the DLNs was significantly higher in mice that received *Cxcl10*-transduced F5 T cells than in mice that received empty vector–transduced F5 T cells (Fig. 6C, *left panel*). However, in the spleen, CXCL10 expression by the F5 donor cells had no effect on the $CD8^+$ T cell response of the recipients (Fig. 6C, *right panel*). We conclude from these experiments that, in the DLNs of influenza virus–infected mice, CXCL10 produced by Ag-specific $CD8^+$ T cells in response to CD27 costimulation acts in a paracrine fashion to promote the generation of a $CD8^+$ effector T cell population.

Cxcl10 *gene transduction cannot rescue CD27-deficient T cell responses at the effector site*

In this model of influenza virus infection, the lung is a bona fide tissue effector site. There is no formation of BALT and no priming of T cells in the lung (34) (data not shown). All $CD4^+$ and $CD8^+$ T cells that accumulate in the lung have an effector phenotype, and the accumulation occurs after T cell priming in the mediastinal DLNs (24, 25, 34). We showed previously that CD27 costimulation provides an autocrine IL-2–signaling pathway that supports the survival of $CD8^+$ effector T cells in the lung, whereas it has no effect at the site of priming (29). In fact, IL-2 gene reconstitution completely rescued the survival defect of *Cd27*$^{-/-}$ $CD8^+$ T cells in the lung in this model (29). Accordingly, we found that *Cd27*$^{-/-}$ F5 T cells were deficient compared with WT F5 T cells in their accumulation in the lung after influenza virus infection (Supplemental Fig. 4). *Cxcl10* gene transduction did not have a significant effect on the accumulation of either WT or *Cd27*$^{-/-}$ F5 T cells in the lung at day 8 postinfection (Supplemental Fig. 4), which is the peak in this experimental setting (29). Likewise, it did not significantly affect the accumulation of *Cd27*$^{-/-}$ recipient $CD4^+$ or $CD8^+$ effector phenotype T cells in the lung at this time point (Supplemental Fig. 4). Thus, CXCL10 could not rescue the survival defect of *Cd27*$^{-/-}$ T cells at the tissue site, as expected from the proven importance of CD27-mediated autocrine IL-2 signaling for effector T cell survival in nonlymphoid tissue (29). We also conclude that effects of CXCL10 on T cell expansion in the DLNs are most likely direct and not a consequence of effects of CXCL10 at the tissue site.

$CD8^+$ T cells require CXCR3 expression to optimally profit from CD27/CD70 costimulation

Because CXCL10 exerts its functional effects via CXCR3, it followed from our observations that the effects of CD27/CD70 costimulation on $CD8^+$ T cells must rely, in part, on CXCR3 expression. To test this, we made use of CD11c-*Cd70* transgenic mice that constitutively express CD70 on DCs. When immunized with MHC class I–restricted peptide in PBS, a situation that is ordinarily tolerogenic, these mice mount a $CD8^+$ T cell response that is highly dependent on CD27/CD70 costimulation (26). Therefore, this model optimally reveals the contribution of CD27/CD70 costimulation during the priming of $CD8^+$ T cells. CD11c-*Cd70* transgenic mice are maintained on a *Cd27*$^{-/-}$ background to avoid the effects of transgenic CD70 on the endogenous T cells.

We previously used i.v. immunization with peptide; however, in this case, we aimed to examine effects in the DLNs. Therefore, we tested the model with s.c. peptide immunization. WT and *Cd27*$^{-/-}$ OT-I donor T cells were transferred at equal ratios into the same

