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IL-12 Produced by Dendritic Cells Augments CD8+ T cell Activation through the Production of the Chemokines CCL1 and CCL17[1]

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Abstract

Interleukin-12 (IL-12) family members are an important link between innate and adaptive immunity. IL-12 drives Th1 responses by augmenting IFN-γ production, which is key for clearance of intracellular pathogens. Interleukin-23 (IL-23) promotes the development of IL-17 producing CD4 + T cells that participate in the control of extracellular pathogens and the induction of autoimmunity. However, recent studies have shown that these cytokines can modulate lymphocyte migration and cellular interactions. Therefore, we sought to determine the individual roles of IL-12 and IL-23 in naïve CD8+ T cell activation by addressing their ability to influence interferon gamma (IFN-γ) production and cellular interaction dynamics during priming by *Listeria monocytogenes* (*Lm*)-infected dendritic cells (DC). We found that IL-12 was the major cytokine influencing the level of IFN-γ production by CD8+ T cells while IL-23 had little effect on this response. In addition, we observed that IL-12 promoted longer duration conjugation events between CD8+ T cells and DC. This enhanced cognate interaction time correlated increased production of the chemokines CCL1 and CCL17 by wild-type but not IL-12 deficient DC. Neutralization of both chemokines resulted in reduced interaction time and IFN-γ production demonstrating their importance in priming naïve CD8 + T cells. Our study demonstrates a novel mechanism through which IL-12 augments naïve CD8+ T cell activation by facilitating chemokine production thus promoting more stable cognate interactions during priming.

Keywords

T cells; Dendritic Cells; Cytokines; Chemokines; Cell Activation

Introduction

It is becoming increasingly clear that the initial signals CD8+ T cells receive during priming impacts the subsequent primary and secondary responses generated in these cells (1–13). Dendritic cells (DC) efficiently deliver the required signals to activate naive CD8+ T cells. Without DC, adaptive immune responses against several pathogens are not elicited (14–17). Their potent ability to stimulate naive T cells depends on their maturation state (14,17). Mature

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DC will present several activating ligands or secreted factors to naïve CD8+ T cells during priming such as peptide-MHC complexes (signal 1), costimulatory molecules (signal 2), and cytokines (signal 3) (18–20). CD8+ T cells encountering mature DC engage in long duration interactions resulting in the generation of highly functional effector cells (21). However, an immature DC has a reduced capacity to prime naïve CD8+ T cells which correlates with shorter duration interactions during activation (21). The full complement of factors delivered by DC that promote long term physical interactions leading to the efficient priming of naïve CD8+ T cells remains to be determined.

Interleukin-12 (IL-12) is produced by mature DC in response to infection by various intracellular pathogens (14,22). Due to the potent effects that IL-12 has on T cell activation, it is largely viewed as an important bridge between the innate and adaptive immune systems (23–29). This cytokine influences several aspects of T cell activation, most notably interferon gamma (IFN-γ) production, which plays a key role in the resolution of intracellular pathogens such as *Listeria monocytogenes* (*Lm*) (30,31). Extensive research has been performed on *Lm*, and the immune responses required for resolving infection are well documented (32). IL-12 is required to resolve high-dose infection; however in the absence of IL-12, increased IFN-γ can compensate (30,33,34). Direct infection of DC with *Lm in vitro* results in production of p40, a cytokine subunit shared by IL-12 and IL-23 (14). Furthermore, neutralization of p40 during priming of CD8+ T cells by *Listeria*-infected DC results in a significant decrease in the amount of IFN-γ produced by these cells (14). IFN-γ plays multiple roles in resolving listerial infection including activating macrophages and increasing target cell sensitivity to lysis by CD8+ T cells (8,30,35,36). Thus, IL-12 production is a key hallmark of resolving *Lm* infection through its augmentation of IFN-γ production.

The IL-12 family is composed of the cytokines IL-12, IL-23, and IL-27 (24–29,37–39). They are grouped together based on structural similarities between the cytokines and/or their receptors (24–29,37–39). Interleukins-12 and 23 share the p40 subunit (24–29,37–39). However, p35 is exclusively associated with IL-12, and a heterodimer of p19/p40 makes up IL-23 (24–29,37–39). The distinct biological functions of each cytokine in this family may be attributed to differences in the composition of their receptors and distinct sets of downstream transcription factors that are activated upon ligation (24–29,37–39). The main role attributed to IL-12 is the ability to induce and augment IFN-γ production by CD4+ T cells (driving a Th1-type response) as well as NK and CD8+ T cells (24–29,37–39). IL-12 has been shown to increase CD8+ T cell cytolysis, survival, proliferation, and influence the ability of these lymphocytes to migrate to inflammatory foci (24–29,37–39). A more recently recognized activity of IL-23 is the ability to stimulate the production of IL-17 by CD4+ T cells. These Th17 cells are critical for the development of autoimmune diseases and immunity to certain extracellular pathogens (24–29,37–45). However, the role of IL-23 in the activation of naïve CD8+ T cells is still being investigated.

Since IL-12 and IL-23 have very potent effects on specific immune responses, we wanted to determine the individual contributions of IL-12 and IL-23 on CD8+ T cell priming by dendritic cells. In addition, we wanted to identify the mechanism(s) through which these cytokines modulated naïve CD8+ T cell activation. To approach these goals we employed DC generated from mice lacking both IL-12 and IL-23 (p40−/−) or only IL-12 (p35−/−). We found that IL-12, but not IL-23, augmented IFN-γ production by CD8+ T cells. We also found that IL-12 promoted longer duration interactions between CD8+ T cells and *Listeria*-infected DC. To address the potential mechanisms through which IL-12 regulated T cell-DC interactions, we examined chemokine production in the presence or absence of IL-12. The production of CCL1 and CCL17 was increased by DC in the presence of IL-12. Further studies determined that these chemokines directly increased the duration of conjugation as well as IFN-γ production by CD8+ T cells.

Materials and Methods

Mice

C57BL/6, IL-12p35−/−BL/6-IL12a$^{tm/Jm}$, IL-12p40−/−BL/6-IL12b$^{tm/Jm}$, B6.129S1 *Il12rb2*^{tm1Jm}/J, OT-1 and OT-2 TCR transgenic mice specific for OVA (257–264) presented by 2K^b and OVA$_{(329–337)}$ presented by I–A^b were purchased from The Jackson Laboratory (Bar Harbor, ME). P14 TCR transgenic mice specific for the LCMV glycoprotein (GP) peptide 33–41 were kindly provided by Dr. Jason M. Grayson (Wake Forest University School of Medicine, Winston-Salem, NC). All mice were maintained and bred in the animal facility at Wake Forest University School of Medicine.

Antibodies

Neutralizing Antibodies—To neutralize the activity of IL-12 and IL-23, anti-p40 (clone C15.6; Biosource International, Camarillo CA) or just IL-23, anti-p19 (clone G23-8, eBioscience, San Diego CA) neutralizing antibodies (10 μg/ml) were added prior to T cell addition and maintained throughout the assay unless otherwise indicated. Neutralizing antibodies against the chemokines TARC/CCL17 (clone 110904; R & D Systems, Jamul CA) and TCA-3/CCL1 (Cat. No. AF845; R & D Systems, Jamul CA) were added at 10μg/ml prior to T cell addition and maintained throughout the assay.

