In Situ IL-12/23p40 Production during Mycobacterial Infection Is Sustained by CD11b^{high} Dendritic Cells Localized in Tissue Sites Distinct from Those Harboring Bacilli[1]

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Although IL-12/23p40 is known to play a major role in host resistance to *Mycobacterium* spp, the cellular source, tissue localization, and regulation of p40 production during mycobacterial infection in vivo has been unclear. In this study, we used IL-12/23p40eYFP (yet40) reporter mice to track expression of the cytokine following *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) infection. We found that in spleens of these mice, p40 production is initiated by a transient burst from CD11b^{low}CD11c^{+} dendritic cells (DC) which are later replaced at the onset of granuloma formation by CD11b^{high}CD11c^{+} DC as the major source of the cytokine. The latter subset was also found to be the key producer of DC-derived p40 in nonlymphoid tissue and in both spleen and liver optimal production of the cytokine was regulated by endogenous TNF-α. Although BCG and p40-expressing DC were both observed in splenic white pulp, p40^{+} DC rarely colocalized with bacilli. Indeed, in vitro flow cytometry and confocal microscopy indicated that the presence of intracellular bacteria is not required for p40 production by DC and Transwell experiments confirmed that soluble mycobacterial components are sufficient for inducing cytokine expression by these cells. Moreover, when stimulated with LPS, DC directly infected with BCG showed impaired IL-12p40 production in vitro. Together, our findings establish CD11b^{high} DC as a major source of IL-12/23p40 during mycobacterial infection in situ and implicate both soluble mycobacterial products and TNF-α in stimulating sustained production of p40 by these cells. ***The Journal of Immunology,*** **2009, 182: 6915–6925.**

Mycobacterial infections caused by *Mycobacterium tuberculosis* (MTB),[4] *Mycobacterium bovis*, and atypical mycobacterial spp. such as *Mycobacterium avium* constitute a major cause of morbidity and mortality. Infection with MTB has an annual incidence of 1.6 million deaths and 8.8 million new cases (1). As many as 5–10% of tuberculosis cases may be due to *M. bovis* infection (2, 3) while *M. avium* spp. are major agents of disseminated disease and death in AIDS patients (4).

Host control of mycobacterial infection relies heavily on IFN-γ-producing CD4^{+} T cells (Th1 cells) that activate infected macrophages to kill intracellular mycobacteria (5). In this context, IL-12 is required for the generation and maintenance of protective Th1 responses (6). Bioactive IL-12 is composed of an inducible p40 subunit and a largely constitutive p35 subunit (6). The p40 subunit can also associate with p19 to generate IL-23 and has additional function as a p40 homodimer (7). IL-12/23p40 (for the sake of simplicity herein referred to as IL-12p40) is critical for resistance to mycobacterial infection in both experimental models and humans (8). Importantly, mice genetically deficient in IL-12p40 display defective granuloma formation, Th1 development and maintenance, and control of mycobacterial infection (9, 10). Bioactive IL-12 rather than IL-23 appears to account for most of these effects (10, 11), although IL-12p40 alone has been shown to mediate T cell priming (12) and to restore resistance to mycobacteria-infected IL-12p40$^{-/-}$/IL-12p35$^{-/-}$ mice (13).

In vitro observations clearly indicate that phagocytes, primarily dendritic cells (DC) and macrophages but also neutrophils, produce IL-12p40 as a consequence of pattern recognition receptor triggering (14). Pattern recognition receptors detect conserved molecular patterns in infecting microbes and generate signals for activation of NF-κB and other transcription factors (15). In the case of murine MTB as well as *M. bovis* bacillus Calmette-Guérin (BCG) infection, TLR2, TLR9, the C-type lectin dectin-1, and nucleotide-binding oligomerization domain protein 2 have all been shown to contribute to DC-derived IL-12 production in vitro (16–20).

Although IL-12p40 production can be readily assayed in vitro, the tools available for its measurement in vivo have been inadequate and have precluded its direct detection during mycobacterial infection. The recent generation of mice with a knock-in IL-12p40

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[4] Abbreviations used in this paper: MTB, *Mycobacterium tuberculosis*; DC, dendritic cell; eYFP, enhanced yellow fluorescent protein; LysM, lysozyme M; BCG, bacillus Calmette-Guérin; RFP, red fluorescent protein; STAg, soluble tachyzoite from *Toxoplasma gondii*; SP, splenic; SpDC, splenic DC; MHC, MHC class II; BMDC, bone marrow-derived DC; MOI, multiplicity of infection.

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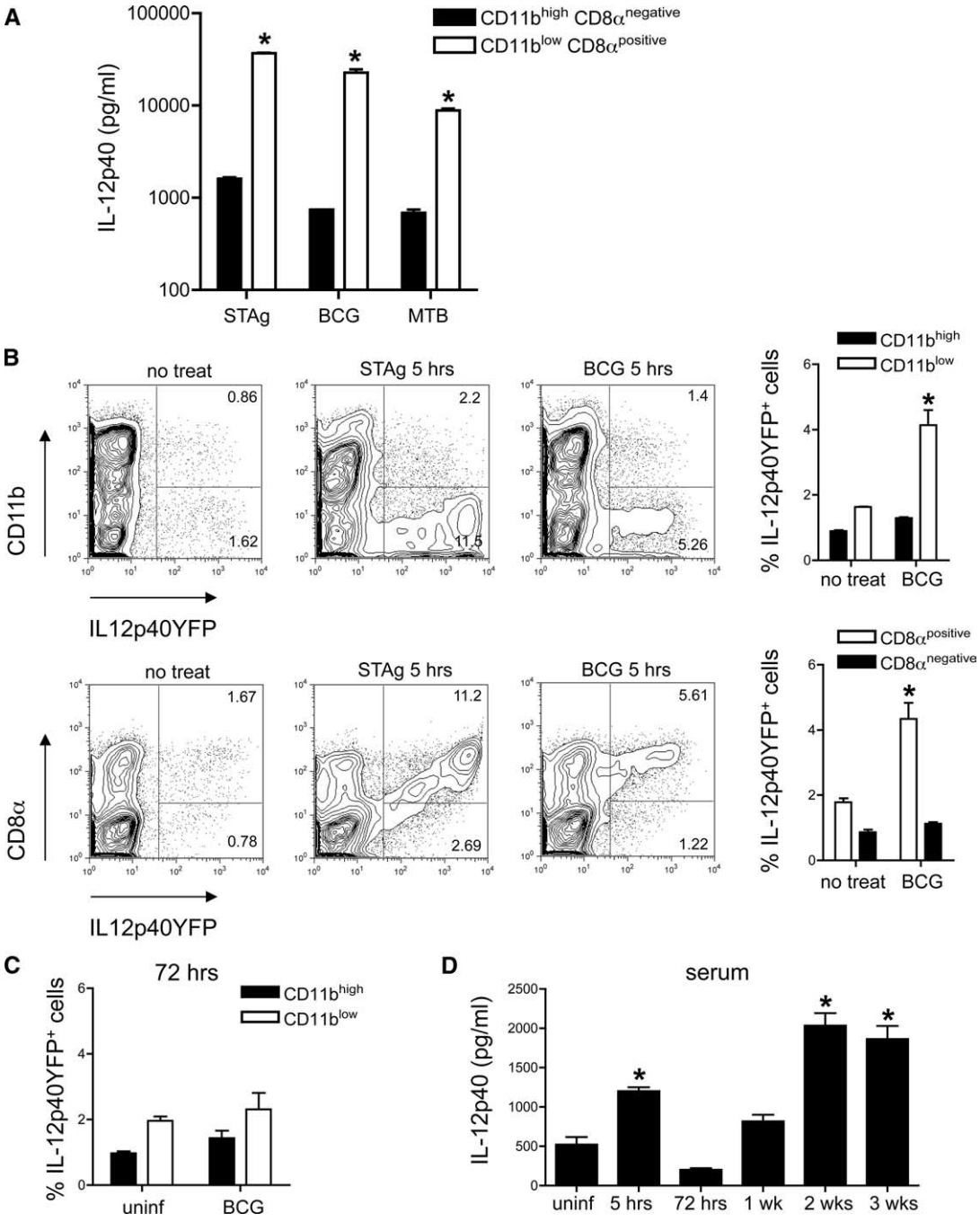