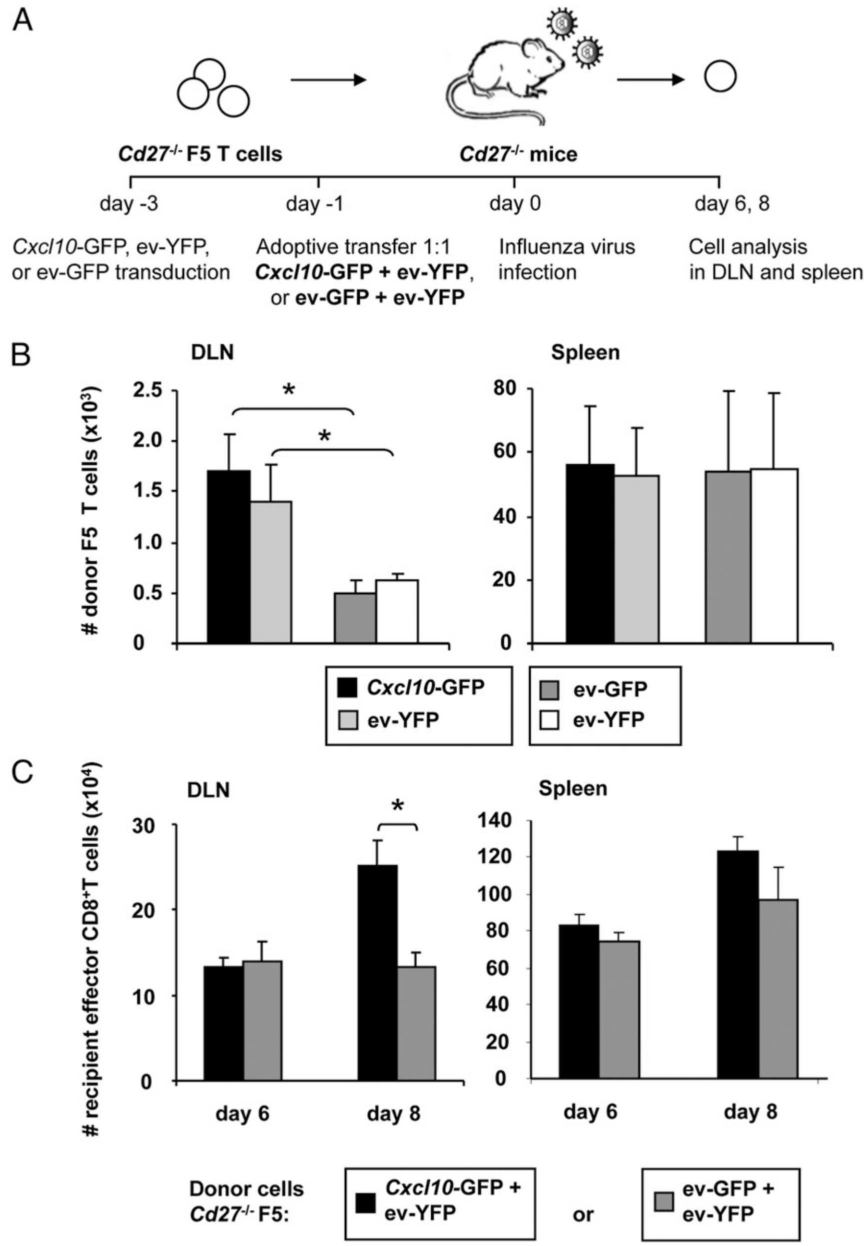

FIGURE 6. CXCL10 produced by virus-specific $CD8^+$ T cells acts in a paracrine fashion to promote the response of other virus-specific $CD8^+$ T cells. (**A**) Experimental design. *Cd27*$^{-/-}$ F5 T cells were retrovirally transduced with *Cxcl10*-IRES-GFP vector or empty vector (ev)-IRES-YFP and adoptively transferred into *Cd27*$^{-/-}$ recipient mice in a 1:1 ratio. Alternatively, *Cd27*$^{-/-}$ F5 T cells were retrovirally transduced with (ev)-IRES-GFP or (ev)-IRES-YFP and adoptively transferred into *Cd27*$^{-/-}$ recipient mice in a 1:1 ratio. Mice were infected with influenza virus; at day 6 or 8 postinfection, cells were harvested from DLNs and spleen, enumerated, stained, and analyzed by flow cytometry for expression of CD8, CD62L, GFP, and YFP. (**B**) The F5 T cell response at day 6 in the DLNs (*left panel*) and spleen (*right panel*): absolute numbers of GFP^+ or YFP^+ F5 T cells recovered from recipients of the indicated donor cell groups. (**C**) The recipient $CD8^+$ T cell response at days 6 and 8 in the DLNs (*left panel*), and spleen (*right panel*): absolute numbers of recipient $CD62L^-$ effector $CD8^+$ T cells recovered from recipients of the indicated donor cell groups. Data are mean + SEM of four mice/group. *$p < 0.05$, Student *t* test.

Cd27$^{-/-}$*;Cd70*tg recipient mice that were subsequently immunized s.c. with $OVA_{257–264}$ peptide in PBS in the left flank. The OT-I T cell response was read out over the next 13 d in blood (Fig. 7A). Only the CD27-proficient OT-I T cells were able to respond, thereby validating that the system is reliant on CD27/CD70 costimulation (Fig. 7B).

Next, WT and *Cxcr3*$^{-/-}$ OT-I T cell responses were compared in this setting by transfer of both cell types into the same *Cd27*$^{-/-}$*; Cd70*tg recipient mice. In the blood, the *Cxcr3*$^{-/-}$ OT-I T cell response was significantly lower than the WT OT-I T cell response, but it was higher than the response of *Cd27*$^{-/-}$ OT-I T cells (Fig. 7B). Moreover, in the left inguinal–DLN on day 7, the *Cxcr3*$^{-/-}$ OT-I T cell response was significantly lower than the WT OT-I T cell response (Fig. 7C). There was also a significant difference in the right inguinal lymph node, whereas the responses in the spleen were not significantly different. From these results, we conclude that, in a CD27/CD70-dependent response, the accumulation of Ag-specific $CD8^+$ T cells in the DLNs is partially dependent on their expression of CXCR3. Furthermore, these data support the idea that CXCL10, produced by $CD8^+$ T cells in response to CD27/CD70 costimulation, promotes effector $CD8^+$ T cell accumulation at the site of priming by acting directly on Ag-specific $CD8^+$ T cells.

Discussion

In this study, we found that CD27/CD70 costimulation induces *Cxcl10* gene transcription in mouse $CD8^+$ T cells. In response to CD27 triggering, $CD8^+$ T cells upregulated *Cxcl10* mRNA expression with rapid kinetics, both in the presence and absence of CD28 costimulation. Moreover, CD27 costimulation increased *Cxcl10* mRNA levels independent of new protein synthesis. This strongly suggests that *Cxcl10* is a CD27 target gene. CD27/CD70 costimulation also induced *Cxcl10* gene transcription and protein production in human $CD8^+$ T cells (data not shown). The conserved rapid upregulation of CXCL10 expression suggested an important contribution of this chemokine during the priming phase of the $CD8^+$ T cell response.