Surface and Intracellular Cytokine Staining Antibodies—Fluorescent antibodies measuring the expression of the mouse DC costimulatory molecules CD40 (Clone 3/23), CD80 (clone 16-10A1), and CD86 (GL1) and the phenotypic marker, CD11c were purchased from BD Biosciences (San Diego, CA). Interferon gamma production by CD8+ OT-1 T cells was measured using fluorescent antibodies to CD8 (clone 53–6.7; BD Pharmingen, San Diego CA) and IFN-γ APC (clone XMG1.2; BD Pharmingen, San Diego CA).

Dendritic Cell Propagation

Bone marrow-derived DC were generated as previously described (14). Briefly, bone marrow was removed from the tibias and femurs of 8- to 10-week-old C57BL/6 (or strain indicated) mice. Red blood cells were lysed, and the progenitor cells (5×10^5/ mL) were resuspended and plated in RPMI 1640 containing 10% FCS supplemented with 10 ng/mL GM-CSF (generated from a recombinant baculovirus expression system). Dendritic cells were cultured for 6 days at 37°C in 5% CO_2 and given fresh medium and cytokine on days 2 and 4. DC used for the described experiments were between 90–95% CD11c+ and expressed low levels of CD40, CD80, and CD86 which are characteristic of immature DC (data not shown).

In vivo Infection of mice

C57BL/6 (Wild type) or IL-12 deficient (p35−/−) DC were mock treated (PBS only) or infected with 1 LD_{50} of *Lm* strain 10403S delivered intravenously (5×10^5). At 18 hours post infection, mice were sacrificed and inguinal, lumbar, and mesenteric lymph nodes as well as spleens were harvested. RNA was isolated for chemokine analysis from DC following enrichment for CD11c + cells.

In vitro Infection of DC

For T cell priming assays, DC were seeded at 2×10^4 per well and infected with wild-type *Listeria* (strain 10403S) at a multiplicity of infection (MOI) of 1. Four hours post infection, OVA peptide$_{257–264}$ was added at a concentration of 0.1 ng/mL (or as indicated) with chloramphenicol (10μg/mL) and gentamicin (10μg/mL). Twenty-four hours post infection OT-1 were added at 10:1 T to DC. For some experiments OT-1 T cells were stained with CFSE prior to culture with DC.

Time-Lapse Video Microscopy

DC were prepared and infected as in T cell priming assays with the following modifications. Day 6 wild-type, IL-12p35−/−, or IL-12p40−/− dendritic cells (10^6) were seeded in T25 flask and infected with *L. monocytogenes* at an MOI of 1. After four hours antibiotics were added (chloramphenicol and gentamicin, 10 μg/mL), and ovalbumin peptide was added at a concentration of 0.1 ng/mL. At 24 hours post infection, OT-1 T cells were added to the flask at a ratio of 10:1 T cells to DC. For neutralization experiments, isotype control protein or neutralizing antibodies to CCL1, CCL17, or IL-12/23 p40 were added to the flask at a concentration of 10 μg/mL. The total time of conjugation between individual CD8+ T cells and DC was determined by measuring the ligation (on) and detachment (off) times of a T cell with DC conjugate. At least 50 interactions per experiment were monitored. Interaction times are expressed as dissociation curves between T cell and DC with the percent of T cells in conjugate versus total time of conjugation with DC (in minutes). A more gradual slope indicates longer duration interactions between a population of T cells and DC. Time-lapse phase contrast images were recorded with an exposure time of 5 second frame intervals using an Olympus 1×70 (Olympus America, Inc., Melville, NY) enclosed with an incubation chamber set at 37° C. Video recordings were captured over a 48 hour period.

ELISAs

Culture supernatants from either DC infection/maturation assays or T cell priming assays were collected at the indicated times. These supernatants were then probed for the presence of the following cytokines using ELISA kits according to the manufacturer's instructions: TCA3 (CCL1) and TARC (CCL17) (DuoSet® ELISA, R & D System, Minneapolis MN), IL-12p40 (OptEIA kit, BD Biosciences), IL-23 (eBioscience), IFN-γ (OptEIA kit, BD Biosciences).

Cytokine/Chemokine Protein Array

Wild-type, IL-12p35−/−, and IL-12p40−/− were infected with *L. monocytogenes* at an MOI 1. Twenty-four hours post infection, supernatants were harvested and probed for various cytokines and chemokines according to the manufacturer's instructions (Raybiotech Mouse Cytokine Antibody Array 3).

Real-Time Reverse Transcriptase PCR

Real-Time PCR was performed according to the manufacture's instructions (Applied Biosystems; Forster City, CA). Briefly, RNA was isolated from bone-marrow derived DC or DC enriched from the spleens and lymph nodes of *Lm*-infected mice using the RNAqueous Kit (Ambion, Austin TX). Once isolated, 20 ng of RNA of each sample was used in each reaction. Primer and probe sets used to detect CCL1, CCL17, and ActB messages were purchased from Applied Biosystems (Forster City, CA). Taqman Universal PCR Master Mix was purchased from Applied Biosystems (Forster City, CA), and nuclease free water was purchased from Ambion (Austin, TX). The reverse transcriptase used in these studies was MMLV-RT which was purchased from Invitrogen (Carlsbad, CA). The reactions were performed and analyzed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems; Forster City, CA).

Column Enrichment of Ex Vivo DC

Dendritic cells were enriched (CDllc+ selection) from the spleens and lymph nodes of *Lm*-infected mice according to the manufacturers instructions (Miltenyi Biotec, Auburn, CA). LS columns were used for the spleen and MS columns were used for the lymph nodes.

Results

IL-12, but not IL-23 Significantly Augments IFN-γ Production by CD8+ T cells

Previous work from our lab and others has shown that IL-12 production increases IFN-γ production by CD8+ T cells (3,14,28,29,46–48). In addition, it has been reported that IL-12 augments the proliferative capacity of these cells (3,18,19). These conclusions were drawn primarily from neutralization studies in which the p40 subunit of the cytokine was targeted; therefore, the individual contributions of IL-12 or IL-23 to these responses could not be distinguished. To address the relative contributions of IL-12 and IL-23 to CD8+ T cell activation, we primed naïve CFSE-stained OT-1 T cells with either wild-type, p35−/− (no IL-12), or p40−/− (no IL-12 or IL-23) DC. Prior to incubation with T cells, DC were infected with *Listeria* to induce cytokine production and were pulsed with OVA peptide. Infection of DC with *Listeria* resulted in low levels of IL-23 production from wild-type (53 pg/mL) and p35−/− DC (44 pg/ mL), but not from p40−/− DC (7 pg/mL vs. 10 pg/ mL produced by uninfected DC; data not shown).