FIGURE 1. $CD11b^{low}CD8\alpha^{+}$ DC produce IL-12p40 in response to mycobacteria in vitro and are the initial source of the cytokine in the spleen following BCG infection in vivo. *A*, Splenocytes were isolated from C57BL/6 wild-type (WT) mice and $CD11b^{high}CD8\alpha^{-}$ and $CD11b^{low}CD8\alpha^{+}MHC\text{-}II^{+}CD11c^{+}$ cells sort purified by FACS. These cells were then stimulated with STAg, live *M. bovis* BCG, or live MTB and levels of IL-12p40 in overnight culture supernatants were measured by ELISA. The experiment shown is representative of two performed. Bars represent the SEM. ∗, Indicates statistically significant differences between IL-12p40 levels in $CD11b^{high}$ vs $CD11b^{low}$ DC. *B*, yet40 mice were injected i.v. with STAg or live BCG. Splenocytes were isolated 5 h (*B*) or 72 h (*C*) later and stained for CD11c and CD11b or CD11c and CD8α. Representative contour dot plots for CD11b (*upper panels*) or CD8α (*lower panels*) and IL-12p40YFP expression gated on $CD11c^{+}$autofluorescent^{low} cells are shown. Bar graphs on the *right* depict the mean frequency of IL-12p40YFP expression of the contour dot plot-defined populations on the *left* based on two naive and four infected mice. Error bars represent the SEM. The experiment shown is representative of four performed. ∗, Indicates statistically significant differences between the frequency of IL-12p40YFP production in $CD11b^{low}$ vs $CD11b^{high}$ DC or $CD8\alpha^{+}$ vs $CD8\alpha^{-}$ SpDC in BCG-infected yet40 mice. *D*, Levels of IL-12p40 in the serum of wild-type mice were measured by ELISA at different time points after i.v. inoculation of BCG. Bars represent the SEM. ∗, Statistically significant increase in IL-12p40 levels in infected mice compared with uninfected controls. uninf, Uninfected.

allele modified to express a bicistronically linked enhanced yellow fluorescent protein (eYFP) now allows the efficient tracking of IL-12p40-expressing cells in vivo (21). In the current study, we have used IL-12p40eYFP reporter (yet40) mice to characterize the cellular source, localization, and regulation of IL-12p40 production following infection with BCG. The spleen was chosen as the focus for this work because of its role as a major secondary lymphoid organ that generates host-protective responses to bacteria

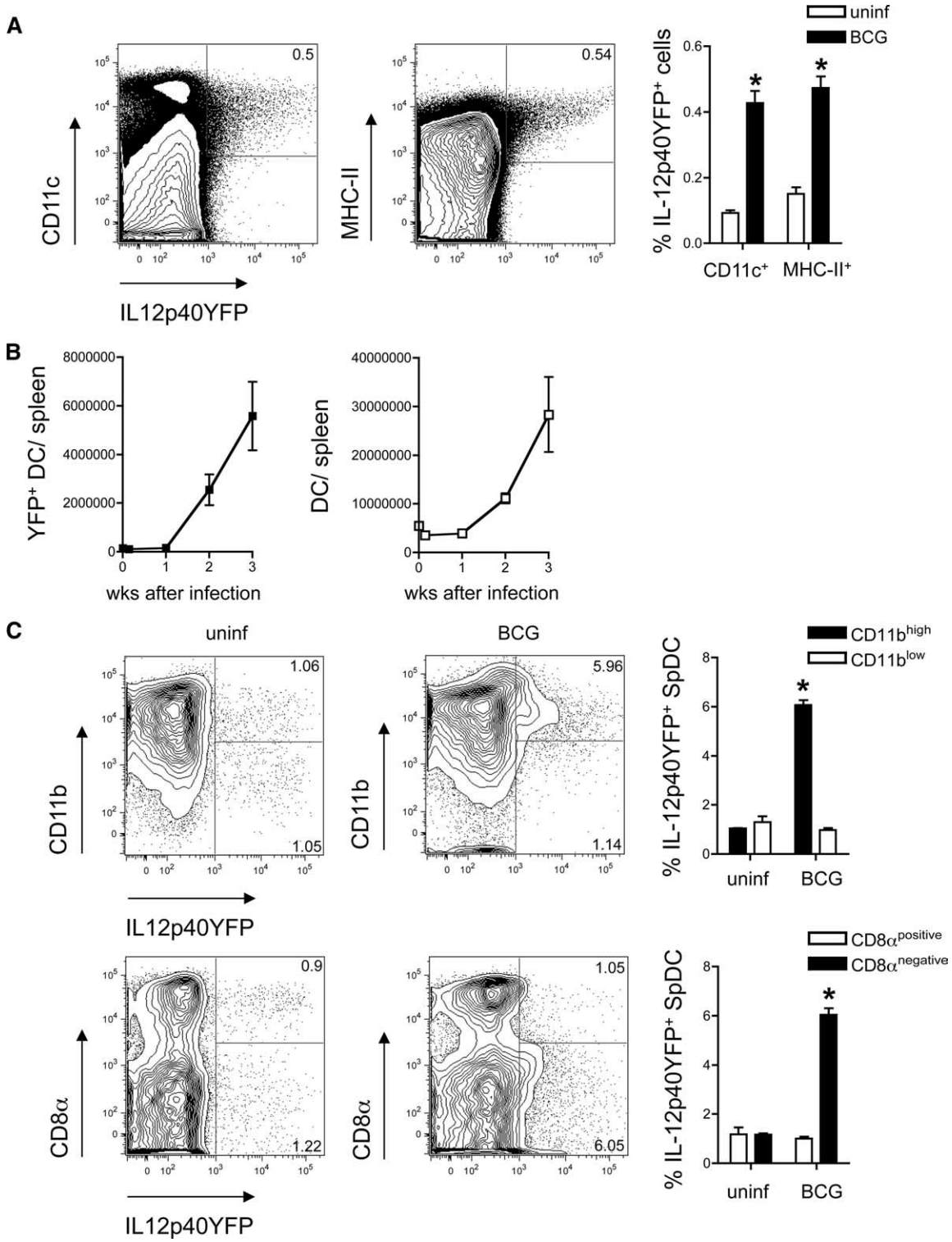

FIGURE 2. IL-12p40 production in the spleen of BCG-infected yet40 mice is maintained by CD11b^{high}CD8α^- DC. *A*, yet40 mice were injected i.v. with live BCG, splenocytes were isolated at 3 wk after infection, and then stained for CD11c and MHC-II. Representative contour dot plots showing expression of CD11c and IL-12p40YFP on the *left panel* and MHC-II and IL-12p40YFP expression on the center panel are shown. Gates are based on autofluorescent^{low} cells. *B*, The absolute number of IL-12p40YFP-expressing splenic CD11c$^+$MHC-II$^+$autofluorescent^{low} cells (SpDC) or total SpDC at different time points after BCG infection is graphed. Two naive and three to four BCG-infected yet40 mice were used per time point. *C*, Same procedure as in *A* but here showing expression of CD11b or CD8α along with IL-12p40YFP on splenic CD11c$^+$MHC-II$^+$autofluorescent^{low} cells. Bar graphs on the *right* depict the mean frequency of IL-12p40YFP expression of the contour dot plot-defined populations on the *left* based on two naive and four infected mice. Error bars represent the SEM. The experiment shown is representative of two performed. ∗, Statistically significant differences between the frequency of IL-12p40YFP production in CD11b^{low} vs CD11b^{high} SpDC or CD8α^+ vs CD8α^- SpDC in BCG-infected yet40 mice. uninf, Uninfected.