Cxcl10 gene transduction improved, but did not fully correct, the deficient clonal expansion of *Cd27*$^{-/-}$ F5 T cells in DLNs

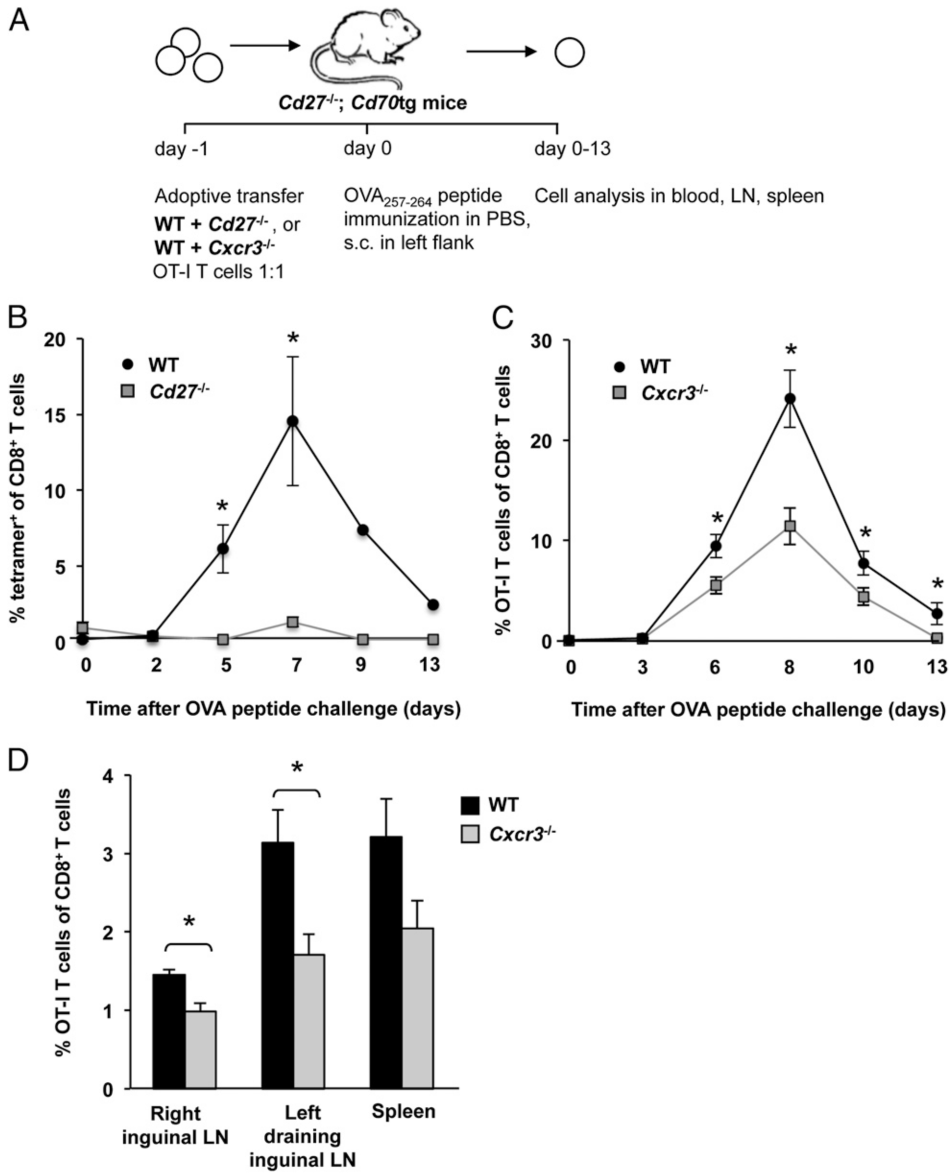

FIGURE 7. $CD8^+$ T cells require CXCR3 expression for clonal expansion in a CD27/CD70-dependent model of peptide immunization. (**A**) Experimental design. WT OT-I (CD45.1) and *Cd27*$^{-/-}$ OT-I (CD45.2) or WT OT-I (CD45.1) and *Cxcr3*$^{-/-}$ (GFP^+) OT-I T cells were adoptively transferred, at a 1: 1 ratio of 10,000 cells/genotype, into *Cd27*$^{-/-}$;*Cd70*tg recipient mice that transgenically express CD70 on steady-state DCs. Mice were challenged s.c. in the left flank with $OVA_{257–264}$ peptide in PBS 1 d later. OT-I T cell responses were read out subsequently in blood on days 0–13 and the left and right inguinal lymph node on day 7. (**B**) Comparison of WT and *Cd27*$^{-/-}$ OT-I T cell responses. At the indicated days after immunization, the percentages of $CD8^+$ H-$2K^b$/$OVA_{257–264}$ $tetramer^+$ OT-I T cells of WT ($CD45.1^+$) or *Cd27*$^{-/-}$ ($CD45.2^+$) origin were determined in the blood. (**C** and **D**) Comparison of WT and *Cxcr3*$^{-/-}$ OT-I T cell responses. (**C**) Percentages of WT ($CD45.1^+$) or *Cxcr3*$^{-/-}$ (GFP^+) $CD8^+$ OT-I T cells were determined in the blood at the indicated days after immunization (*right panel*). Data are mean + SEM of three mice/group, and the experiment is representative of two experiments. (**D**) On day 7, the percentages of WT ($CD45.1^+$) or *Cxcr3*$^{-/-}$ (GFP^+) $CD8^+$ OT-I T cells were determined in the left and right inguinal lymph nodes (LN) and in the spleen. Data are mean + SEM of five mice/group, and the experiment is representative of two experiments. *$p < 0.05$, Student *t* test.

