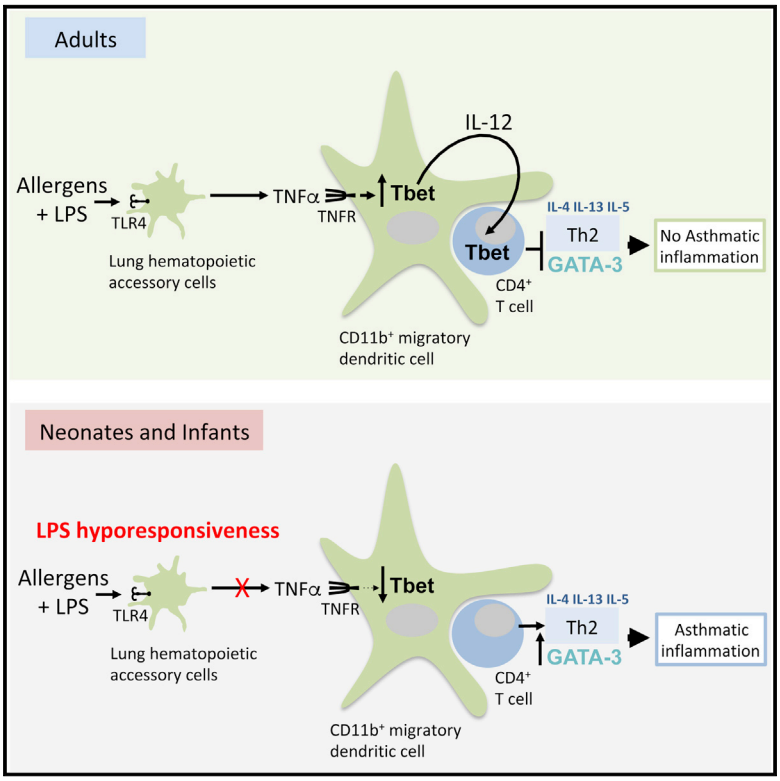
Article

Immunity

Impaired Tumor-Necrosis-Factor-α-driven Dendritic Cell Activation Limits Lipopolysaccharide-Induced Protection from Allergic Inflammation in Infants

Graphical Abstract



Highlights

- Infant mice require high doses of LPS to suppress Th2-driven allergic asthma to HDMs
- T-bet^{+} lung CD11b^{+} DCs inhibit Th2 cell development after HDM + LPS sensitization
- T-bet expression on CD11b^{+} DCs depends on TNF-α production and TNFR signaling
- Infants have an impaired ability to induce TNF-α and T-bet^{+}CD11b^{+} DCs in response to LPS

Authors

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In Brief

Children have a higher risk of developing asthma in low-LPS "clean" environments than adults. Bachus et al. demonstrate that infant mice require higher doses of LPS than adult mice to prevent Th2-dependent allergic responses because of the decreased ability to induce LPS-driven TNF-α production and TNFR-mediated CD11b^{+} dendritic cell activation for Th2 cell suppression.

Bachus et al., 2019, Immunity *50*, 225–240
January 15, 2019 Published by Elsevier Inc.
https://doi.org/10.1016/j.immuni.2018.11.012

CellPress

Immunity
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Impaired Tumor-Necrosis-Factor-α-driven Dendritic Cell Activation Limits Lipopolysaccharide-Induced Protection from Allergic Inflammation in Infants

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https://doi.org/10.1016/j.immuni.2018.11.012

SUMMARY

Infants have a higher risk of developing allergic asthma than adults. However, the underlying mechanism remains unknown. We show here that sensitization of mice with house-dust mites (HDMs) in the presence of low-dose lipopolysaccharide (LPS) prevented T helper 2 (Th2) cell allergic responses in adult, but not infant, mice. Mechanistically, adult CD11b$^+$ migratory dendritic cells (mDCs) upregulated the transcription factor T-bet in response to tumor necrosis factor-α (TNF-α), which was rapidly induced after HDM + LPS sensitization. Consequently, adult CD11b$^+$ mDCs produced interleukin-12 (IL-12), which prevented Th2 cell development by promoting T-bet upregulation in responding T cells. Conversely, infants failed to induce TNF-α after HDM + LPS sensitization. Therefore, CD11b$^+$ mDCs failed to upregulate T-bet and did not secrete IL-12 and Th2 cell responses normally developed in infant mice. Thus, the availability of TNF-α dictates the ability of CD11b$^+$ mDCs to suppress allergic Th2-cell responses upon dose-dependent endotoxin sensitization and is a key mediator governing susceptibility to allergic airway inflammation in infant mice.