On day 3 of priming, CD8+ T cell IFN-γ production and proliferation were measured (Figure 1A). Proliferation of CD8+ T cells was not influenced by IL-12 or IL-23. However, in the absence of IL-12, we observed a significantly decreased the level of IFN-γ produced on a per cell basis (2-fold reduction in mean fluorescence intensity, MFI). In the absence of both IL-12 and IL-23 (p40−/−) we observed the most pronounced decrease in the IFN-γ MFI; yet, it was not significantly less than that observed in the absence of IL-12 (p35−/−) alone (Figure 1B). In addition, the amount if IFN-γ secreted by CD8+ T cells over the three day priming period was reduced 3-fold compared to wild-type in the absence of IL-12 (Figure 1C). Again, we observed no significant difference between the p35- and p40-deficient DC in their ability to influence IFN-γ secretion over this period.

To more specifically address the role of IL-23, this cytokine was neutralized using an anti-p19 antibody. IL-23 neutralization did not significantly decrease OT-1 IFN-γ production on a per cell basis when primed by WT DC (control IFN-γ MFI 262, anti-p19 IFN-γ MFI 271, figure 1D). In contrast, when both IL-12 and IL-23 was neutralized (anti-p40), IFN-γ production by OT-1 was significantly decreased (control IFN-γ MFI 262, anti-p40 IFN-γ MFI 74.6). Neutralization of IL-23 alone or IL-12 and IL-23 in combination did not alter proliferation (Figures 1D and 1E). In addition, these results suggest that IL-23 does not augment IFN-γ production by CD8+ T cells and confirms that IL-12 is critical mediator of this process.

We next wanted to address the possibility that these observations could be attributed to changes in costimulatory molecule expression or antigen presenting capacity of DC in the absence of IL-12 or IL-12/23. So, we measured the upregulation of costimulatory molecules (CD86, CD80, and CD40) and the antigen presentation capacity of all DC was determined by an OVA-specific T cell hybridoma, B3Z following listerial infection. We observed similar levels of costimulatory molecule upregulation and antigen presentation by *Lm*-infected wild-type, p35 −/−, and p40−/− DC (data not shown).

IL-12 Promotes Long Duration Interactions between CD8+ T cells and Lm-infected DC

Several studies have monitored the dynamics of interactions between T cells and antigen presenting cells to determine how these interactions impact T cell proliferation and function (21,49–51). What has become clear is that full activation of naïve CD8+ T cell requires long duration interactions with DC (21). Since IL-12 production peaks within 12 hours of listerial infection and is known to influence lymphocyte migration during inflammation (52,53), we decided to test the effect of IL-12 on the duration of interactions between CD8+ T cells and DC. Dendritic cells were infected in a flask for four hours at which time OVA peptide and

antibiotics were added. Twenty hours post-infection, anti-p40 neutralizing antibodies were added to the culture. After 2 hours, OT-1 T cells were added to the culture. Time lapse video microscopy was then performed over a 48 hour period, and the on/off times of individual T cell/DC interactions were measured. Wild-type DC promoted long duration interactions with CD8+ T cells with 50% of the interactions lasting 127 minutes (Figure 2A). Only transient interactions occurred in the absence of antigen. Neutralization of the p40 subunit of IL-12 and IL-23 significantly reduced the conjugation time between CD8+ T cells and DC with 50% of the interactions lasting only 27 minutes (Figure 2A).

In order to determine the relative contributions of IL-12 and IL-23 to this interaction, CD8+ T cells were primed by wild-type, p35−/−, or p40−/− DC and the duration of conjugation was determined as described above. Here we also observed that in the absence of IL-12 and IL-23 (p40−/−), the majority of interactions were of short duration with 50% of the interactions lasting only 16 minutes (Figure 2B). Again, there was no significant difference in the duration of interaction in the absence of IL-12 (p35−/−) versus both IL-12 and IL-23 (p40−/−). This observation indicated that IL-12 could promote long duration interactions between CD8+ T cells and DC, and that IL-23 did not significantly impact this interaction.

IL-12 augments CCL1 and CCL17 production by Lm-infected DC

IL-12 is not widely regarded as a chemotactic mediator, yet we observed enhanced cell-cell interaction when it was present. Therefore, we decided to test if IL-12 affected the expression of chemokines by DC. To identify candidate chemokines produced by DC in an IL-12 dependent manner, we employed the use of a cytokine/chemokine protein array. Wild-type, p35−/−, and p40−/− DC were infected with *Lm* at an MOI of 1, and 24 hours post infection the culture supernatants were subjected to this multiplex analysis. Interestingly, expression of the two chemokines, CCL1 and CCL17, was markedly reduced in the absence of IL-12 as well as IL-12/23 (data not shown). However, the lack of IL-12 and IL-23 did not appear to significantly alter the production of other chemokines as strongly, including MCP-1, MIP-1α, MIP-1γ, or RANTES (data not shown).

We next wanted to more quantitatively measure the induction of CCL1 and CCL17 by DC in the presence or absence of IL-12 or IL-23. As a first step, RNA was isolated from either wild-type, p35−/−, p40−/−, or DC lacking the IL-12 receptor β2 chain (IL-12R−/−) at 24 hours post infection with *Lm*. Real-time reverse transcriptase PCR analysis was performed to measure these chemokine messages. We found that CCL1 message was significantly reduced in the absence of IL-12, IL-12/23, and the IL-12R (all four-fold reduced) compared to the levels found in wild-type DC (Figure 3A). We also observed reduced CCL17 message in the absence of IL-12, IL-12/23, and the IL-12R (eight-fold reduced) compared to the levels generated in *Lm*-infected wild-type DC (Figure 3A). Taken together, these results indicate that IL-12 (but not IL-23) enhances the production of CCL1 and CCL17 message by *Lm*-infected DC and that this enhancement is dependent on signaling through the IL-12 receptor.

To more directly determine the role of IL-12 in augmenting CCL1 and CCL17 protein production, ELISA analysis was performed on the supernatants of WT or p35−/− DC infected with *Listeria* at 24 hours post infection. We found that p35−/− DC produced significantly less CCL17 (three-fold reduction) at 24 hours post infection than wild-type DC (Figure 3B). The amount of CCL1 produced by p35−/− DC was also reduced by two-fold compared to WT DC (Figure 3C); however, CCL1 secretion was very modest overall. Additionally, we found significant reductions in secreted CCL17 (three-fold) and CCL1 (greater than two-fold) if DC lacked expression of the IL-12 receptor (Figures 3B and 3C). Importantly, addition of recombinant IL-12 to *Lm*-infected p35−/− DC restored CCL1 and CCL17 production to levels comparable to wild-type DC (Figures 3B and 3C), further demonstrating that this is an IL-12-dependent enhancement. In contrast, the addition of recombinant IL-23 to *Lm*-infected wild-

type or IL-12 deficient DC did not alter CCL1 or CCL17 production (data not shown). Interestingly, the expression of the chemokine receptors for CCL1 (CCR8) or CCL17 (CCR4) by CD8+ T cells remained unchanged whether IL-12 was added exogenously or provided by DC (data not shown). These results reveal that IL-12 augments the production of CCL1 and CCL17 by *Lm*-infected DC at both the RNA and protein levels.