(22), including the stimulation of IL-12-dependent antimycobacterial immunity (23, 24). Our findings demonstrate that DC, and not macrophages, are the major source of IL-12p40 during mycobacterial infection. Although production of the cytokine is initiated by CD11b^{low} DC, it is maintained during chronic infection by a population of CD11b^{high} DC that arises at the onset of granuloma

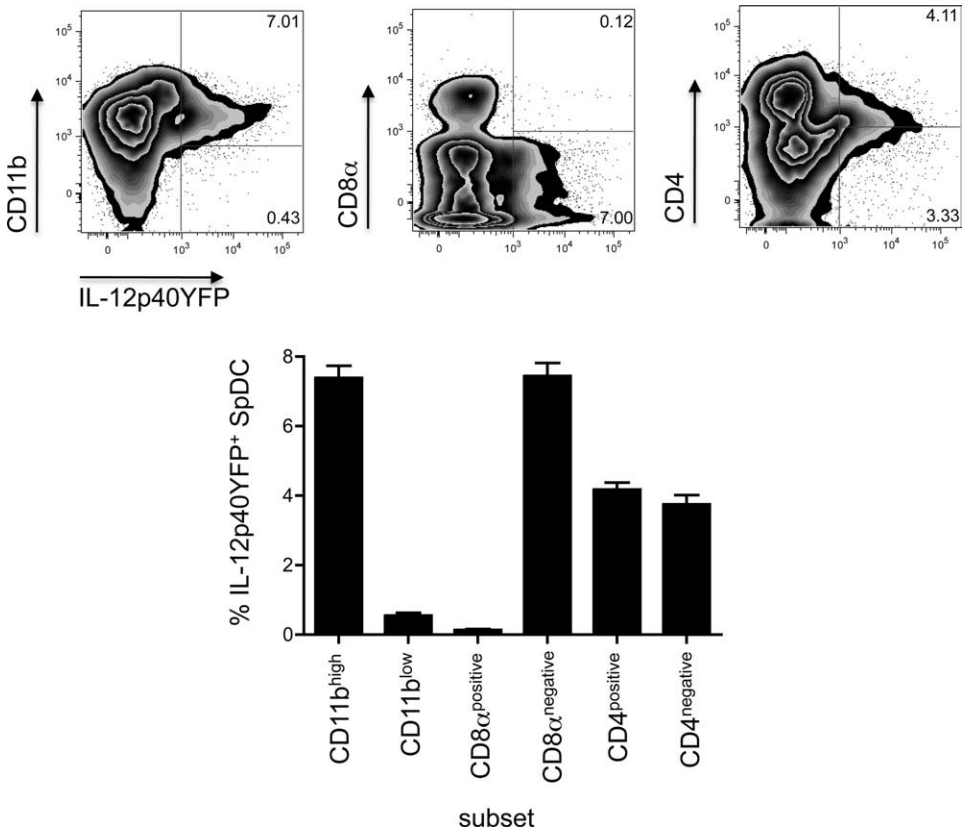

FIGURE 3. Both $CD4^+$ and $CD4^-$ subpopulations of SpDC contribute to IL-12p40 production in BCG-infected spleen. yet40 mice were injected i.v. with live BCG, splenocytes were isolated 3 wk after infection, and stained for DC markers. Representative contour dot plots show expression of CD11b, CD8α, or CD4 along with IL-12p40YFP on $SpCD11c^+MHC\text{-}II^+autofluorescent^{low}$ cells. Bar graph depicts the mean frequency of IL-12p40YFP$^+$ SpDC within each subset based on four infected mice. Error bars represent the SEM. The experiment shown is representative of two performed.

formation and depends on TNF-α for its optimal function. Interestingly, these IL-12p40$^+$ cells do not colocalize with mycobacteria in situ and instead appear to depend primarily on soluble mycobacterial factors rather than direct infection for their cytokine activity. This IL-12p40-producing DC population may play an important role is sustaining host resistance and granulomatous responses during mycobacterial infection.

Materials and Methods

Mice

C57BL/6 IL-12/23p40eYFP (yet40) mice were generated as previously described (21). C57BL/6 lysozyme M (LysM)-enhanced GFP knock-in mice (25) were a gift from S. Graf (Albert Einstein University, New York, NY). C57BL/6 mice were obtained from Taconic Farms. All animals were maintained in specific pathogen-free conditions at an American Association of Laboratory Animal Care-accredited animal facility at the National Institute of Allergy and Infectious Diseases, National Institutes of Health (Bethesda, MD). Both male and female mice between 8 and 12 wk old were used.

Generation of mycobacterial stocks

M. bovis BCG strain Pasteur expressing the red fluorescent protein DsRed (BCG-RFP) has been previously described (26). BCG-RFP, wild-type BCG strain Pasteur, and MTB strain H37Rv were expanded to log phase in Middlebrook 7H9 liquid medium supplemented with albumin-dextrose-catalase (Difco), washed, aliquoted in PBS, and stored at -80^oC until further use. BCG-RFP was grown in the presence of hygromycin (Sigma-Aldrich). Expression of RFP by this strain is observed in vivo without detectable loss for at least 5 wk (Ref. 27 and data not shown). Quantification of bacterial stocks for infection and bacterial loads in organs was performed by culture on 7H11 agar supplemented with oleic acid-albumin-dextrose-catalase (Difco).

Inoculation of mice

For experiments using BCG or BCG-RFP, animals were inoculated i.v. with 3×10^6 CFU of bacteria. In the case of MTB infection, animals were exposed to a low dose of ~100 CFU of MTB via the aerosol route by using a nose-only exposure unit (CH Technologies). In certain experiments, separate groups of mice were injected i.v. with 20 μg of soluble tachyzoite Ag from *Toxoplasma gondii* (STAg) (28) as a positive control for IL-12p40 induction.

Generation of single-cell suspensions from tissue

Spleens, livers or lungs were aseptically removed and injected with 0.4 mg/ml Liberase CI solution (Roche Biochemicals) for 30 min at 37°C. Tissues were then gently homogenized through a 100-μm cell strainer and the homogenate was resuspended in PBS containing 5 mM EDTA and pelleted at 1200 rpm for 5 min. An intermediate step involving a 33% Percoll gradient centrifugation (2000 rpm for 20 min) was performed for livers and lungs to enrich for leukocytes. Erythrocytes were then lysed using ACK lysing buffer (Cambrex) and the resulting single-cell suspensions were washed and counted by trypan blue exclusion before further processing.