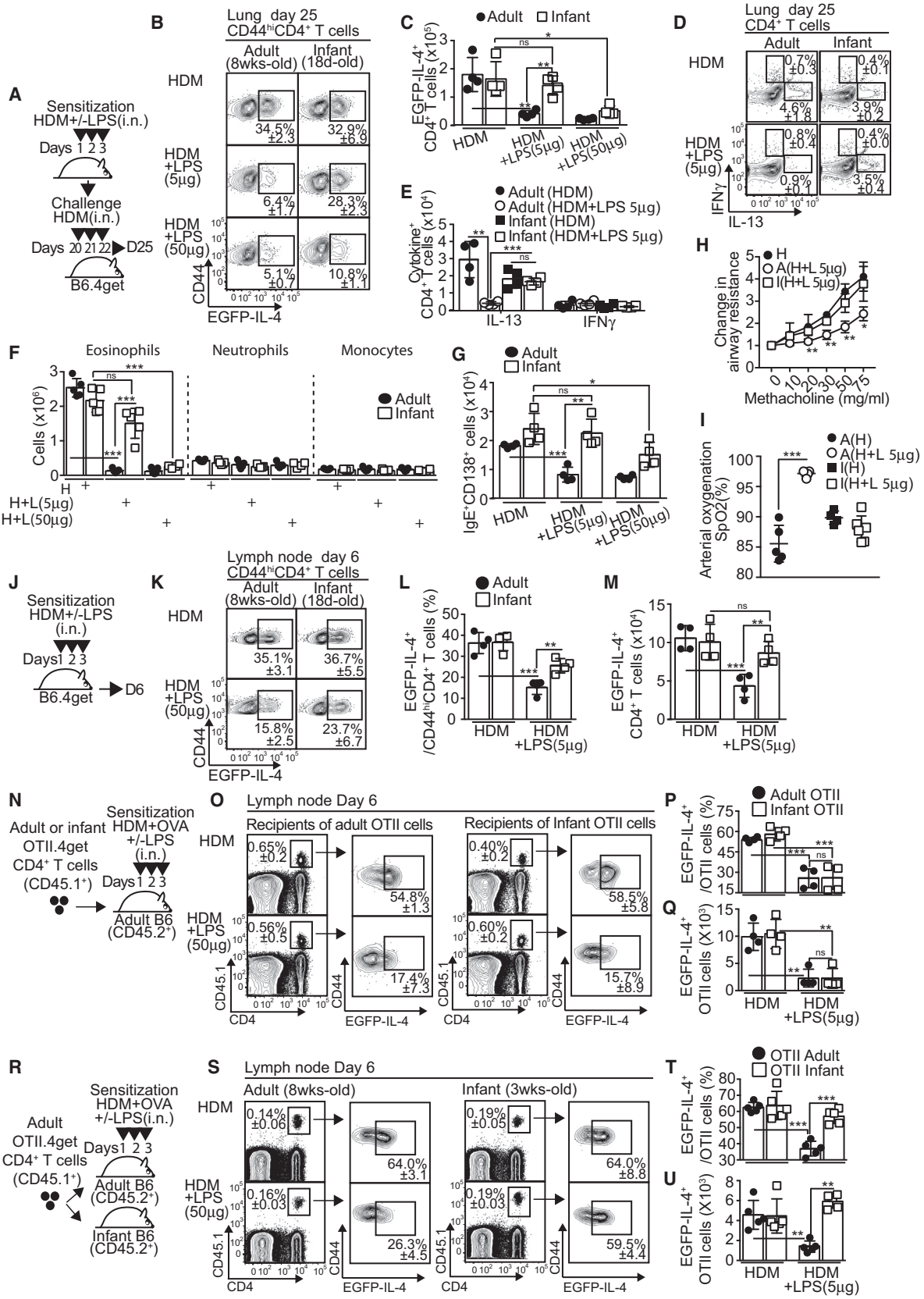
INTRODUCTION

Asthma is the most common chronic disease of childhood and currently affects ~10% of school-aged children in the US (Masoli et al., 2004). About 95% of those children begin developing asthma in very early childhood, normally before they turn 5 years of age (Masoli et al., 2004). Most cases of pediatric asthma are triggered by early sensitization to common environmental allergens, which ultimately leads to the activation of T helper 2 (Th2) cells and the development of chronic Th2-cell-driven lung inflammation (Reynolds and Finlay, 2017). Despite the epidemiological and mechanistic studies, however, the underlying mechanisms for the high susceptibility to airway allergic inflammation and asthma development in infants remain elusive.

The incidence of allergic airway disease has increased over the past decades in industrialized countries (Masoli et al., 2004). Although numerous environmental factors associated with this change of lifestyle might have contributed to this rise, the hygiene hypothesis proposes that the decreased exposure to microbial products is one of the main drivers of allergic airway disease. Supporting this idea, exposure to microbial products, such as lipopolysaccharide (LPS), protects from the development of experimental-allergen-induced asthma (Daan de Boer et al., 2013; Schuijs et al., 2015). In addition, infants sensitized to common circulating allergens in a "clean" environment are at a higher risk of developing asthma later in life than infants exposed to high levels of enviromental endotoxin (Gereda et al., 2000; Reynolds and Finlay, 2017; Schuijs et al., 2015; Stein et al., 2016; Zhu et al., 2010), thus suggesting a particular requirement for high endotoxin exposure during infancy for allergic asthma protection, although this requirement is not fully understood.

Dendritic cells (DCs) are the main antigen-presenting cells for T cell activation and polarization. In lymph nodes (LNs), DCs can be broadly divided into LN-resident DCs (rDCs) and migratory DCs (mDCs), which differentiate in peripheral tissues and migrate constantly through afferent lymphatics to the draining LN. Both rDCs and mDCs contain two major subsets: IRF8-dependent CD103$^+$ DCs (recently named cDC1s) and IRF4-dependent CD11b$^+$ DCs (recently named cDC2s) (Guilliams et al., 2014). Although the roles of the various DC subsets are not fully understood, CD11b$^+$ mDCs have been shown to be critical for the development of Th2 cell responses to common allergens (León, 2017; Plantinga et al., 2013). In contrast, CD103$^+$ mDCs are likely to prevent the development of allergic inflammation (Conejero et al., 2017). Whether alterations in individual DC subsets during infancy contribute to asthma susceptibility remains unexplored.

Here, we show that CD11b$^+$ mDCs from adult mice upregulated the transcription factor T-bet and produced interleukin-12 (IL-12) after house-dust-mite (HDM) sensitization in the presence of LPS. As a consequence, CD4$^+$ T cells interacting with CD11b$^+$



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mDCs in adult mice upregulated T-bet, which precluded Th2 cell differentiation and subsequent pathogenic allergic responses to HDMs. We found that the upregulation of T-bet on $CD11b^+$ mDCs was dependent on TNF-α production and TNFR signaling. Adults rapidly produced TNF-α in response to low-dose LPS sensitization; however, infant mice had an impaired ability to produce TNF-α in response to LPS. As a result, $CD11b^+$ mDCs from infant mice failed to upregulate T-bet and IL-12 in low-LPS conditions. Thus, $CD4^+$ T cells interacting with $CD11b^+$ mDCs in infants failed to receive IL-12 signals, did not upregulate T-bet, and fully differentiated into pathogenic Th2 cells. Thus, whereas low-LPS exposure during HDM sensitization protected adult mice from developing Th2-driven allergic asthma, infant mice required higher doses of LPS. Collectively, our data demonstrate that LPS prevents Th2-dependent allergic responses with different thresholds in adults and infants and provide a plausible mechanism underlying the higher susceptibility to allergic airway inflammation observed in children.