Neutralization of CCL1 and CCL17 significantly reduces the duration of interaction between CD8+ T cells and Lm-infected DC

In order to determine if the chemokines CCL1 and CCL17 affect the physical interaction between T cells and DC, time lapse video microscopy was used to measure the duration of interaction between T cells and DC in the presence of neutralizing antibodies against CCL1 and CCL17. The neutralization of these chemokines resulted in a significant decrease in the duration of conjugation between the OT-1 CD8+ T cells and DC compared to control samples (Figure 4A). To determine if this phenomenon could be generalized to other CD8+ T cells, not just OT-1, we included an additional source of naïve CD8+ T cells, the P14 TCR transgenic cells, specific for the LCMV peptide, $GP_{(33–41)}$ presented by $H\text{-}2D^b$. As in the case of the OT-1, we also observed a significant decrease in the duration of interaction of the P14 T cells with DC in the absence of CCL1 and CC17 (Figure 4B). Thus, the diminished T cell/DC interaction time observed in the absence of IL-12 seems to correlate with similar decreases in interaction times observed when these two chemokines are neutralized.

Neutralization of CCL1 and CCL17 significantly reduces the amount of IFN-γ produced by CD8+ T cells

Based on the observation that IL-12 augmented the production of the chemokines CCL1 and CCL17, and that neutralization of these chemokines reduces T cell/DC interaction time, we decided to determine if the neutralization of these chemokines also affected IFN-γ production by CD8+ T cells. OT-1 and P14 CD8+ T cells were primed by DC in the presence of the indicated neutralizing antibodies (Figures 4C and 4D). The neutralization of CCL1 or CCL17 alone resulted in only a modest (not statistically significant) reduction in the amount of IFN-γ produced on a per cell basis by OT-1 and P14 T cells (Figures 4C and 4D). However, the neutralization of CCL1 and CCL17 in combination did significantly reduce IFN-γ production by both T cell populations (two-fold less than the control). These observations indicate that CCL1 and CCL17 together augment IFN-γ production by CD8+ T cells but that neither one alone significantly impacts this response.

Neutralization of CCL1 and CCL17 does not reduce IFN-γ by CD4+ T cells

To determine if CCL1 and CCL17 also augmented IFN-γ production by CD4+ T cells, OVA-specific, OT-2 transgenic CD4+ T cells were primed by *Lm*-infected DC in presence of neutralizing antibodies against CCL1, CCL17, or both. Parallel experiments with OT-1 T cells were used as positive controls. On day 3 of priming, IFN-γ production was measured by ELISA. We found that neutralization of IL-12/23 (anti-p40) significantly reduced IFN-γ production by both OT-1 and OT-2 T cells (Figures 5A and 5B). IFN-γ production by CD8+ T cells was also significantly reduced when CCL1 and CCL17 were neutralized in combination. However, the single or double neutralization of these chemokines did not significantly alter IFN-γ production by CD4+ T cells. These results indicate that unlike CD8+ T cells, CD4+ T cell production of IFN-γ is not augmented by CCL1 and CCL17.

Interleukin-12 Augments CCL1 and CCL17 Production from DC Induced by listerial Infection in Vivo

Having determined that CCL1 and CCL17 production was enhanced by IL-12 *in vitro*, we wanted to determine if this enhancement was also observed *in vivo*. To address this question,

wild-type or IL-12 deficient (p35−/−) mice were intravenously infected with *Lm* (1 LD_{50}). At 18 hours post infection lymph nodes (pooled mesenteric, inguinal, and lumbar) were harvested. Dendritic cells were enriched from these organs using CD11c+ positive selection (cells were >85% CD11c+). Once isolated, we purified RNA and determined the message levels for CCL1 and CCL17 using real time PCR.

We observed increased message levels for CCL1 and CCL17 in DC isolated from *Lm*-infected wild-type and p35−/− mice compared with mock treated mice (Figure 6). As a normalization control, actin message levels were measured and were found to be equivalent in DC isolated from infected wild-type and p35−/− mice (data not shown). In *Lm*-infected mice, we observed a greater than six-fold decrease in the message levels for CCL17 and a four-fold decrease in CCL1 levels in DC isolated from the lymph nodes of IL-12 deficient mice compared with wild-type mice (Figure 6). These data indicate that CCL1 and CCL17 production in response to listerial infection in vivo is enhanced by IL-12 to at least the same extent as that observed in vitro.

Discussion

The ability of IL-12 to promote strong Th1 responses by enhancing IFN-γ production from various lymphocytes is well documented (25,27–29,38). However, its ability to influence other parameters of T cell activation, such as the physical interactions between immune cells during priming, has not been well characterized. To our knowledge, this is the first report which shows that IL-12 influences the interactions between CD8+ T cells and dendritic cells during priming and points to a mechanism through which this effect is mediated. In our study, we found that IL-12 produced by DC augmented the production of the chemokines CCL1 and CCL17. These chemokines were found to promote longer duration interactions between CD8+ T cells and *Listeria*-infected DC. These longer duration interactions correlated with increased IFN-γ production by CD8+ T cells. Finally, neutralization of CCL1 and CCL17 resulted in a significant decrease in the production of IFN-γ and in the duration of cognate interaction. Taken together, our findings highlight a novel mechanism in which IL-12 enhances chemokine production, prolonging T cell-DC interactions, and increasing the production of IFN-γ by T cells.

It has been shown that IL-12 can influence T cell migration, based on observations that IL-12-conditioned CD8+ and CD4+ T cells are more responsive to chemokine stimulation (52). Therefore, an alternative explanation to our observed findings was that IL-12 influenced the expression of adhesion molecules or chemokine receptors expressed on CD8+ T cells. In order to address this possibility we primed T cells by *Listeria*-infected wild-type, p35−/−, or p40−/− DC for a period of three days. The surface expression of CCR8 and CCR4 (the receptors for the chemokines CCL1 and CCL17 respectively) as well as CCR5 and CXCR4, two chemokines known to be important for the development of T cell responses (52,54–59) were monitored. We found no difference in the expression of these molecules over the three day priming period, regardless of the presence of IL-12. Additionally, expression of CD11a, CD69, and CD44, known mediators of adhesion during T cell activation (3,60–65), were determined on CD8+ T cell on day 1, day 2, and day 3 of priming by flow cytometry. The expression levels of these molecules were also not different in the presence or absence of IL-12 or IL-23 (data not shown). In addition, we analyzed if IL-12 increased the surface expression of ICAM-1 on *Listeria*-infected DC. Wild-type, p35−/−, or p40−/− DC were infected with *Lm* and on days 1, 2, and 3 post infection the surface expression of ICAM-1 was measured via flow cytometry. The expression of ICAM-1 was not influenced by IL-12 or IL-23 (data not shown).