upon in vivo challenge with influenza virus, in agreement with the known mechanism of action of CD27. CD27 can promote clonal expansion of $CD8^+$ T cells at the site of priming by survival signaling (25). This proceeds, at least in part, via Bcl-x_L and Pim-1, which exert cell-autonomous antiapoptotic and prometabolic effects (27). Accordingly, CD27 costimulation promoted the TCR/CD3-driven clonal expansion of primed $CD8^+$ T cells in vitro, independent of CXCL10. CD27 costimulation also supports the survival of effector $CD8^+$ T cells after the clonal expansion phase via autocrine IL-2 signaling (29). The *Il2* gene is the most important target gene of CD27 costimulation in primed $CD8^+$ T cells (29) (Supplemental Fig. 2). The IL-2 produced in response to CD27 costimulation does not contribute to clonal expansion of primed $CD8^+$ T cells in lymphoid organs, but it supports the survival of $CD8^+$ effector T cells in nonlymphoid tissue (29). In fact, the defective accumulation of *Cd27*$^{-/-}$ $CD8^+$ effector T cells in the lung of influenza-infected mice could be fully rescued by *Il2* gene transduction. This indicates that survival signaling via IL-2 is the main mechanism by which CD27 supports accumulation of $CD8^+$ effector T cells at the tissue site. In agreement, *Cxcl10* gene transduction could not rescue the survival defect of *Cd27*-deficient $CD4^+$ and $CD8^+$ T cells in the lung after influenza virus infection. Our current findings highlight that CD27 costimulation promotes the $CD8^+$ T cell response by effects on T cell survival, as well as by the induction of chemokine expression, which we propose serves to optimize the cellular niche for T cell priming.

Members of the TNFR family are generally implicated in survival signaling. Via TRAF adaptor molecules, CD27 and other TNFR family members link to the NF-κB–signaling pathway (37–40), which mediates expression of various antiapoptotic molecules (41). However, the NF-κB pathway can also induce the expression of chemokines, including CXCL10 (42, 43). From this perspective, it is perhaps not surprising that CD27 directs *Cxcl10* gene expression. In contrast, the only TNF family members that have been implicated in CXCL10 expression are TNF-α (44), CD40L (45, 46). and TWEAK (47), all in nonlymphoid cell types. The finding that a T cell costimulatory receptor induces chemokine expression during priming is novel and begs for a conceptual interpretation. Simultaneous induction of prosurvival molecules and chemokines by TNFR family members suggests that these receptors create specific cellular niches that may support cell proliferation and/or differentiation. Our data indeed suggest that the CXCL10 produced in response to CD27 costimulation acts in dedicated cellular niches. CXCL10 production by $CD8^+$ responder

T cells improved the virus-specific CD8+ T cell response in the DLNs but not in the spleen. We hypothesize that CXCL10 produced by responder CD8+ T cells can only impact on formation of the CTL effector pool by local effects in the lymph node microenvironment.

Chemokines play an important role in the lymph nodes. Under conditions of homeostasis, certain chemokines orchestrate the separation of T and B cells in different zones. Upon infection, de novo expressed chemokines and their receptors direct the migration of T and B cells to optimize specific cellular interactions (48). For instance, naive CCR7+ T cells remain in T cell zone under homeostatic conditions, but CCR4 receptor expression allows activated T cells to migrate toward newly arrived DCs in the paracortex (49). Along the same lines, Yoneyama et al. (20) found that CXCL10 recruits primed CD4+ T cells from the T cell zone to recently immigrated DCs in the paracortex of hepatic lymph nodes after i.v. immunization with heat-killed bacteria. They postulated that sustained contact of the already primed, but uncommitted, CD4+ T cells with the CXCL10-producing DCs in the paracortex of the lymph node allowed for expansion and full Th1 polarization. Indeed, a recent study proves this point (21). CXCR3 was upregulated on CD4+ T cells in DLNs during priming, prior to clonal expansion, and contributed to migration of Ag-specific CD4+ T cells from the T cell zone to the interfollicular and medullary regions, to interaction with DCs, and to Th1 differentiation. CXCL10 was expressed in the medulla of the DLNs and in scattered cells in the T cell zone, and CXCL10 produced by DCs and possibly other hematopoietic cells was required for optimal Th1 cell formation (21). These studies were the first to implicate cell migration directed by CXCL10 in the fate of Ag-specific T cells at the priming site, in this case CD4+ T cells. We now add that CXCL10 performs such a function for CD8+ T cells, in accordance with a recent finding that CXCL10 produced by DCs can support the generation of a CD8+ T cell response (50).