RESULTS

LPS Inhibits Th2-Cell-Mediated Immunity to Inhaled HDMs with Different Thresholds in Infant and Adult Mice

To test whether LPS influenced Th2 cell response to HDM sensitization in infancy and adulthood, we intranasally (i.n.) sensitized adult ($\geq$8-week-old) and infant (18-day-old) IL-4 reporter B6.4get mice with HDMs in the presence or absence of different amounts of LPS (sensitization phase; Figure 1A). We then challenged them i.n. with HDMs (challenge phase; Figure 1A) and determined the frequency (Figure 1B) and number (Figure 1C) of $GFP^+CD44^{hi}CD4^+$ T cells in the lungs. GFP^+CD4^+ T cells similarly accumulated in the lungs of adult and infant mice that had been sensitized with HDMs (Figures 1B and 1C) but failed to accumulate in adult mice that had been sensitized with HDMs in the presence of either low (5 μg; LPS^{lo}) or high (50 μg; LPS^{hi}) doses of LPS (Figures 1B and 1C). Similarly, LPS^{hi} significantly prevented the accumulation of GFP^+CD4^+ T cells in infant mice (Figures 1B and 1C). Importantly, however, LPS^{lo} exposure failed to prevent the expansion of GFP^+CD4^+ T cells in infant mice (Figures 1B and 1C). As a result, GFP^+CD4^+ T cells largely accumulated in the lungs of HDM + LPS^{lo}-sensitized infants relative to adult counterparts (Figure 1C). We next sensitized and challenged adult and infant C57BL/6J (B6) mice as described in Figure 1A and quantified IL-13^+ and IFN$^+$ $CD4^+$ T cells in the lungs. As predicted, IL-13^+CD4^+ T cells were less abundant in adults that had been initially sensitized with HDM + LPS^{lo} than in those initially sensitized with HDMs only (Figures 1D and 1E). Conversely, this effect was not observed in infants (Figures 1D and 1E). LPS^{lo} sensitization did not induce IFN-γ^+CD4^+ T cells in the lungs (Figures 1D and 1E), but inhalation of LPS^{hi} resulted in IFN-γ^+CD4^+ T cells (Figures S1A and S1B). These data show that relatively higher amounts of endotoxin exposure are necessary for preventing the accumulation of Th2 cells in infants. In agreement with these results, whereas LPS^{lo} administration at the time of initial sensitization prevented eosinophilic airway inflammation (Figures 1F, S1C, and S1D), airway goblet cell hyperplasia (Figure S1F) and accumulation of IgE^+CD138^+ antibody-secreting cells (IgE^+ ASCs) in the lung-draining, mediastinal lymph node (mLN) (Figures 1G and S1E) in adult challenged mice, it failed to do so in infants. Moreover, LPS^{lo} sensitization in adult, but not in infant, mice reduced airway responsiveness to methacholine (Figure 1H) and improved peripheral-blood arterial oxygen saturation (SpO2) (Figure 1I). Similar differences from adults were observed in neonatal (7-day-old), early infant (14-day-old), and infant (18-day-old) mice (Figures S2A–S2J). Altogether, these data indicate that exposure to relatively low endotoxin amounts, in a defined time window from the neonatal to infant period, fails to prevent the development of Th2 cell responses and allergic inflammation to inhaled HDMs.

The Environment of Infants Is More Resistant to LPS and More Prone to Prime HDM-Specific Th2 Cell Responses Than That of Adults

The development of allergen-specific Th2 effector cells that migrate to the lungs requires T cell priming in the mLN, which occurs after initial sensitization (Ballesteros-Tato et al., 2016). Therefore, we next enumerated $GFP^+CD44^{hi}CD4^+$ T cells in the mLN of adult and 18-day-old infant B6.4get mice during the sensitization phase (Figure 1J). The frequency (Figures 1K and 1L) and number (Figure 1M) of GFP^+CD4^+ T cells were significantly lower in HDM + LPS^{lo}-sensitized adult mice than in HDM-sensitized adult mice. In contrast, LPS^{lo} did not prevent the accumulation of GFP^+CD4^+ T cells in infants (Figures 1K–1M). Similar results were found in 7- and 14-day-old mice (Figure S2K). Lung neutrophilia were not examined because they are unlikely to significantly affect Th2 cell priming. These results suggest that low-dose LPS prevents Th2 cell priming in the mLN after HDM sensitization in adult, but not in neonatal or infant, mice. Next, we tested whether the differential capacity of low-dose LPS to prevent Th2 cell priming in adults and infants is

Figure 1. LPS^{lo} Sensitization in Infants Does Not Prevent HDM-Induced Th2 Cell Priming or Development of Lung Th2 Cell Responses in Adult Life

(A–I) B6.4get (A–C) or B6 (D–I) adults (>8 weeks old) and infants (18 days old) were i.n. treated with 50 μg HDMs plus different concentrations of LPS for 3 days. On day 20, mice were i.n. challenged with 50 μg HDMs daily for 3 days and analyzed on day 25 (A). Shown are frequencies and numbers of IL-4^+ ($EGFP^+$) (B and C) and IL-13^+ and IFN-γ^+ (D and E) $CD4^+$ T cells in the lungs. Numbers of eosinophils, neutrophils, and monocytes in the lungs are shown in (F). Numbers of IgE^+ ASCs in the mLN are shown in (G). Changes in lung resistance (cm H_2O/mL/s) from baseline were analyzed via mechanical ventilation with the flexiVent system (H). Blood oxygen saturation (SpO2) was determined by pulse oximetry (I).

(J–M) B6.4get adults and 18-day-old infants were i.n. treated with 50 μg HDM ± 5 μg LPS for 3 days and analyzed on day 6 (J). Shown are frequencies (K and L) and numbers (M) of $EGFP^+CD4^+$ T cells in the mLN.

(N–U) Adult B6 mice were adoptively transferred with 25 × 10^3 $CD45.1^+$ OT-II.4get cells from adult or infant donor mice (N–Q), or adult and 18-day-old infant B6 mice were adoptively transferred with 25 × 10^3 $CD45.1^+$ OT-II.4get cells from naive adult donor mice (R–U). Recipients were i.n. treated with 50 μg HDM + 5 μg OVA ± 5 μg LPS for 3 days and analyzed on day 6. Shown are frequencies (O, P, S, and T) and numbers (Q and U) of donor $EGFP^+$ OT-II T cells in the mLN.