It has been determined that long duration interactions between CD8+ T cells and DC precedes effective activation of these cell *in vivo* (21). The majority of these extended interactions occur

within the initial 48 hours of encounter (21). This observation correlates with our results, which illustrate that the expression of the IL-12 receptor by CD8+ T cells peaks within the first two days of priming and is down-regulated after this period (data not shown). These observations may explain why CD8+ T cells are more sensitive to IL-12 within the first two days of activation when T cells are engaged in long duration interaction with DC.

The roles of CCL1 and CCL17 in immune responses are only beginning to be delineated, yet several recent reports indicate that these chemokines play important roles in T cell activation. Following *in vivo* infection with *Lm*, Alferink *et al* observed an accumulation of CCL17-producing DC in the lymph nodes and nonlymphoid organs of infected mice (66). In addition, BMDC producing CCL17 were better stimulators of naïve CD4+ T cell proliferation and IFN-γ production *in vitro*. Mice deficient in CCL17 production exhibit delayed allograft rejection and reduced hypersensitivity responses *in vivo*. Based on the localization of CCL17- producing DC and their ability to promote Th1 immune responses by CD4+ T cells, these DC are likely to be critical in the induction of anti-listerial CTL responses. Additionally, patients with atopic dermatitis were found to have significantly higher CCL1 levels when compared with the levels found in normal skin and other skin diseases (67). This chemokine, along with CXCL12, induced migration of T cells and DC, providing a potential mechanism that would be important in linking innate and adaptive immune responses, perhaps resulting in disease progression (67). Thus, our observation that these chemokines enhance IFN-γ production by CD8+ T cells adds to the growing body of literature demonstrating the importance of these chemokines in the development of T cell responses.

Interestingly, while CCL1 and CCL17 were shown to regulate the level of IFN-γ produced by CD8+ T cells, we observed little effect of these chemokines on this response in CD4+ T cells. This difference may be due to distinct repertoires of chemokine receptors expressed by the two T cell types or to differential requirements for the duration of cognate interaction. These questions are the focus of ongoing study.

Even though IL-12 augmented IFN-γ production through the mechanisms described above, we found that it did not play a significant role in increasing T cell proliferation. Thus we have concluded that transient interaction between CD8+ T cells and antigen bearing DC are sufficient to induce T cell proliferation, which is in agreement with published literature (21). One potential explanation why IL-12 does not play a significant role in augmenting T cell proliferation when these lymphocytes are primed only by DC could be due to DC potency as antigen presenting cells (14,15,17,68–70). *Listeria*-infected DC express high levels of costimulatory molecules such as CD80 and CD86 which augments CD8+ T cells proliferation and cytokine production (14,17). In addition, *Lm*-infected DC secrete cytokines such as IFN-β that can also be a potent signal 3 to T cells (14,18–20,71). Therefore, we feel that the effect of IL-12 on CD8+ T cell proliferation changes based on other factors present during priming (the level of costimulation, other cytokines, and chemokines) that could play redundant roles with IL-12 in augmenting this process (72–75).

Since IL-12 shares the p40 subunit with IL-23, we hypothesized that IL-23 could potentially augment the duration of interaction between T cells and DC correlating with the increased IFN-γ production we observed. In our study, we found that IL-23 produced by DC did not augment IFN-γ production by CD8+ T cells or influence the interactions between CD8+ T cells and DC during priming. From these observations, we conclude that IL-12 has a greater impact on naïve CD8+ T cell activation than does IL-23. However, since IL-23 activates multiple immune cells, its *in vivo* role in the control of infection by intracellular bacterial pathogens and T cells activation warrants further investigation.

The observation that CCL17 is produced by DC in response to *Lm* infection *in vivo* was demonstrated several years ago (66). Our *in vivo* data confirm this finding and extend our understanding of the mechanistic basis of this response by demonstrating that this chemokine is produced by DC in an IL-12 dependent manner in response to listerial infection (Figure 6). This result suggests that following *in vivo* infection with *Listeria*, CD8+ T cells are likely to remain in long term cognate interaction with antigen-bearing DC due to IL-12 and CCL1 and CCL17 production, enhancing their expression of IFN-γ and improving the ability of the host to clear the infection.

Our study has demonstrated a previously unrecognized mechanism through which IL-12 regulates CD8+ T cell activation. Our data, along with previously published studies, support the hypothesis that the delivery of IL-12 by DC to CD8+ T cells occurs most efficiently when these immune cells are in close proximity and when a large percentage of CD8+ T cells express the receptor for this cytokine. These observations further support our model in which IL-12 (through the increased production of CCL1 and CCL17) increases the physical interactions between CD8+ T cells and DC when the T cells are most responsive to this cytokine. However, it remains to be determined if IL-12 and IL-23 produced by DC affect the generation of potent memory or secondary effector CD8+ T cell responses in the context of a bacterial infection. In addition, it will be important to determine if CCL1 and CCL17 govern naive CD8+ T cell activation and generation of memory CTL *in vivo*, which could have major implications for vaccine design.

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Abbreviations used in this paper

DC, dendritic cells; IL-12, interleukin-12; IL-23, interleukin-23; IFN-γ, interferon gamma; *Lm*, *Listeria monocytogenes*; CFSE, carboxy-fluorescein diacetate succinimidyl ester; TCA3/ CCL1, T cell activating gene 3; TARC/ CCL17, Thymus and activation-regulated chemokine; MFI, mean fluorescence intensity; ICS, intracellular cytokine staining.