Isolation, generation, and infection of DC

Splenic (Sp) single-cell suspensions were generated from naive mice as described above and $SpCD11c^+$ cells were enriched by magnetic selection using Miltenyi Biotec CD11c microbeads according to the instructions of the manufacturer. This yielded, as previously observed (19), a population of ~90% $CD11c^+MHC\text{-}II^+$ SpDC. Bone marrow-derived DC (BMDC) were generated using recombinant GM-CSF (BioSource International) from 6- or 7 day-old cultures as previously described (16). To avoid macrophage or granulocyte contamination, BMDC were further enriched for CD11c expression by magnetic selection (Miltenyi Biotec) which yielded a population of ~98–99% $CD11c^+$ cells. For in vitro flow cytometric experiments, unstimulated yet40 BMDC or SpDC were incubated overnight with BCG-RFP at the different multiplicities of infection (MOI) indicated in the figure legends. For Transwell experiments, naive yet40 BMDC were placed in the upper chamber of a 0.4-μm-wide Transwell (Costar). Bone marrow-derived macrophages generated with L929-conditioned medium as previously described (29), or BMDC were added to the lower chamber in the presence or absence of BCG-RFP. After overnight incubation, BMDC in the upper chambers were subjected to FACS analysis for IL-12p40YFP expression.

Flow cytometry

Single-cell suspensions from tissues, BMDC, or SpDC were incubated with various combinations of fluorochrome-conjugated rat anti-mouse mAbs specific for CD4 (L3T4), CD8 (53-6.7), CD11b (M1/70), CD11c

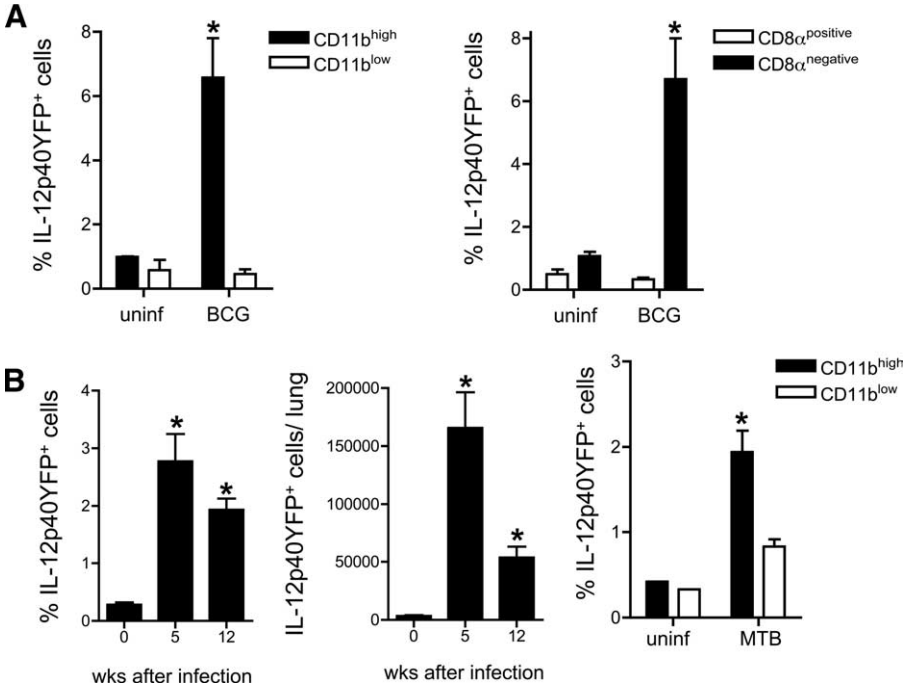

FIGURE 4. IL-12p40 production in nonlymphoid tissue following BCG or MTB infection. *A*, yet40 mice were injected i.v. with live BCG. The mean frequency of IL-12p40YFP expression by hepatic $CD11c^+$ MHC-II^+autofluorescent^{low} cells in naive and 3-wk BCG-infected yet40 mice were calculated based on expression of CD11b or CD8α. *B*, yet40 mice were aerogenically infected with a low dose of MTB. Pulmonary leukocytes were then isolated at different time points after infection and stained for CD11c and MHC-II (*left panel* and *center panel*) or CD11c and CD11b (*right panel*). Populations shown are gated on autofluorescent^{low} cells. Error bars represent the SEM. The data shown are representative of two separate experiments performed. *, Statistically significant differences between uninfected and infected mice or between the frequency of IL-12p40YFP production in $CD11b^{low}$ vs $CD11b^{high}$ DC or $CD8\alpha^+$ vs $CD8\alpha^-$ DC in mycobacteria-infected yet40 mice.

(HL3), CD40 (HM40-3), CD80 (16-10A1), CD86 (GL1), MHC-II I-A/I-E (M5/114.15.2), Gr-1 (RB6-8C5; all from BD Pharmingen), and F4/80 (BM8; Caltag Laboratories) for 45 min in FACS buffer containing 0.5 mg/ml anti-mouse FcγIII/II receptor (2.4G2; BD Pharmingen). Data were collected using a FACSCalibur with CellQuest (BD Immunocytometry Systems) or using an LSR-II with Diva (BD Pharmingen). Data were analyzed with FlowJo software (Tree Star).

Quantification of IL-12p40 in culture supernatants and serum

In vitro cytokine production assays using isolated $SpCD11c^+$ cells, $CD11b^{high}CD8\alpha^-$, and $CD11b^{low}CD8\alpha^+$ MHC-II^+ $SpCD11c^+$ cells purified on a FACSVantage flow cytometer or in vitro-generated $CD11c^+$ BMDC were performed as previously described using live mycobacteria at a MOI of 1 or 0.5 μg/ml STAg as the stimulus (19). After overnight incubation, IL-12p40 was measured in culture supernatants by sandwich ELISA (30). In some experiments, IL-12p40 levels in the serum of naive and BCG-infected mice was also measured by sandwich ELISA.

Microscopy

Spleens or livers were excised from naive and infected animals, fixed overnight with 4% paraformaldehyde/PBS, followed by dehydration in 30% sucrose/PBS before embedding in Tissue-Tek OCT freezing medium (Sakura Finetek). Sixteen- to 20-μm-thick sections were cut on a CM3050s cryostat (Leica Microsystems) and adhered to Superfrost Plus slides (VWR). Sections were permeabilized and blocked in PBS containing 0.3% Triton X-100 (Sigma-Aldrich) and 10% goat serum (Jackson ImmunoResearch Laboratories). This was followed by incubation with different combinations of Abs, which include Alexa Fluor 647-conjugated rat anti-CD3 (17A2; BD Pharmingen) and Alexa Fluor 488-conjugated rabbit polyclonal anti-GFP (Invitrogen). Incubation with unconjugated rat anti-mucosal addressin cell adhesion molecule 1 (MECA-367; BD Pharmingen) was followed by staining with Alexa Fluor 647-conjugated secondary Abs (Invitrogen). Slides were counterstained with Hoechst 33342 and mounted with Prolong Gold (both from Invitrogen). Microscopy of $CD11c^+$ BMDC incubated with BCG-RFP was performed by infecting cells overnight on poly-L-lysine glass coverslips (BD Biosciences). Cells were counterstained and mounted as above. Three-dimensional image stacks of tissue sections or BMDC were acquired on an LSM 510 (Zeiss MicroImaging,) or SP5 (Leica Microsystems) confocal microscope. Images are displayed as two-dimensional maximum intensity projections. Large composite sections of splenic tissue were obtained by image tiling using Zeiss AIM software with the Multitime macro on the LSM 510 microscope.

Statistical analyses

The significance of differences in data group means was analyzed by Student's *t* test with a cutoff of $p < 0.05$.