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (unpaired Student's t test). Data are representative of at least two independent experiments (mean and SD of four or five mice per group). Please also see Figures S1 and S2.

dictated by intrinsic differences in the CD4$^+$ T cell compartment (Figures 1N–1Q). We transferred 4get.OT-II TCR-transgenic CD4$^+$ T cells from adult or infant mice into congenic adult recipients (Figure 1N). Recipients were then i.n. sensitized with HDM + endotoxin-free ovalbumin (HDM + OVA) ± LPS^{lo}, and donor 4get.OT-II (CD45.1$^+$) cells were analyzed for the expression of GFP-IL-4 in the mLN (Figures 1O–1Q). Data showed that the differential capacity of LPS^{lo} to prevent Th2 cell development in adults and infants was not due to intrinsic differences in the CD4$^+$ T cells. We next transferred 4get.OT-II cells from adult mice into adult or infant recipients (Figure 1R). Those were then sensitized with HDM + OVA ± LPS^{lo}, and donor-derived GFP$^+$ cells were analyzed (Figures 1S–1U). LPS^{lo} failed to prevent the accumulation of donor-derived GFP$^+$ 4get.OT-II cells when developing in infant, but not in adult, recipients (Figures 1S–1U). i.n. HDM + LPS delivery was similarly effective in adults and infants (Figures S2L–S2N). Similar results were obtained when sensitization was given intratracheally (i.t.) rather than i.n. (Figures S2O–S2Q). Thus, whereas low-LPS prevents Th2 cell differentiation in adult mice after HDM sensitization, the environment of the infants is more resilient to LPS; hence, infants are at higher risk for priming allergen-specific Th2 cell responses in the presence of low doses of endotoxin.

Infants Do Not Induce T-bet Expression in T Cells under LPS^{lo} Sensitization

T-bet suppresses the Th2-cell-associated program (Zhu et al., 2012). To test whether CD4$^+$ T cells developing in an infant or adult environment differently upregulate T-bet, we transferred 4get.OT-II cells from adult mice into either adult or infant recipients, sensitized them, and analyzed the dynamics of T-bet expression (Figures 2A–2C and S3A). As expected, LPS^{lo} treatment prevented the accumulation of 4get.OT-II GFP$^+$ cells in adult mice, but not infant mice, from day 5 (Figure 2B). Importantly, 4get.OT-II cells expressed higher amounts of T-bet when developing in adults sensitized with HDM + OVA + LPS^{lo} than when developing in non-LPS-sensitized recipients (Figure 2C). Further, this upregulation of T-bet occurred concomitantly with the downregulation of GATA-3 (Figures S3B–S3D). In contrast, 4get.OT-II cells failed to upregulate T-bet in HDM + OVA + LPS^{lo}-sensitized infants (Figure 2C). These results indicate that, although LPS^{lo} promotes T-bet expression in CD4$^+$ T cells differentiating in adult mice, CD4$^+$ T cells fail to upregulate T-bet when developing in infants. We next investigated whether T-bet upregulation to LPS is necessary for preventing Th2 cell differentiation. Thus, we co-transferred wild-type (WT) (CD45.2$^+$CD45.1$^+$) and *Tbx21*$^{-/-}$ (CD45.2$^+$) 4get.OT-II cells into CD45.1$^+$ adult recipients, sensitized them, and analyzed the progeny from the WT and *Tbx21*$^{-/-}$ donors (Figure 2D). As expected, the frequency of GFP$^+$ cells within the WT 4get.OT-II cells was lower in the mLN of HDM + OVA + LPS^{lo}-sensitized mice than in HDM + OVA-sensitized mice (Figures 2E and 2F). In contrast, the frequency of GFP$^+$ cells within the *Tbx21*$^{-/-}$ 4get.OT-II cells was equivalent in both groups (Figures 2E and 2F). We next analyzed the frequency of IL-13$^+$ cells within the donor WT and *Tbx21*$^{-/-}$ OT-II cells in the lung after the challenge phase (Figure 2G). As predicted, the frequency of IL-13$^+$ WT OT-II cells was lower in mice that had been initially sensitized with HDM + OVA + LPS^{lo} than in those that had been sensitized with HDM + OVA (Figures 2H and 2I). In contrast, the frequency of IL-13$^+$*Tbx21*$^{-/-}$ OT-II cells was equivalent in both groups (Figures 2H and 2I). Thus, expression of T-bet by CD4$^+$ T cells in response to low-dose endotoxin is important for suppressing the endogenous Th2 cell differentiation program in response to HDMs.

Lung mDCs from Adult and Infant Mice Differ in Their Ability to Induce T-bet in Responding T Cells

Lung mDCs are essential for priming Th2 cells to HDMs (León, 2017). Thus, we tested whether LPS^{lo} administration differentially affects adult and infant mDCs. Given that expression of T-bet by CD4$^+$ T cells was important to suppressing Th2 cell development after HDM + LPS^{lo} sensitization (Figures 2A–2I), we first tested the ability of infant and adult DCs to activate naive CD4$^+$ T cells and promote T-bet expression. mLN CD11c$^+$ DCs were co-cultured with CFSE-labeled naive OT-II cells (Figure 2J). O-TII proliferated equally when primed with CD11c$^+$ cells from adults or infants (Figure 2K). OT-II cells primed by CD11c$^+$ cells from HDM + OVA + LPS^{lo}-sensitized adults upregulated T-bet in relation to OT-II cells primed by CD11c$^+$ cells from HDM + OVA-sensitized adults (Figures 2L and 2M). In contrast, OT-II cells primed by CD11c$^+$ cells from HDM + OVA + LPS^{lo}-sensitized infants failed to upregulate T-bet (Figures 2L and 2M). These results indicate that DCs from infant and adult mice differ in their ability to induce T-bet expression in T cells after HDM + LPS^{lo} sensitization. We next characterized mLN mDC subsets in adult and infant mice as previously described (Ballesteros-Tato et al., 2010). Frequency (Figure S3E) and number (Figure S3F) of CD103$^+$mDCs decreased after HDM + LPS^{lo} sensitization in adult mice. In contrast, CD11b$^+$ mDCs largely accumulated (Figures S3E and S3G). Similar results were obtained in infants (Figures S3E–S3G). We next assessed the capacity of DC subsets to capture and transport HDM-derived antigens (Ags) into the mLN. Thus, we i.n. sensitized adults and infants with Alexa-647-labeled HDM + LPS^{lo} and analyzed Alexa-647$^+$CD11c$^+$ DCs in the mLN (Figures S3H–S3K). Although some CD103$^+$ mDCs were Alexa-647$^+$, the vast majority of HDM-bearing, Alexa-647$^+$CD11c$^+$ DCs were CD11b$^+$ mDCs in both adults and infants (Figures S3H–S3K). Moreover, the frequency (Figure S3H) and number (Figures S3I–S3K) of HDM-bearing, Alexa-647$^+$ CD11b mDCs and CD103$^+$mDCs were identical in infants and adults. Thus, the differences in Th2 cell responses between adults and infants after HDM + LPS^{lo} sensitization were not due to differences in the number, or the capacity, of mDC subsets to capture and transport HDM-derived Ags into the mLN but to their functional capacity to induce T-bet expression in T cells.