Interestingly, recent findings on the role of CXCR3 in fate determination of primed CD8+ T cells further support this notion (51, 52). CXCR3 binds CXCL9, CXCL10, and CXCL11, but C57BL/6 mice only express CXCL9 and CXCL10 (52). Therefore, findings on CXCR3 function in C57BL/6 mice reflect the role of one or both of these chemokines. Kurachi et al. (51) and Hu et al. (52) found that CXCR3 on CD8+ T cells promoted their commitment to an effector fate, rather than a memory fate. Both studies used acute infection with a systemic virus and monitored CD8+ T cell responses in the spleen. CXCR3 was rapidly upregulated on CD8+ T cells during the first few days of priming, and its expression affected the localization of CD8+ T cells within the spleen in the priming phase. WT CD8+ T cells were initially found in the T zone, but they relocalized to the marginal zone where they formed clusters with DCs. This redistribution was impaired when the CD8+ T cells lacked expression of CXCR3. It was suggested that this redistribution within the priming organ and the consequent longer or shorter contact with Ag-presenting DCs determined the long-term fate of the CD8+ T cells (51, 52).

Our data are in full agreement with the concept that CXCR3 and CXCL10 play a role in the localization of primed CD8+ T cells at the site of priming and, thereby, affect the CD8+ T cell response. CXCL10 produced by primed CD8+ T cells in response to CD27/CD70 costimulation may serve to attract recently primed CD8+, either to profit from each other's company or to profit from sustained colocalization with Ag-presenting DC, which support clonal expansion and effector cell formation. Our data indicate that primed CD4+ T cells can also profit from CXCL10 production by primed CD8+ T cells. Notably, gene-expression profiling also revealed that CD27 induced expression of CCL4 and XCL1 in primed CD8+ T cells (Supplemental Fig. 2), which suggests attraction of CD4+ T cells (53) and cross-priming DCs (54). This is in perfect agreement with known functions of CD27 in priming at the T cell–DC interface (26, 55) and orchestrating CD4+ T cell help for the CD8+ T cell response (29, 55, 56). We did not find a significant phenotype for CXCL10 or CXCR3 expression in the spleen, which is in line with the localized nature of Ag delivery, wherein the DLN is the first site that receives the Ag from the site of infection or immunization.

In conclusion, our data substantiate that CXCL10 plays a role in the priming phase of the T cell response. We present the novel concept that a T cell costimulatory receptor induces the synthesis of a chemokine to influence T cell fate. CXCL10 production by primed CD8+ T cells does not directly affect CD8+ T cell survival or proliferation, yet it impacts on effector T cell generation in vivo. These findings connect very well with the recently identified role for CXCR3 in effector development of CD8+ T cells and emphasize that chemokines can indirectly affect cell fate by orchestrating cell–cell communication.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