Results

$CD11b^{low}$ DC are the major subset producing IL-12p40 in response to mycobacteria in vitro and initiate production of the cytokine in the spleen following BCG infection in vivo

In studies using a soluble extract of *T. gondii* (STAg) as the stimulus, our laboratory previously identified a key role for $CD11b^{low}CD8\alpha^+$ SpDC as a source of IL-12p40 in vitro and, importantly, for initiating IL-12 production in vivo (31). Interestingly, FACS-sorted $CD11b^{low}CD8\alpha^+$ SpDC also secreted high levels of IL-12p40 as measured by ELISA when stimulated in vitro with live *M. bovis* BCG or live MTB (Fig. 1*A*). To examine whether mycobacteria are capable of generating a similar response in vivo, yet40 reporter mice were i.v. injected with STAg or live BCG and IL-12p40YFP expression was determined by flow cytometry after 5 h. As predicted, STAg triggered a robust IL-12p40 response by splenic $CD11b^{low}CD11c^+$ cells. BCG infection also resulted in a similar albeit weaker response confined largely to $CD11b^{low}$ SpDC (Fig. 1*B*). In agreement with the fact that CD11b and CD8α segregate between two subsets of SpDC (32), IL-12p40-producing DC triggered by either STAg or BCG were found to be $CD8\alpha^+$ (Fig. 1*B*). Furthermore, this BCG-induced IL-12p40 response was transient and was no longer detected at 72 h after inoculation (Fig. 1*C*). A similar drop in IL-12p40 expression was observed in sera of infected mice at the same time point (Fig. 1*D*).

IL-12p40 production is maintained by $CD11b^{high}$ DC following the onset of granuloma formation and is regulated by TNF-α

Although the initial burst of IL-12p40 production was transient, infection-induced production of the cytokine was again evident at later time points after BCG inoculation (Fig. 2, *A* and *B*). This "secondary" IL-12/23p40 response was detected primarily in $CD11c^+$MHC-II^+ DC with no contribution by $CD11c^-$ $F4/80^+CD11b^+$ Gr-1^{low} (monocytic) or $CD11c^-F4/80^{low}CD11b^{high}$ Gr-1^{high} (granulocytic) cell populations (Fig. 2*A* and data not shown). The response was first observed 2 wk after infection (Fig. 2*B*) and correlated with increased detection

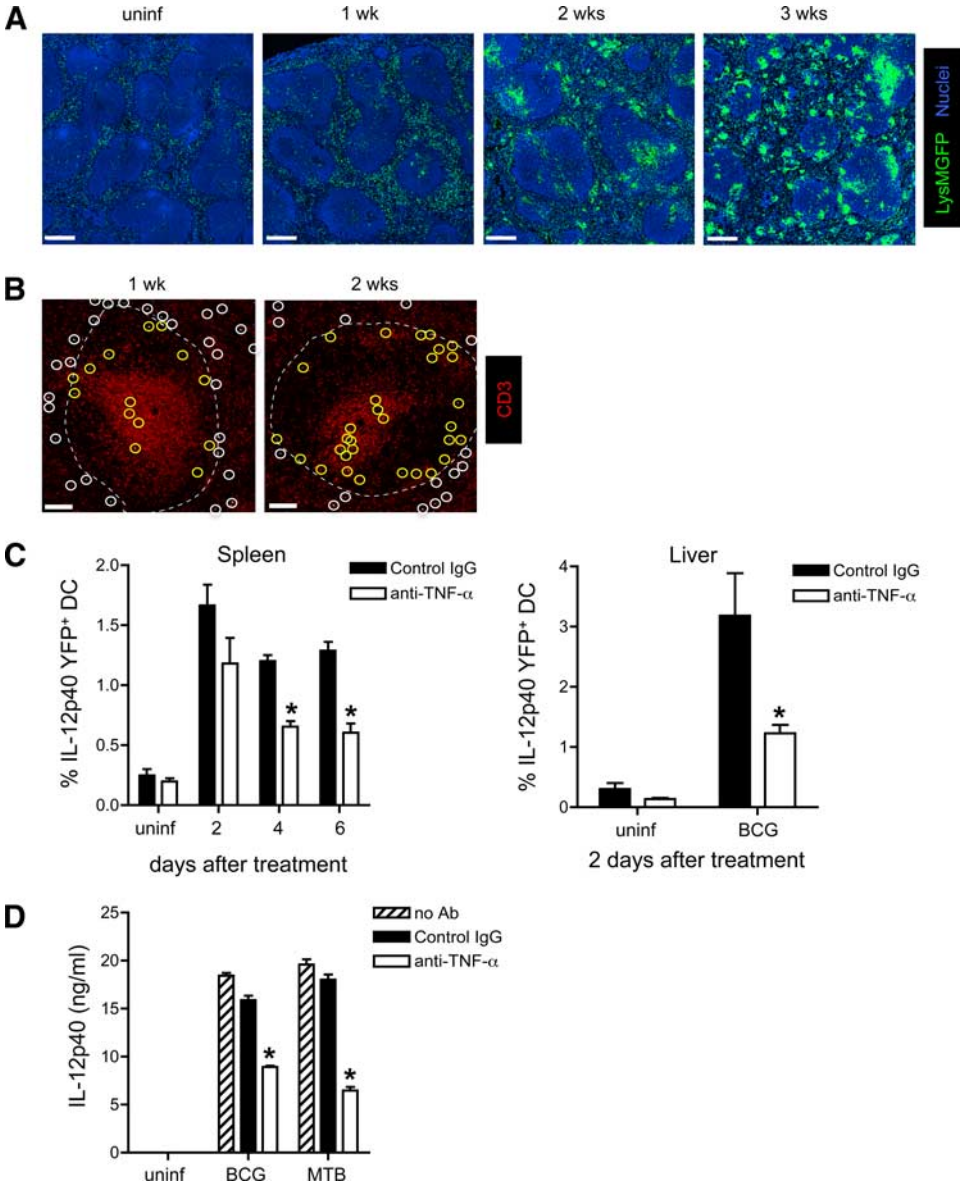

FIGURE 5. Detection of IL-12p40 production by CD11b^{high} DC correlates with the onset of splenic granuloma formation and is regulated by TNF-α. *A* and *B*, LysM-GFP mice were inoculated i.v. with BCG-RFP, spleens were obtained at different weeks after infection, sectioned, and stained for CD3, nuclei were counterstained with Hoechst, and sections were visualized by confocal microscopy. *A*, Micrograph demonstrates distribution of LysM-GFP (green), indicating macrophages associated with granulomas and nuclei (blue). Scale bar, 300 μm. *B*, Appearance of BCG-RFP within the T zone (CD3 in red) of the white pulp delineated by white dashed lines at 1 and 2 wk after infection. BCG within the white pulp are circled in yellow and outside the white pulp in white. Scale bar, 100 μm. *C*, yet40 mice were inoculated i.v. with BCG. Beginning at 3 wk after infection, mice were i.p. injected with 1 mg of control IgG (GL113) or anti-TNF-α (XT/22-11) on alternate days and sacrificed 2, 4, or 6 days after the first treatment. Splenocytes (*left panel*) or hepatic leukocytes (*right panel*) were isolated and stained for CD11c and MHC-II. The frequency of IL-12p40YFP-expressing CD11c$^+$MHC-II$^+$autofluorescent^{low} cells was determined by flow cytometry. *D*, BMDC were obtained from naive wild-type mice, incubated with control IgG or anti-TNF-α, and stimulated with live *M. bovis* BCG or live MTB. Levels of IL-12p40 in overnight culture supernatants were measured by ELISA. Bars represent the SEM. $*$, Statistically significant differences between control and anti-TNF-α-treated groups. uninf, Uninfected.

of IL-12p40 in sera of BCG-infected mice (Fig. 1*D*). In direct contrast to the initial response seen immediately following BCG inoculation, this second wave of IL-12p40 production was mediated almost entirely by splenic CD11c$^+$MHC-II$^+$CD11b^{high}CD8α^- cells (Fig. 2*C*). This population of CD11b^{high}IL-12p40$^+$ DC was phenotyped as both CD4$^+$ and CD4$^-$ (Fig. 3). CD11b^{high} DC were also found to be a major source of IL-12p40 production in nonlymphoid tissue as demonstrated in BCG-infected liver (Fig. 4*A*) and in MTB-infected lung (Fig. 4*B*).