T-bet Expression by CD11b$^+$mDCs Is Required for Preventing Th2 Cell Responses to HDMs

Few reports have suggested that intrinsic T-bet expression might fine-tune the capacity of DCs to differentially induce Th1 and Th2 cells (Lipscomb et al., 2009; Lugo-Villarino et al., 2003). Thus, we next used T-bet-ZsGreen reporter mice (Zhu et al., 2012) to investigate whether infant and adult mDCs upregulate T-bet in response to HDM + LPS sensitization (Figures 3A–3D). CD11b$^+$ mDCs from HDM + LPS^{lo}-sensitized adult mice upregulated T-bet expression after 48 hr in comparison with that of HDM-sensitized mice (Figures 3A and 3C). In

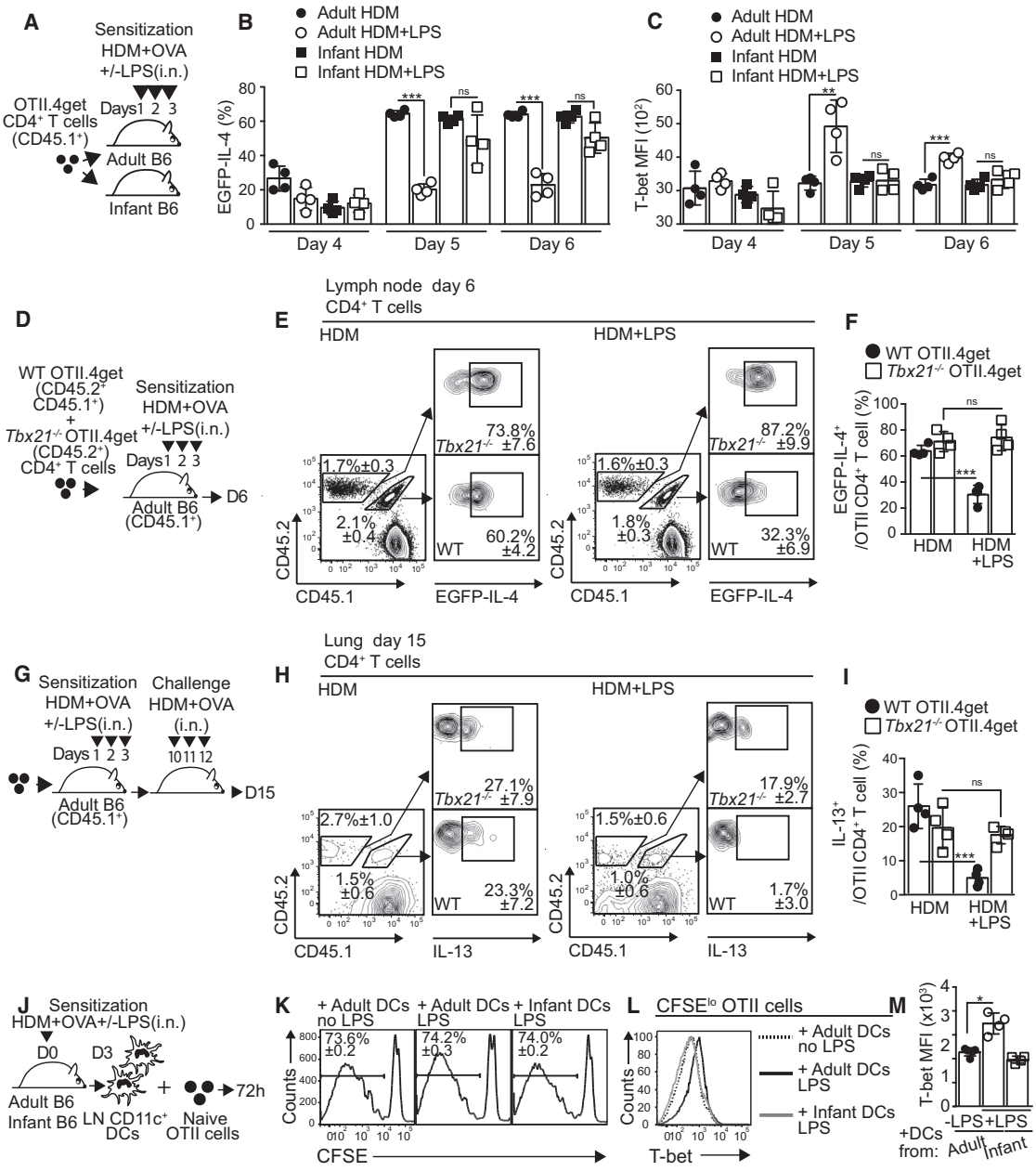


Figure 2. T-bet Induction by Responding T Cells Is Required for Suppressing the Endogenous Th2 Cell Differentiation Program

(A–C) Adult and 18-day-old infant B6 mice were transferred with 25 × 10^3 CD45.1$^+$ OT-II.4get cells at day 0, i.n. treated with 50 μg HDM + 5 μg OVA ± 5 μg LPS for 3 days starting on day 1 (A) and analyzed on days 4, 5, and 6. Shown are frequencies of EGFP$^+$ cells (B) and mean fluorescence intensity (MFI) of T-bet expression (C) in donor mLN OT-II cells.

(D–I) Adult CD45.1$^+$ B6 mice were co-transferred with 15 × 10^3 CD45.1$^+$CD45.2$^+$ OT-II.4get cells + 15 × 10^3 CD45.2$^+$*Tbx21*$^{-/-}$ OT-II.4get cells and treated as described above (D). Shown are frequencies of donor EGFP$^+$ WT and *Tbx21*$^{-/-}$ OT-II cells in the mLN at day 6 (E and F). On day 10, recipients were i.n. challenged with 50 μg HDM + 5 μg OVA for 3 days and analyzed on day 15 (G). Shown are frequencies of donor IL-13$^+$ WT and *Tbx21*$^{-/-}$ OT-II cells in the lungs (H and I).