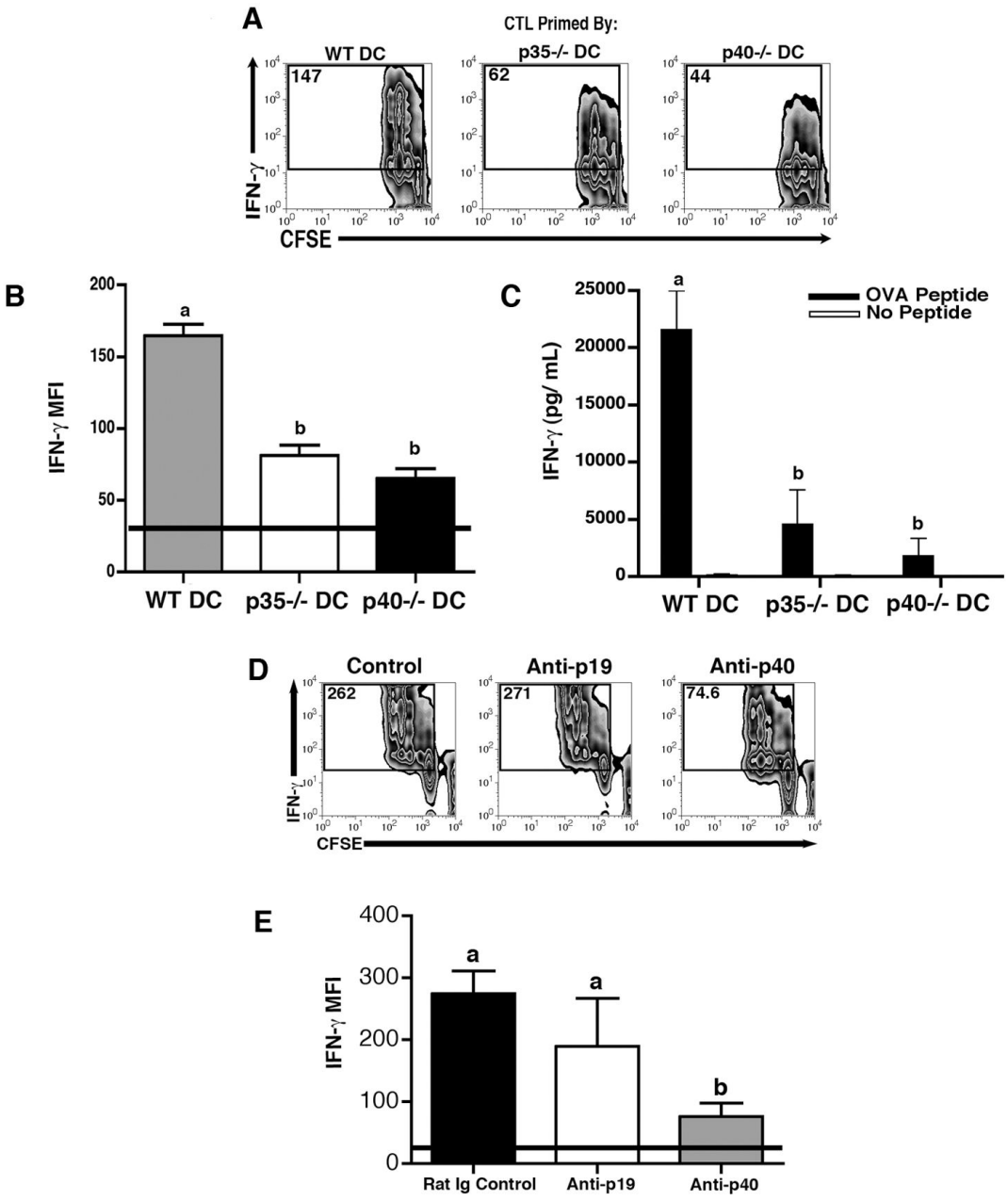

FIGURE 1. IL-12, but not IL-23 augments IFN-γ production by naïve CD8+ T cells

A, CFSE-stained OT-1 T cells were primed by *Listeria*-infected OVA-pulsed wild-type, p35 −/−, or p40−/− DC and IFN-γ production was determined by ICS staining on day 3 of priming. IFN-γ MFI of T cells is denoted in the upper left corner. Contour plots are representative of 10 independent experiments. *B*, The combined data from eight independent flow cytometric experiments is graphed. The solid line indicates the amount of IFN-γ T cells produced when primed in the absence of OVA peptide. The mean ± the SD is shown. *C*, IFN-γ production by CD8+ T cells during priming measured by ELISA. CD8+ T cells were primed by either wild-type, p35−/−, or p40−/− DC and the amount of IFN-γ that accumulated over three days was determined via ELISAs. The average concentration of IFN-γ from three independent

experiments is graphed. *D*, CFSE-stained OT-1 T cells were primed by *Listeria*-infected OVA-pulsed DC in the presence of neutralizing antibodies against IL-23 (anti-p19) or IL-12/23 (anti-p40) and IFN-γ production was determined by ICS staining on day 3 of priming. Contour plots are representative of eight independent experiments. *E*, The combined data from 10 independent experiments is graphed. The solid line indicates the amount of IFN-γ T cells produced when primed by wild-type DC in the absence of OVA peptide. The mean ± the SD is shown. Significance compared to the Rat Ig or WT DC controls was determined using a Student's t-test with *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

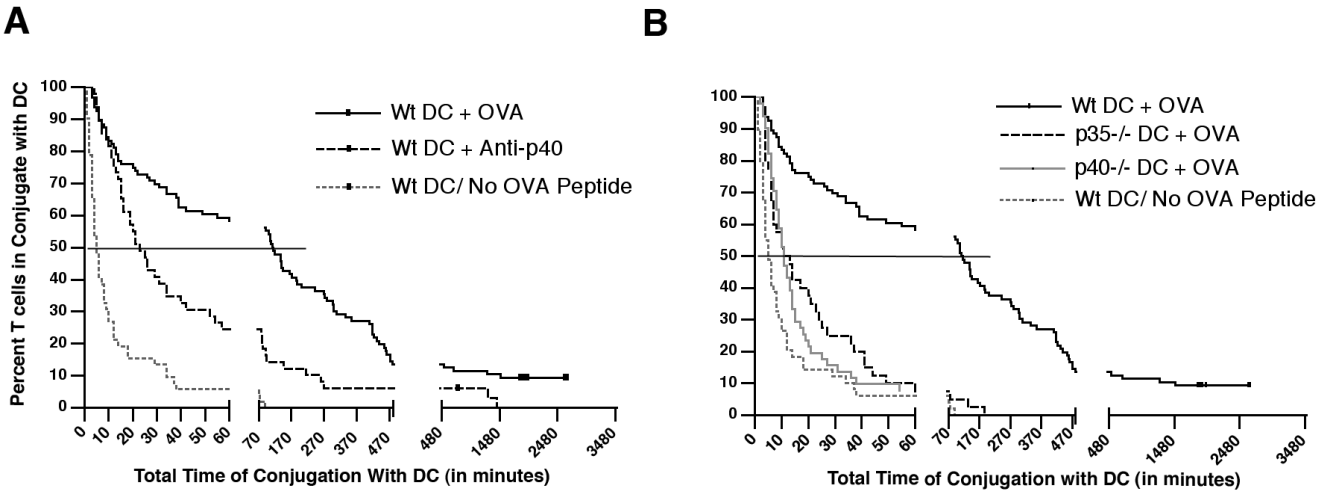

FIGURE 2. IL-12 promotes long duration interactions with DC

A, OT-1 were primed by *Listeria*-infected, OVA-pulsed DC for 48 hours *in vitro* with control or anti-p40 neutralizing antibodies. The duration of interaction between T cells and DC were determined via time lapse video microscopy. *B*, OT-1 were primed by wild-type, p35−/−, or p40−/− DC as described above and the duration of interaction between T cells and DC were determined via time lapse video microscopy. Dissociation curves are combined data from at least three independent experiments per condition. The solid line in *A* and *B* represents 50% of the interactions analyzed. Significance compared to the WT DC + OVA control samples was determined by Cox Proportional Hazard Regression Analysis. The neutralization of IL-12/23 (anti-p40) resulted in a significant decrease in the duration of interaction of OT-1 ($p < 0.01$) with DC, which was further reduced in the absence of IL-12 (p35−/− DC; $p < 0.001$) or IL-12/23 (p40−/− DC; $p < 0.001$).