Interestingly, the appearance of IL-12p40-producing CD11b^{high} DC in the spleen correlated kinetically with the onset of granulomas in that organ (Fig. 5*A*). Since it is known that local TNF-α synthesis is important for granuloma formation in liver of BCG-infected mice (33), we asked whether TNF-α might also play a role in regulating IL-12p40 production by DC in vivo. Although the initial burst of BCG-triggered IL-12p40 production by CD11b^{low} SpDC was not affected by TNF-α neutralization (data not shown), the same blockade in yet40 mice with established BCG infection lead to a reduction in DC-derived IL-12p40 production in both

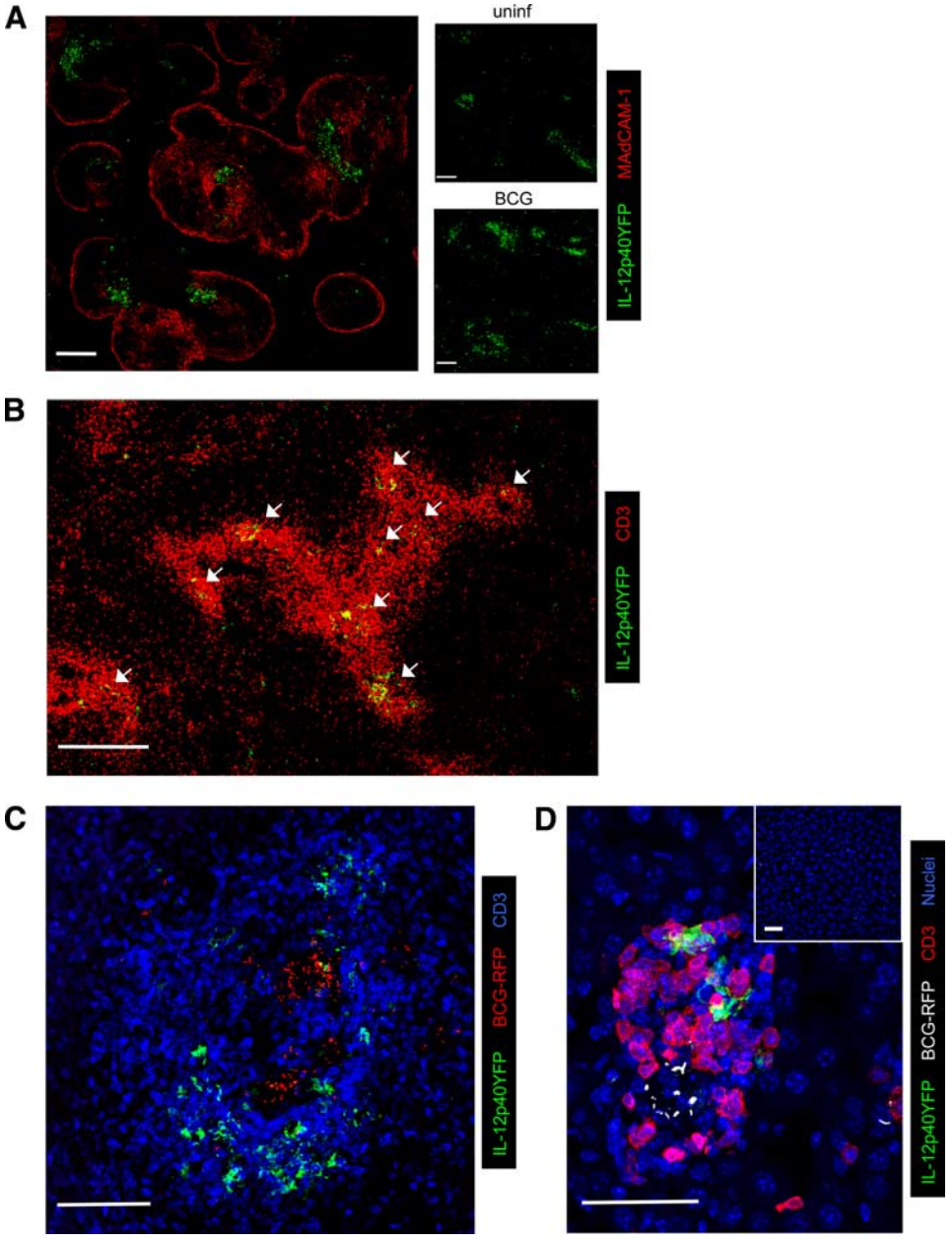

FIGURE 6. In situ localization of IL-12p40-producing cells vs bacteria in spleen and liver following BCG infection. *A*, yet40 mice were inoculated i.v. with live BCG and spleens obtained 3 wk after infection. Sections were then stained with anti-YFP (green) and anti-mucosal addressin cell adhesion molecule 1 (red) Abs and analyzed by confocal microscopy. Scale bar, 300 μm. The *smaller panels* on the *right* show IL-12p40YFP (green) expression in uninfected (uninf) and 3-wk infected (BCG) yet40 mice. Scale bar, 200 μm. *B*, As in *A* but stained for CD3 (red). White arrows indicate regions of IL-12p40YFP expression (green) within the splenic T zone. Scale bar, 300 μm. *C*, Higher magnification of a splenic T zone region showing $CD3^+$ cells (blue), BCG-RFP (red), and IL-12p40YFP (green). Scale bar, 100 μm. *D*, Micrograph of a hepatic granuloma 3 wk after infection showing nuclei counterstained with Hoechst (blue), BCG-RFP (white), CD3 (red), and IL-12p40YFP (green). The *inset* on the *upper right* shows a liver section from a uninfected yet40 mouse stained for the same markers. Scale bar, 50 μm.

spleen and liver (Fig. 5*C*) and in DC stimulated with live BCG or live MTB in vitro (Fig. 5*D*). This regulatory effect of TNF-α on IL-12p40 production in vivo was seen at times before other previously described changes associated with the absence of TNF-α, such as alterations in immune cell populations, granuloma number, and size or mycobacterial growth (34) (data not shown).

IL-12p40-producing DC in spleen and liver are closely associated with T cells but distal to mycobacteria

We next assessed the distribution of IL-12p40 in mycobacteria-infected spleen and found that IL-12p40-producing cells are present largely in the white pulp (Fig. 6*A*) and in particular in the T cell zone (Fig. 6*B*), where they are found in close apposition to T cells (Fig. 6*C*). Since the accumulation of mycobacteria in the T zone (Fig. 5*B*) kinetically coincided with the detection of IL-12p40 production by $CD11b^{high}$ SpDC (Fig. 2, *B* and *C*) and granuloma formation (Fig. 5*A*), we asked whether BCG colocalized with IL-12p40 by infecting yet40 animals with BCG expressing the RFP DsRed (BCG-RFP). Interestingly, IL-12p40-expressing cells were typically located at sites distal to mycobacteria (Fig. 6*C*). Similarly, in hepatic granulomas $IL\text{-}12p40^+$ cells were found colocalized with T cells but not with fluorescent mycobacteria (Fig. 6*D*). Together, these data indicate that DC, the major detectable cellular source of IL-12p40 in vivo, do not colocalize with BCG in spleen and liver.