(J–M) Day-3 CD11c$^+$ mLN DCs from HDM + OVA ± LPS-treated adults and 18-day-old infants were co-cultured for 3 days with CFSE-labeled OT-II cells (J). Shown are frequencies of CFSE^{lo} OT-II cells (K), a representative overlaid histogram of T-bet expression (L), and MFI of T-bet expression (M) in CFSE^{lo} OT-II cells. *p < 0.05, **p < 0.01, ***p < 0.001 (unpaired Student's t test). Data are representative of three independent experiments (mean and SD of four mice per group). Please also see Figure S3.

contrast, CD11b$^+$ mDCs from HDM + LPS^{lo}-sensitized infants only modestly upregulated T-bet expression (Figures 3A and 3C). Notably, CD103$^+$ mDCs (Figures 3B and 3D) failed to express T-bet. Similar results were obtained when T-bet expression was analyzed by intracellular staining (Figures 3E and 3F). Members of the IFN regulatory factor (IRF) family play essential roles in the functional activation of DCs (Gao et al., 2013; Takaoka et al., 2005; Taki et al., 1997; Williams et al., 2013).

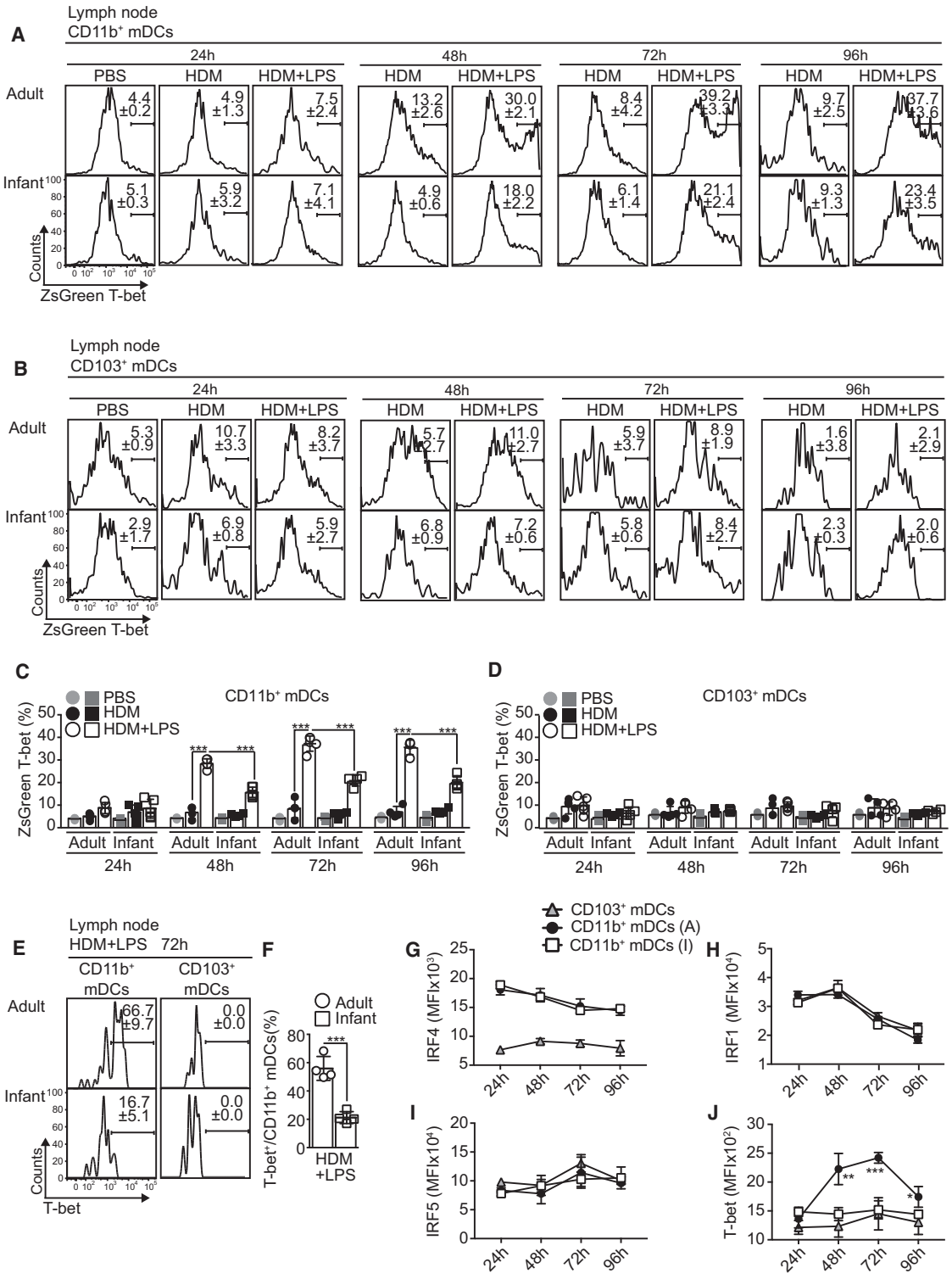


Figure 3. Infant CD11b$^+$ mDCs Fail to Upregulate T-bet Expression in Response to LPS^{lo} Sensitization

(A–D) T-bet-ZsGreen reporter adults and 18-day-old infants were i.n. treated with HDM ± 5 μg LPS. Shown are frequencies of T-bet-expressing (ZsG$^+$) CD11b$^+$ mDCs (A and C) and CD103$^+$ mDCs (B and D).

(E–J) B6 adult and 18-day-old infant mice were i.n. treated with HDM + 5 μg LPS. Shown are frequencies of T-bet expression at 72 hr (E and F) and kinetics of IRF4 (G), IRF1 (H), IFR5 (I), and T-bet (J) expression in mLN CD11b$^+$ and CD103$^+$ mDCs.

***p < 0.001 (unpaired Student's t test). Data are representative of at least two independent experiments (mean and SD of three or four mice per group).