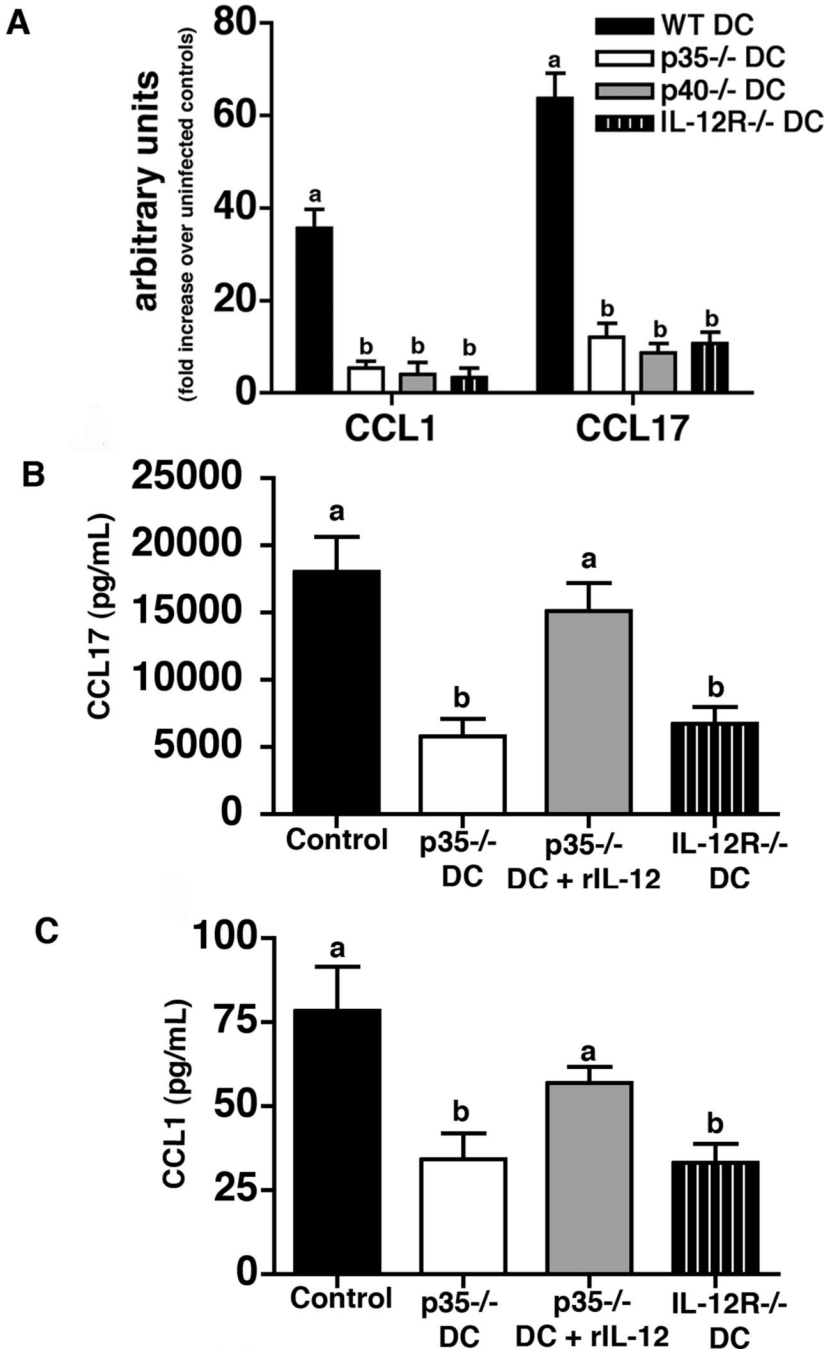

Figure 3. IL-12 increases the *expression* of CCL1 and CCL17 message and secretion in *Lm*-infected DC

Wild-type, p35−/−, p40−/−, and IL-12R−/− DC were mock treated or infected with *Lm* (MOI 1), and RNA was isolated from these DC at 24 hours post infection. *A*, CCL1 and CCL17 message levels were determined via real-time PCR. Data represents combined results from 3 independent experiments and the mean ± SD is shown. (a) indicates that samples was significantly different (b). *B and C*, Wild-type, p35−/−, and IL-12R−/− DC were infected with *Lm* at an MOI of 1. Chemokine secretion was measured 24h later by ELISA. In some experiments recombinant IL-12 was added back to IL-12 deficient DC (p35−/−) in order to determine the effects this treatment had on CCL17 (*B*) and CCL1 production (*C*). Significance

was determined compared to the WT DC control using a Student's t-test with *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