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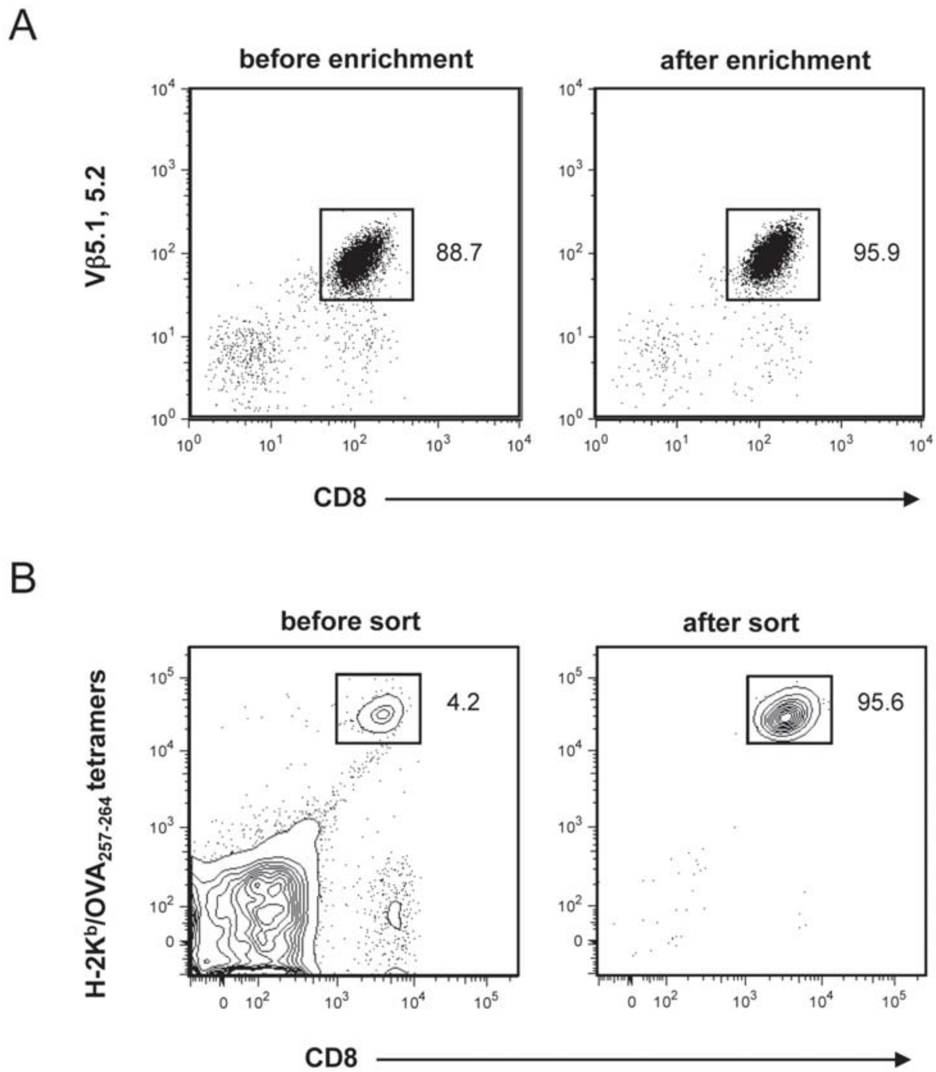
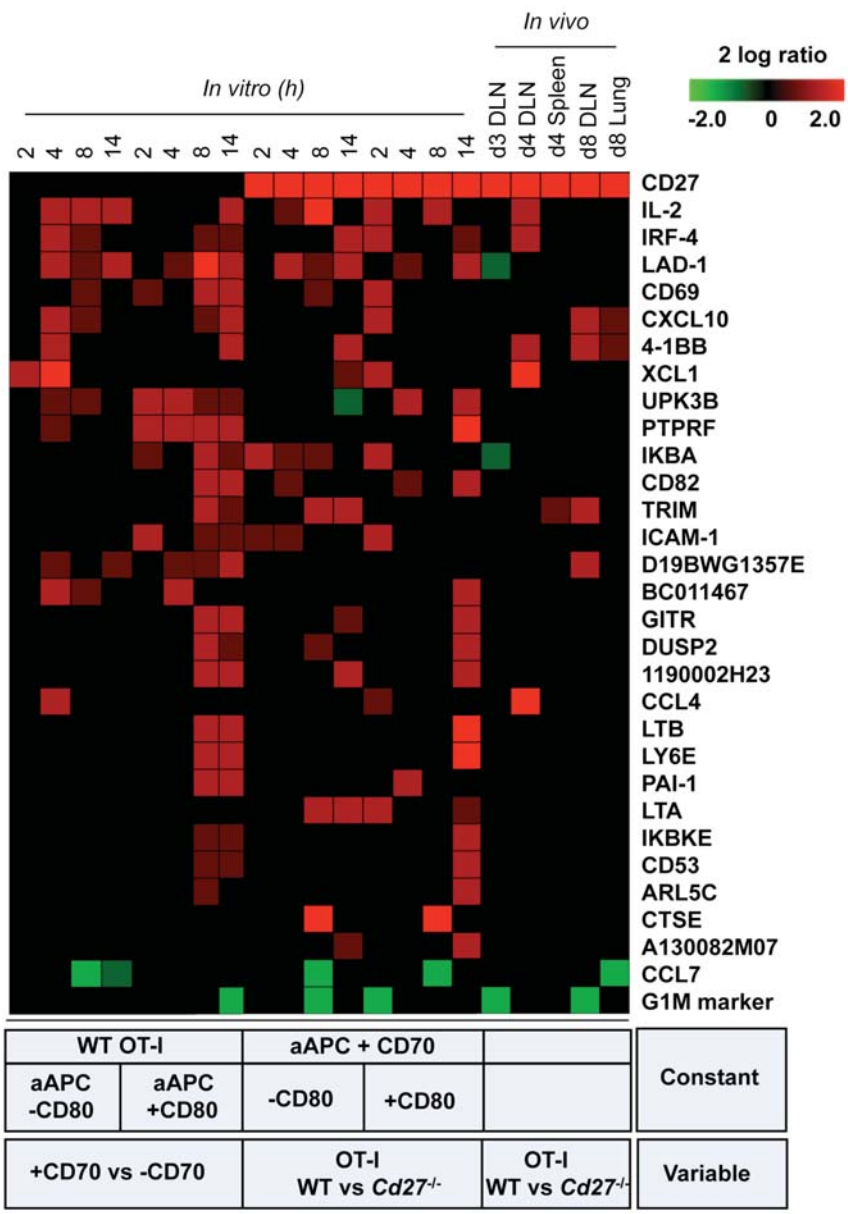
Supplementary Figure 1