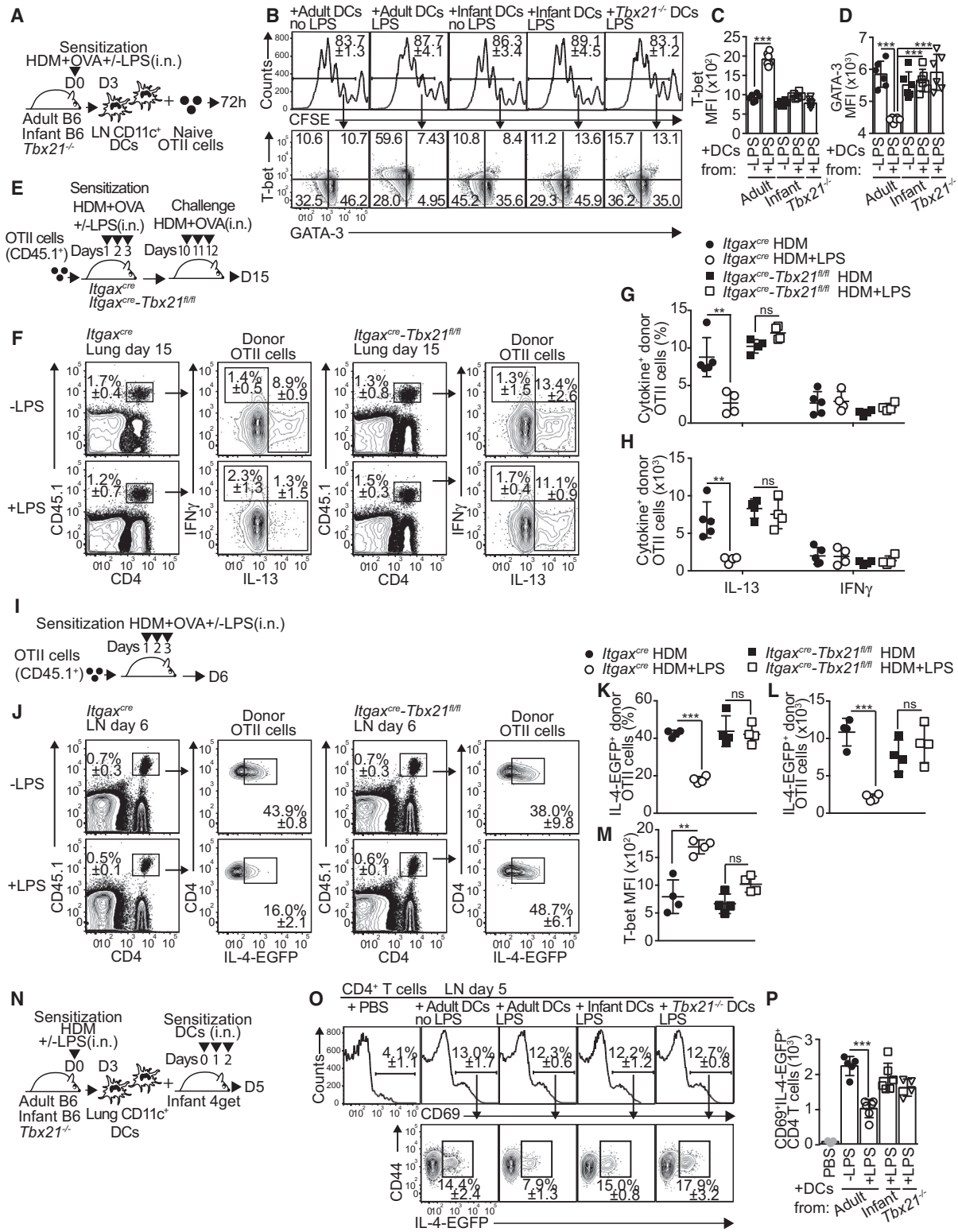


Figure 4. T-bet Expression by DCs Is Required for Preventing Allergen-Specific Th2 Cell Responses upon LPS^{lo} Exposure

(A–D) Day-3 CD11c$^+$ mLN DCs from HDM + OVA ± LPS-treated adult and 18-day-old infant B6 mice or *Tbx21*$^{-/-}$ mice were co-cultured for 3 days with CFSE-labeled OT-II cells (A). Shown are frequencies of CFSE^{lo}OT-II cells (B), frequencies of T-bet and GATA-3 expression (B), and MFI of T-bet (C) and GATA-3 (D) expression in CFSE^{lo}OT-II cells.