Direct infection of DC by mycobacteria is not necessary for IL-12p40 production

We next performed a series of in vitro experiments to more formally address the requirements for direct infection on DC IL-12p40 production. Coculture of BMDC with BCG-RFP at different MOI followed by flow cytometric analysis revealed that while IL-12p40 production was detected in DC associated with BCG, the majority of IL-12p40-producing cells were those containing few or no bacteria (Fig. 7*A*, *upper panels*). Similar observations were made using SpDC (Fig. 7*A, lower panels*), arguing that this phenomenon is not restricted to in vitro-generated DC populations. Importantly, confocal microscopy confirmed that a large

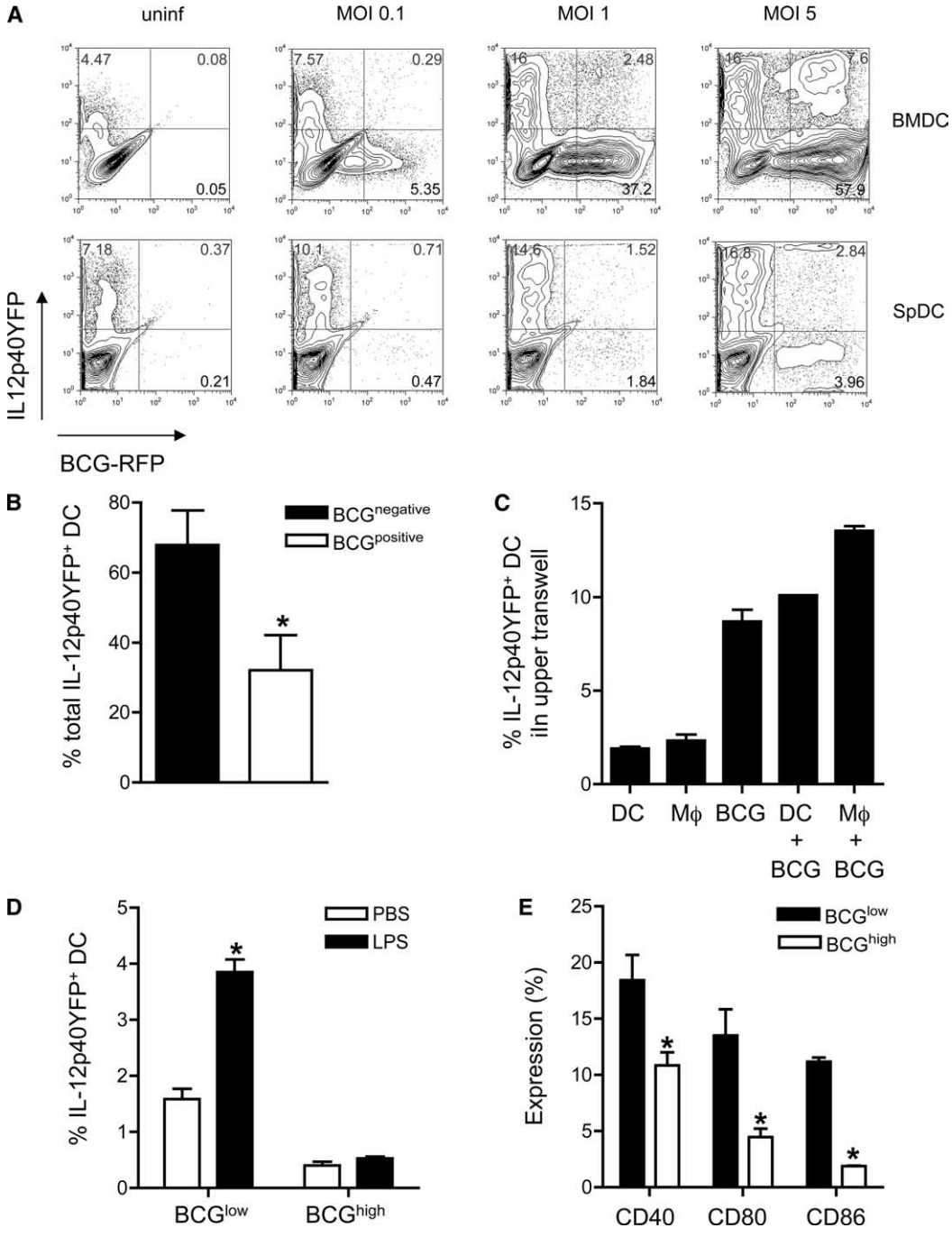

FIGURE 7. Direct infection is not required for mycobacterial-triggered IL-12p40 production by DC in vitro. *A*, BMDC (*upper panels*) or SpCD11c$^+$ cells (*lower panels*) were obtained from naive yet40 mice. The DC populations were then cultured overnight with BCG-RFP at a MOI of 0.1, 1, or 5 and flow cytometry was performed. Representative contour dot plots showing IL-12p40YFP and BCG-RFP are presented. *B*, Confocal microscopy was used to determine the frequency of IL-12p40YFP$^+$ BMDC colocalizing or not with BCG-RFP. Quantification of at least 11 different three-dimensional stacks using a $\times$20/1.4 aperture objective on a confocal microscope was performed. *C*, Naive yet40 BMDC were added to the upper chamber of a 0.4-μm-wide Transwell. BMDC, bone marrow-derived macrophages (Mϕ), BCG-RFP, or either cell type in the presence of BCG at a MOI of 5 (DC+ BCG, Mϕ + BCG) were added to the lower chambers. Following overnight incubation, the frequency of IL-12p40YFP$^+$ DC in the upper chambers was quantified by flow cytometry. *D*, BMDC were generated from naive yet40 mice and cocultured with BCG-RFP for 12 h, after which cultures were supplemented with either 100 ng/ml LPS or PBS. Flow cytometry was performed 12 h later and the frequency of IL-12p40YFP$^+$ BMDC was determined in the BCG-infected (BCG^{high}) population as well as in the subpopulation containing few or no bacteria (BCG^{low}). *E*, BMDC generated from naive wild-type mice were incubated overnight with BCG-RFP at a MOI of 1 and surface expression of CD40, CD80, and CD86 was determined by flow cytometry in the BCG-infected (BCG^{high}) as well as in the subpopulation containing few or no bacteria (BCG^{low}). Bars represent the SEM. $*$, Statistically significant differences between BCG$^+$ and BCG$^-$ BMDC, between PBS- vs LPS-stimulated BCG^{high} or BCG^{low} BMDC, or between BCG^{high} and BCG^{low} BMDC. The experiments shown are representative of at least two performed. uninf, Uninfected.

proportion of the DC observed were positive for IL-12p40 but negative for BCG (Fig. 7*B*). Furthermore, cell viability experiments using LIVE/DEAD exclusion dye (Invitrogen) failed to reveal increased death of infected BMDC, arguing against this possible explanation for the reduced IL-12p40 expression of the BCG^{high} subpopulation (data not shown).

Soluble mycobacterial factors are sufficient for triggering IL-12p40 production by DC

The observation that most IL-12p40$^+$ DC are not directly infected with mycobacteria suggested that soluble bacterial factors might serve as a trigger for cytokine production. Transwell experiments in which DC in an upper chamber were separated by a 0.4-μm-wide membrane from a lower chamber containing BCG, macrophages, DC, or either cell type in the presence of BCG were performed to test this hypothesis. The inclusion of APC in the lower chambers was to additionally investigate the paracrine effects of soluble host factors secreted by APC in direct contact with BCG for IL-12p40 production by DC in the upper chambers. Interestingly, the presence of BCG in the lower chamber was found to be sufficient to induce IL-12p40 production by DC, arguing against an absolute requirement for direct infection or soluble host factors from APC exposed to bacteria (Fig. 7*C*).