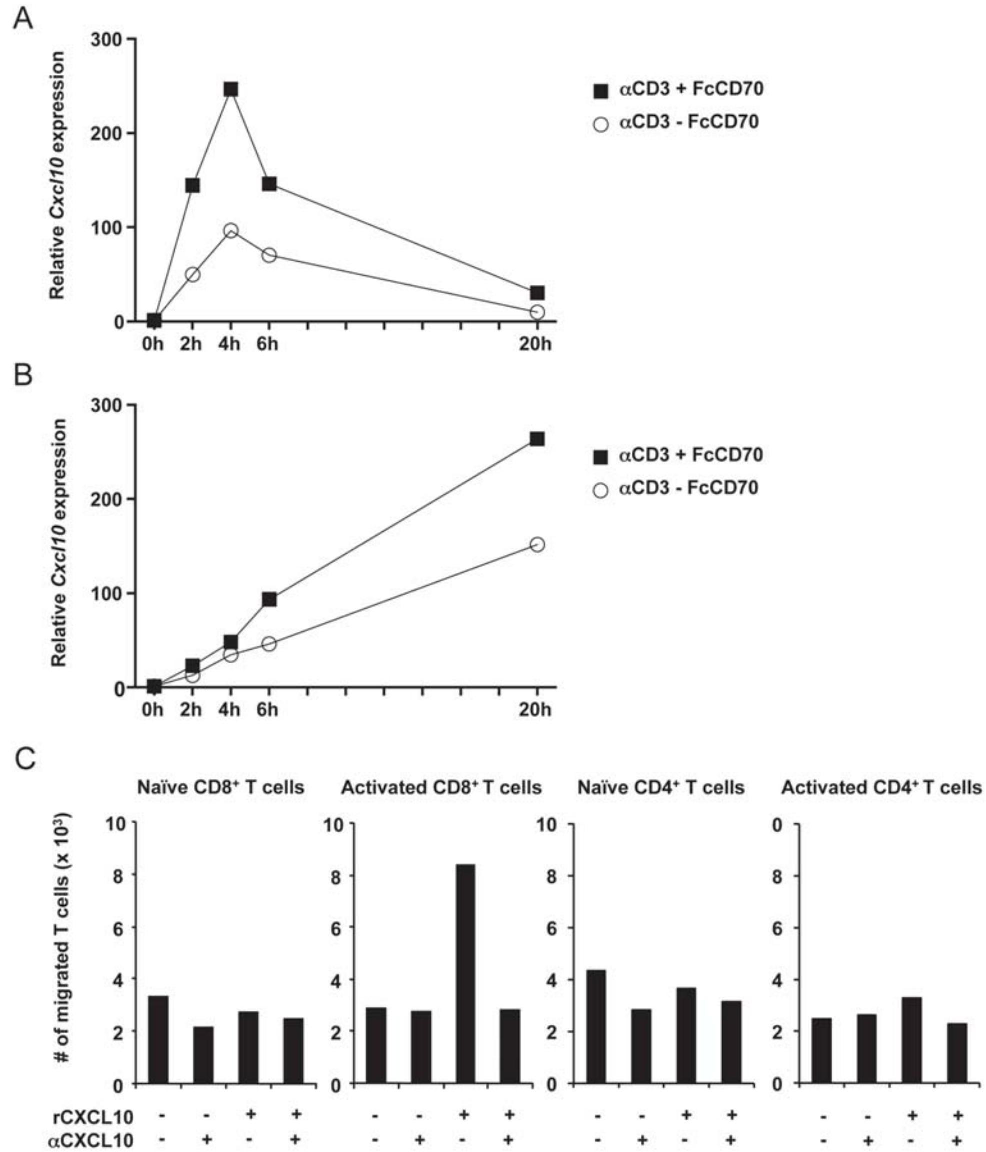
Supplementary Figure 1. Purity of the OT-I T cells used for gene expression profiling. (**A**) *In vitro* activation. OT-I T cells that had been purified as described (29) before adding them to aAPC were further purified after activation by aAPC and prior to gene expression profiling using MACS. OT-I cell purity was determined before and after this enrichment using anti-CD8 mAb and mAbs to Vβ5.1/5.2 to detect the OT-I TCR. Purity was always higher than 95% after enrichment. A representative analysis is shown. (**B**) *In vivo* activation. OT-I T cells were activated *in vivo* by immunizing OT-I mice with OVA protein. After 3, 4 or 8 days, the OT-I T cells were isolated from DLN, spleen or lung by flow cytometric sorting after staining with H-2K^b/OVA$_{257\text{-}264}$ tetramers and anti-CD8 mAb. The resulting purity after sorting was always greater than 95%. A representative analysis is shown of cells from spleen before and after sorting.