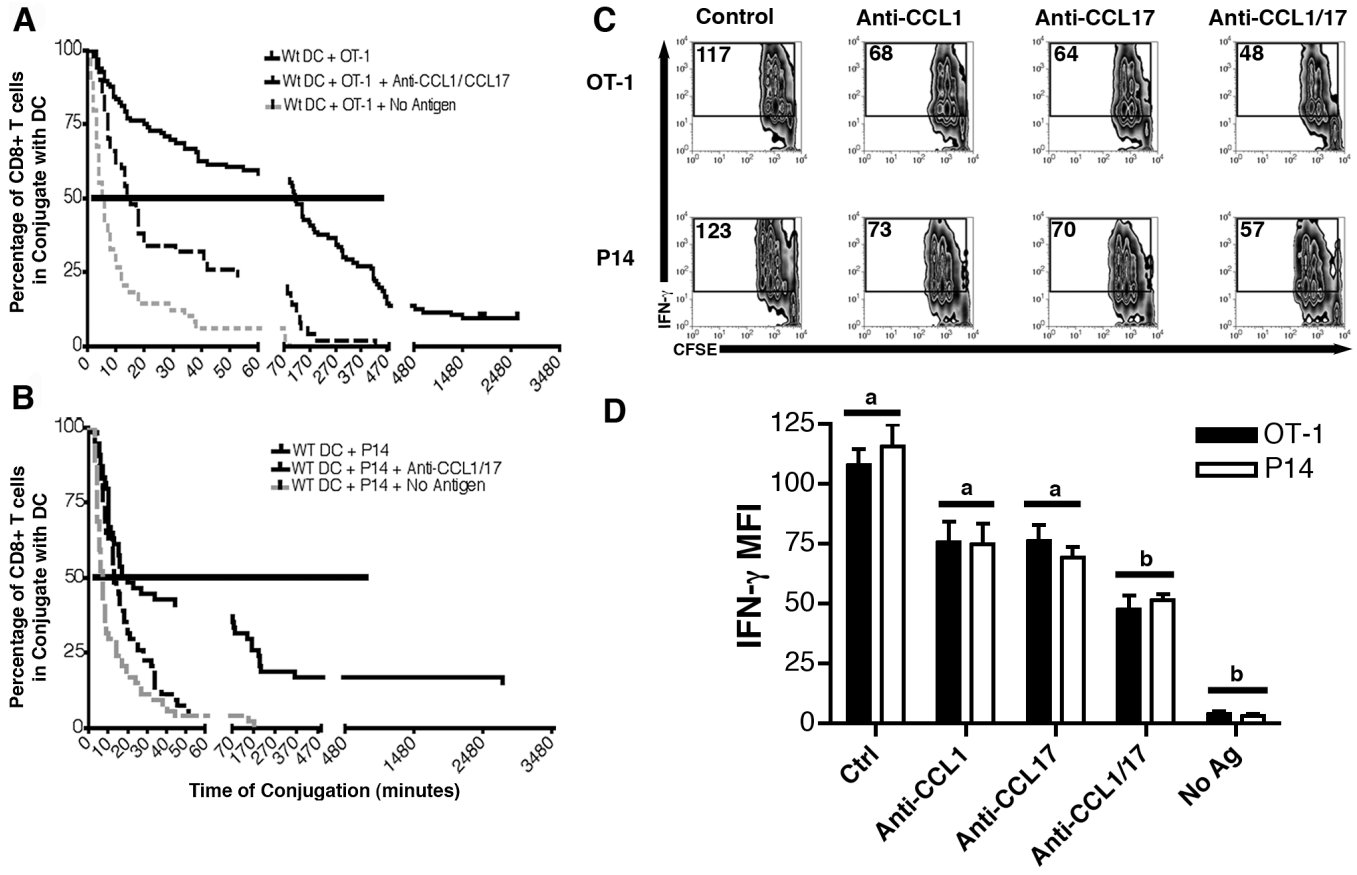

FIGURE 4. CCL1 and CCL17 augment T cell/DC interaction time and IFN-γ production by CD8 + T cells

A and B OT-1 and P14 were primed by OVA or GP33-41 pulsed *Listeria*-infected DC for 72 hours *in vitro* with control or anti-CCL1/CCL17 neutralizing antibodies. The duration of interaction between T cells and DC were determined via time lapse video microscopy. Dissociation curves are combined data from at least three independent experiments per condition. The solid line represents 50% of the interactions analyzed. Significance compared to the control samples was determined by Cox Proportional Hazard Regression Analysis. The neutralization of CCL1/17 resulted in a significant decrease in the duration of interaction of OT-1 ($p<0.01$) and P14 ($p<0.01$) with DC compared to controls. *C*, OT-1 and P14 were primed by *Listeria*-infected, OVA or GP33-41 peptide pulsed DC for 72 hours *in vitro*. Control, anti-CCL1, anti-CCL17, or a combination of anti-CCL1/17 neutralizing antibodies was added when T cells were added with DC. *D*, The IFN-γ MFI of T cells was determined at 72 hours via ICS staining. Compiled data represent 8 independent experiments for the OT-1 T cells and 4 independent experiments for the P14 T cells. The relative statistical significance of either OT-1 or P14 CD8+ T cell responses were determined compared to the same T cell in the absence of neutralization using a Student's t-test with *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

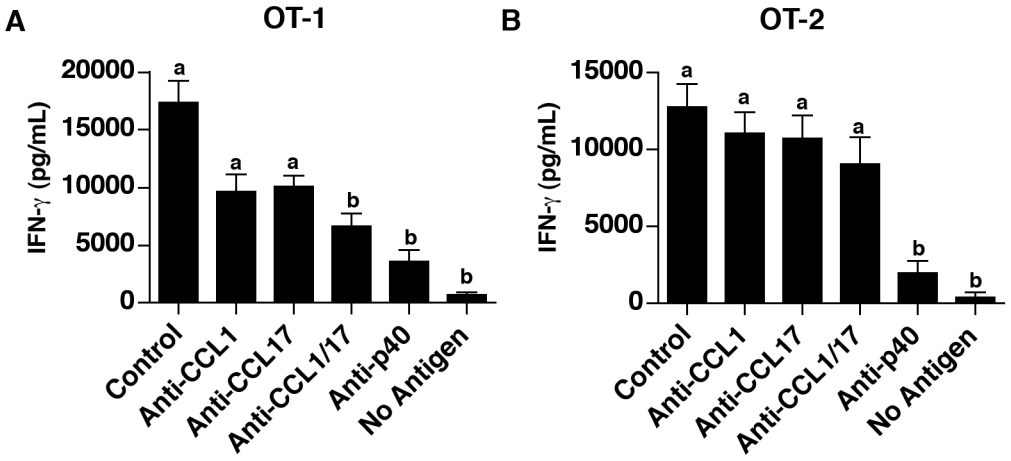

Figure 5. CCL1 and CCL17 do not augment IFN-γ production by CD4+ T cells

OT-1 and OT-2 T cells were primed by *Lm*-infected DC in the presence or absence of anti-p40, anti-CCL1, anti-CCL17, or a combination of anti-CCL1/17 neutralizing antibodies *in vitro* for 3 days. On day 3 of priming, supernatants were filtered and assayed for the concentration of IFN-γ via ELISAs. A, Histograms represent combined data from 5 independent experiments for OT-1 and *B*, three independent experiments for OT-2 T cells. Significance of neutralized samples was calculated in comparison to control OT-1 CD8+ T cells or OT-2 CD4+ T cells without neutralization using a Student's t-test with *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

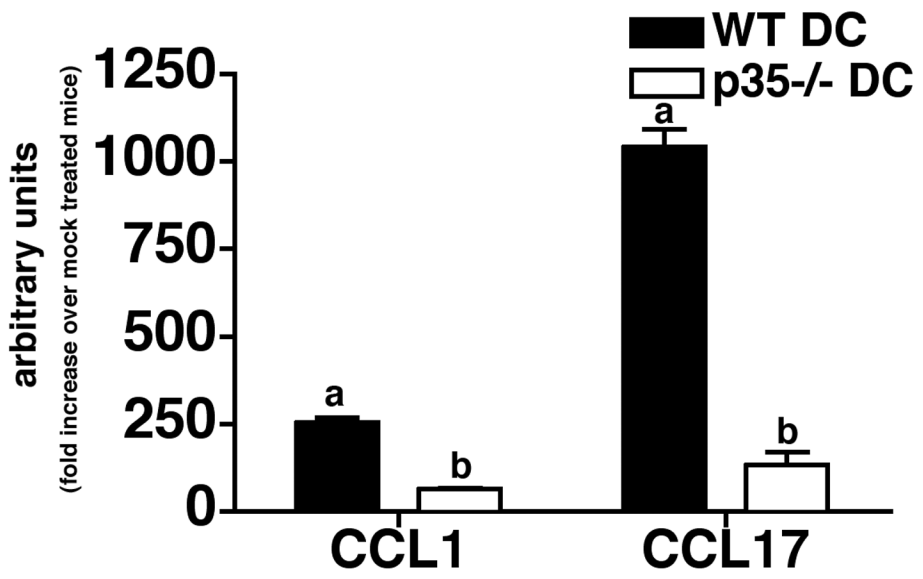

Figure 6. IL-12 augments CCL1 and CCL17 message in DC during *Lm* infection *in vivo*

Wild-type and IL-12 deficient (p35−/−) DC were mock treated (PBS) or infected with 1 LD_{50} of *Lm* intravenously. At 18 hours post infection, RNA was isolated from DC enriched from the lymph nodes of treated mice. Four mice per group were analyzed and data represent the mean ± SD. CCL1 and CCL17 message levels were determined via real-time PCR. Significance was assessed compared to DC isolated from WT mice using a Student's t-test for each gene with *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.