An additional interpretation of the preferential association of IL-12p40 production with uninfected DC is that IL-12p40 production is actively inhibited in infected DC. In support of this hypothesis, we found that when BMDC cultures previously exposed to live BCG were stimulated with LPS, enhanced IL-12p40 expression was observed but not from those DC infected with bacteria (Fig. 7*D*). Further supporting an inhibitory role of BCG infection on DC function, the expression of costimulatory markers CD40, CD80, and CD86 were found to be lower on infected DC compared with uninfected DC (Fig. 7*E*). Taken together, these observations suggests that DC IL-12p40 production following live mycobacterial exposure may involve stimulation of the cytokine by soluble mycobacterial products in uninfected DC accompanied by suppression of its production in infected DC.

Discussion

Although the p40-containing cytokines IL-12 and IL-23 have been shown to play important roles in the control of infection and in vaccine-induced resistance against mycobacteria, the cellular sources of these protective cytokines in vivo as well as the regulation of IL-12/23p40 responses in situ are poorly understood. In this study, we used yet40 reporter mice, flow cytometry, and in situ high-resolution static imaging to track the expression and localization of the p40 subunit by various cell populations in the spleen and other target tissues during mycobacterial infections. Our findings indicate that the majority of YFP$^+$ cells seen in infected animals are CD11c$^+$MHC-II$^+$ DC and that CD11b^{high}CD11c$^+$ MHC-II$^+$ cells are the predominant DC subpopulation that maintains production of IL-12p40 during long-term infection.

Interestingly, immediately after infection YFP expression was detected primarily in CD11b^{low} rather than CD11b^{high} SpDC, a finding that mirrored the role of the former cells as potent producers of IL-12p40 in splenocytes stimulated with mycobacteria in vitro (Fig. 1*A*) as well as their previously described function in the response to *T. gondii* extract STAg (31). Similar to what was observed following STAg injection, we found that IL-12p40-producing DC at this early time point after BCG infection were CD8α^+. This early burst of IL-12p40 production from CD11b^{low}CD8α^+ DC was transient, in agreement with an earlier study where IL-12p40 production was detected ex vivo in culture supernatants from DC but not macrophages obtained from BCG-infected spleen (35). Nevertheless, by 2 wk of infection, CD11b^{high}CD8α^- DC became the major source of the cytokine. Consistent with this finding, IL-12p40 expression was absent in CD11c^{low}MHC-II^{low}B220$^+$PDCA-1$^+$pDC, which are CD11b^{low} (data not shown). CD11b^{high} DC were also found to be the dominant cytokine-producing cells in the liver of BCG-infected mice as well as in the lungs of MTB-infected animals. Previous studies have indicated that persistent IL-12 production is necessary for maintaining protective Th1 responses during MTB infection (10). Our data now implicate CD11b^{high} DC as the cell population that maintains IL-12p40 production during established mycobacterial infection. A more extensive phenotypic analysis of DC p40 production in yet40 mice infected with MTB is needed to confirm this hypothesis.

During BCG infection, the initial detection of IL-12p40 production by CD11b^{high} SpDC coincided with a global increase in SpDC numbers and with the formation of granulomas, suggesting a role for mycobacterial-induced inflammation in the expansion of IL-12p40$^+$ cells in the spleen. The spleen is known to contain precursor populations that give rise to DC under steady-state conditions (36). In contrast, under inflammatory conditions, SpDC can arise from infiltrating monocytes (37) and these DC closely resemble the monocyte-derived CD11c^{int}CD11b^{high}Mac-3$^+$ TNF/iNOS-producing DC observed during *Listeria monocytogenes* infection (38). Although BCG infection clearly triggers the recruitment in tissues of large numbers of lysozyme M$^+$ inflammatory monocytes (Fig. 5*A* and Ref. 27), IL-12p40 expression was not detected in splenic CD11c$^-$F4/80$^+$CD11b$^+$Gr-1^{low} cells, indicating that recently recruited monocytes are not a major source of the cytokine. Nevertheless, it is still possible that the IL-12p40-producing SpDC studied here derive from infiltrating monocytes and since some of these cells are both CD11c^{int} and CD11b^{high} (Fig. 2, *A* and *C*) they may indeed be related in phenotype to Tip-DC. Consistent with this interpretation, it has been shown that when exogenous monocytes are transferred into MTB-infected recipients, they acquire a DC phenotype when recruited to lymph nodes (39).

To more directly address the role of local granulomatous inflammation in supporting DC IL-12p40 production, we asked whether anti-TNF-α treatment, which is known to lead to disruption of granulomas (33, 40, 41), would affect the IL-12 response. We detected a major reduction in the frequency of IL-12p40-producing DC at time points before obvious dissolution of the granulomatous lesions. This effect of TNF-α depletion, which is difficult to formally characterize, could for instance be due to a preferential loss of p40-expressing DC following anti-TNF-α treatment or enhanced TNF-α-mediated differentiation of monocytes into p40$^+$ DC. We believe instead that this observation reflects a direct enhancing effect of TNF-α on IL-12p40 production since in agreement with previous studies on IFN-γ-primed macrophages (42), neutralization of TNF-α partially ablated mycobacterial-induced IL-12p40 production by DC in vitro. Regardless of the exact mechanism involved, our findings raise the possibility that the loss of granuloma integrity seen in mice treated with TNF-α antagonists (43) is due in part to a reduction in IL-12p40-producing CD11b^{high} DC.

Contrary to what was observed in yet40 mice with established BCG infection, TNF-α neutralization did not affect the initial IL-12p40 response triggered 5 h after BCG inoculation (data not shown). Thus, it appears that CD11b^{low} DC, which mediate the early IL-12p40 response, are not subject to the same tissue-derived inflammatory signals such as TNF-α that regulate the IL-12p40 response of CD11b^{high} DC. Nevertheless, IL-12p40 expression by CD11b^{low} DC can be influenced by other cellular factors such as CD40-CD40L (44) and as revealed in a recent study on *Leishmania donovani* infection, by VLA4-VCAM-1 interactions (45).

A striking observation made in our in situ examination of BCG-infected yet40 mice is that 3 wk after infection most IL-12p40-expressing cells were visualized in sites distal to the labeled BCG themselves. This relationship between BCG infection and IL-12p40 expression in individual cells was also evident during the

first 5-h phase of infection, although some IL-12p40$^+$ cells in the white pulp but in close proximity to the marginal zone were found to be infected with BCG (data not shown). Importantly, a further series of experiments performed in vitro indicated that the majority of IL-12p40-producing DC are those containing few or no mycobacteria and that direct infection itself exerts a negative effect on DC-derived IL-12p40 production and DC expression of costimulatory molecules. Suppression of APC function by mycobacteria has been noted previously in a number of studies (46–49), but before the present report has never formally been shown to involve inhibitory effects that specifically target the infected subpopulation. Although not examined here, it is possible that mechanisms similar to those described for APC suppression by mycobacteria also explain the effects on DC IL-12p40 production revealed in our experiments.

The current study establishes CD11b^{high} DC as a major source of IL-12p40 in vivo during mycobacterial infection and suggests that sustained expression of the cytokine by these cells depends not only on pathogen-derived triggers but also on host signals such as TNF-α which are delivered as a consequence of tissue inflammation. This conclusion derives primarily from studies in a BCG model and it will be important to determine whether similar requirements exist during MTB infection where the continued production of IL-12 has been shown to be necessary for Th1-dependent host resistance. If such a role can be confirmed, it will implicate DC as an important target for vaccine design and the loss in DC IL-12 production as a potential factor contributing to pathogenesis.

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Disclosures

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