Supplementary Figure 2



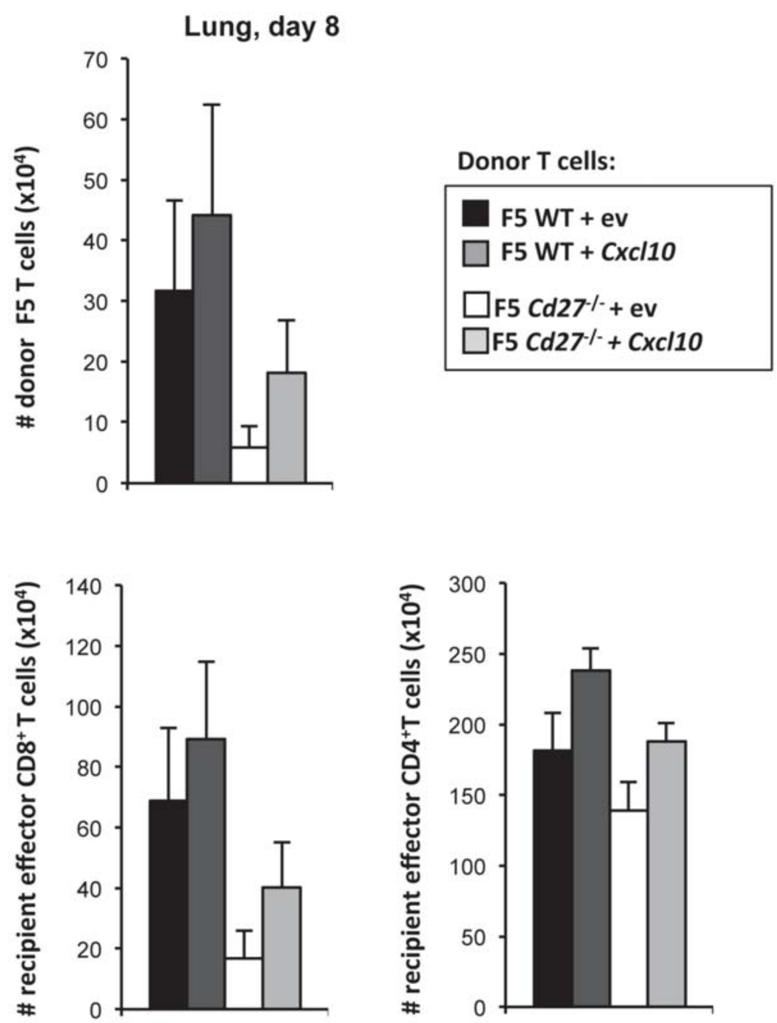
Supplementary Figure 2. The hitlist of CD27-regulated genes in primed CD8^{+} T cells. OT-I T cells were activated *in vitro* or *in vivo*, in the presence or absence of CD27/CD70 costimulation as outlined for Figure 1A. *In vitro*, OT-I T cells were stimulated for 2, 4, 8 or 14 h with aAPC that expressed CD80 (+) or not (-). WT OT-I responder T cells were kept constant and CD70 on aAPC was the variable, or CD70 on aAPC was kept constant and CD27 on WT versus *Cd27*$^{-/-}$ OT-I T cells was the variable. *In vivo*, WT- versus *Cd27*$^{-/-}$ OT-I mice were immunized with OVA protein and T cells were isolated on day 3, 4 or 8 from DLN, on day 4 from spleen and on day 8 from lung. The mRNA extracted from OT-I T cells in the comparative settings was co-hybridized on micro-arrays. A heat map was created to depict the hierarchy of genes differentially expressed with a *P* value below 0.00003 in at least two experimental settings in presence or absence of CD27/CD70 costimulation, according the mRNA signal intensity (^{2}log ratio) and the frequency of occurrence in the various experimental settings. Part of the top two rows of this figure was previously published in “CD27 sustains survival of CTLs in virus-infected nonlymphoid tissue in mice by inducing autocrine IL-2 production”, by Victor Peperzak, Yanling Xiao, Elise A.M. Veraar and Jannie Borst; *J Clin Invest.* 2010; 120(1):168–178 doi:10.1172/JCI40178

Supplementary Figure 3



Supplementary Figure 3. (A,B) Induction of *Cxcl10* mRNA expression by CD27 costimulation does not require new protein synthesis. Purified polyclonal $CD8^+$ T cells from WT mice (n=2, pooled) were stimulated *in vitro* at 500.000 per well in 24-well plates for the indicated periods of time (h) with 1 μg/ml anti-CD3 mAb 145.2C11 alone or in combination with 1 μg/ml FcCD70 in the absence (**A**) or presence (**B**) of 2 μg/ml cycloheximide (CHX). Cells were lysed in TRIzol (Life Technologies) and RNA was isolated according to the manufacturer's protocol. *Cxcl10* mRNA levels were determined by quantitative real time PCR from cell harvested at the indicated time points (h) of culture. *Cxcl10* mRNA levels were related to β–Actin (*Actb*) mRNA levels that were detected using the following primers *Actb* fwd GCTCTTTTCCAGCCTTCCTT *Actb* rev CTTCTGCATCCTGTCAGCAA. Values are means of triplicate samples. Data are representative of 2 independent experiments. (**C**) Validation of the CXCL10 transwell migration assay. Purified naïve or Con A-activated polyclonal $CD8^+$ or $CD4^+$ T cells from WT mice were used as responder cells in the upper chamber. The lower chamber contained medium alone (-), or medium with neutralizing anti-(α)CXCL10 mAb, recombinant (r)CXCL10, or the combination. Shown are the numbers of CD8$^+$ T cells that migrated through the membrane to the lower chamber. Data are means of triplicate samples and representative of 2 independent experiments.

Supplementary Figure 4



Supplementary Figure 4. Effects of reconstitution of the *Cxcl10* gene in *Cd27*$^{-/-}$ F5 T cells on T-cell responses in the lung. The experiment was performed as outlined in Figure 4, wherein influenza-specific F5 CD8$^+$ T cells were transduced with a vector encoding CXCL10 and GFP in an IRES configuration or with empty vector (ev) encoding GFP only mice. Sorted F5 donor T cells were transferred into *Cd27*$^{-/-}$ recipients. At day 8 after influenza virus infection, cells were harvested from lung, enumerated and analyzed by flow cytometry. Shown are absolute numbers (#) of GFP$^+$ CD8$^+$ F5 donor T cells, recipient CD62L$^-$ effector CD8$^+$ T cells and recipient CD62L$^-$ effector CD4$^+$ T cells in mice that had received the indicated donor T cells. Data are mean values of 4 mice per group (+ SEM). Student's *t*-test indicated no significant differences between the indicated groups. The data shown are representative of 3 independent experiments.