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Thus, we also investigated whether HDM + LPS^{lo} sensitization differentially affects the expression of IRF members in infant and adult mDCs. Although CD11b^{+} mDCs from HDM + LPS^{lo}-sensitized adult and infant mice differentially upregulated T-bet expression (Figure 3J), no differences were observed in the expression of IRF4 (Figure 3G), IRF1 (Figure 3H), or IRF5 (Figure 3I). Together, these data indicate that CD11b^{+} mDCs from infant mice fail to upregulate T-bet expression in response to HDM + LPS^{lo} sensitization. To address whether T-bet expression by CD11b^{+} mDCs is required for inducing T-bet expression in T cells, we used CD11c^{+} cells from the mLN of HDM + OVA ± LPS^{lo}-sensitized *Tbx21*$^{-/-}$ mice or adult and infant B6 mice to prime OT-II cells *in vitro* (Figure 4A). OT-II cells proliferated equally well in all conditions (Figure 4B). Compared with non-LPS-sensitized adult-derived DCs, however, only CD11c^{+} cells from the mLN of HDM + LPS^{lo}-sensitized B6 adults, but not B6 infants or *Tbx21*$^{-/-}$ mice, induced significant T-bet expression (Figures 4B and 4C). Correlating with this, OT-II cells activated in the presence of CD11c^{+} cells from HDM + LPS^{lo}-sensitized B6 adults expressed low amounts of GATA-3 (Figures 4B and 4D). These data suggest that T-bet expression by DCs is required for inducing T-bet and preventing GATA-3 expression in responding T cells. To address whether T-bet expression by DCs is required for preventing allergen-specific Th2 cell responses *in vivo*, we crossed *Itgax-cre-EGFP* (Stranges et al., 2007) mice to *Tbx21*$^{fl/fl}$ mice (Intlekofer et al., 2008) to generate mice in which T-bet is conditionally deleted in CD11c^{+} cells (*Itgax*^{cre}-*Tbx21*$^{fl/fl}$). Conditional deletion of T-bet in CD11c^{+} cells did not lead to defects in mDC-subset development (Figures S4A and S4B). Importantly, MHCII^{hi}CD11c^{+} DCs from the mLN of *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ mice, but not CD11c^{-} cells, expressed high amounts of GFP (Figure S4C). All CD11c^{hi} DCs, including CD103^{+} mDCs, CD11b^{+} mDCs, and rDCs, expressed GFP (Figure S4D) and were expected to have equimolar expression with Cre (Stranges et al., 2007). Plasmacytoid DCs expressed lower amounts of GFP than other DC subpopulations (Figure S4D) (Stranges et al., 2007). As predicted, mLN CD11b^{+} mDCs of HDM + LPS^{lo}-sensitized *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ mice failed to express T-bet (Figures S4E and S4F). Thus, we next transferred OT-II cells into either *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ or control mice. Recipients were then sensitized and challenged, and donor OT-II cells were analyzed in the lungs (Figure 4E). As expected, the frequency (Figures 4F and 4G) and number (Figure 4H) of IL-13^{+}OTII cells were reduced in control LPS^{lo}-sensitized mice compared with non-LPS-sensitized mice. Conversely, LPS^{lo} sensitization failed to prevent the accumulation of IL-13^{+} OT-II cells in the lungs of *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ mice (Figures 4F–4H). As a control, LPS^{lo} sensitization did not induce IFN-γ^{+}CD4^{+} T cells (Figures 4F–4H). Likewise, HDM + LPS^{lo} sensitization failed to prevent lung eosinophilia (Figures S4G and S4I) and accumulation of mLN IgE^{+} ASCs (Figures S4H and S4J) in *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ mice. Finally, 4get.OT-II cells were transferred into control or *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ mice, and the frequency of donor GFP^{+}OTII was calculated in the mLN after sensitization (Figure 4I). LPS^{lo} sensitization prevented the accumulation of GFP^{+} OT-II cells in the control recipients, but it failed to do so in *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ mice (Figures 4J–4L). Correlating with these results, LPS^{lo} sensitization induced T-bet upregulation in control, but not *Itgax*^{cre}-*Tbx21*$^{fl/fl}$, mice (Figure 4M). These results indicate that T-bet-expressing DCs are required for the suppression of Th2 cell responses to HDM in the presence of low doses of endotoxin. To further confirm these results, we used CD11c^{+} cells from the lungs of HDM ± LPS^{lo} -sensitized adult B6 and *Tbx21*$^{-/-}$ mice to i.n. sensitize naive infant B6.4get mice (Figure 4N). The frequency (Figure 4O) and number (Figure 4P) of GFP^{+}CD69^{+-} CD44^{hi}CD4^{+} responding T cells were determined in the mLN. As a control, some mice were also sensitized with infant DCs. Purified lung CD11c^{+} cells were enriched over 80% in mDCs (Figure S4L) and were recovered in similar numbers in the mLN of all groups (Figures S4M and S4N). Importantly, from the different donor mDC types, only the donor mDCs from HDM + LPS–-sensitized adult mice were able to prevent the accumulation of activated GFP^{+}CD4^{+} T cells in the infant recipients (Figures 4O and 4P). Collectively, these data suggest that upregulation of T-bet in lung mDCs is required for preventing Th2 cell responses to low-dose endotoxin and HDM sensitization.

CD11b^{+} mDCs from *Tbx21*$^{-/-}$ and Infant Mice Are Defective at Producing IL-12 upon Low-Dose Endotoxin Sensitization

We next made B6:*Tbx21*$^{-/-}$ mixed bone marrow (BM) chimeras to investigate the role of T-bet in DC function. In brief, irradiated B6 (CD45.1^{+}) recipient mice were reconstituted with a 50:50 combination of BM from B6 WT (CD45.1^{+}) and *Tbx21*$^{-/-}$ (CD45.2^{+}) donor mice (Figure 5A). WT:*Tbx21*$^{-/-}$ mice were sensitized with HDM ± LPS^{lo}, and mLN mDCs were analyzed. mDC subsets derived equally from the WT and *Tbx21*$^{-/-}$ progenitors (Figure S5A). Given than IL-12 production is upregulated in DCs after LPS activation (Trinchieri, 2003), we next assessed IL-12 production by mDC subsets. WT CD11b^{+} mDCs largely produced IL-12p70 in day-3 HDM + LPS^{lo}-sensitized mice relative to HDM-sensitized or day-1 HDM + LPS^{lo}-sensitized mice (Figures 5C and S5B). In contrast, sensitization in the presence of LPS^{lo} failed to promote IL-12p70 production by *Tbx21*$^{-/-}$ CD11b^{+} mDCs (Figures 5C and S5B). Conversely, equivalent low numbers of IL-12^{+} WT and *Tbx21*$^{-/-}$ CD103^{+} mDCs were detected in HDM- and HDM + LPS^{lo}-sensitized mice (Figures 5C and S5C). These results indicate that, although

(E–H) *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ and control mice were transferred with CD45.1^{+} OT-II.4get cells and i.n. treated with HDM + OVA ± 5 μg LPS. On day 10, mice were i.n. challenged with HDM + OVA and analyzed on day 15 (E). Shown are frequencies of donor OT-II cells (F) and frequencies (F and G) and numbers (H) of IL-13^{+} and IFN-γ^{+} OTII cells from the lungs.

(I–M) Mice sensitized as described above were analyzed at day 6 (I). Shown are frequencies of donor OT-II cells (J), frequencies (J and K) and numbers (L) of EGFP^{+} cells, and MFI of T-bet expression (M) in donor OT-II cells from the mLN.

(N–P) Day-3 CD11c^{+} lung mDCs from HDM + OVA ± LPS-treated adult and 18-day-old infant B6 mice or *Tbx21*$^{-/-}$ mice were i.n. transferred into B6.4get infant mice on days 0, 1, and 2. Recipient mice were analyzed on day 5 (N). Shown are frequencies (O) and numbers (P) of EGFP^{+} cells in CD69^{+}CD4^{+} T cells from the mLN.

$^{**}p < 0.01$, $^{***}p < 0.001$ (unpaired Student's t test). Data are representative of three independent experiments (mean and SD of four to six mice per group). Please also see Figure S4.

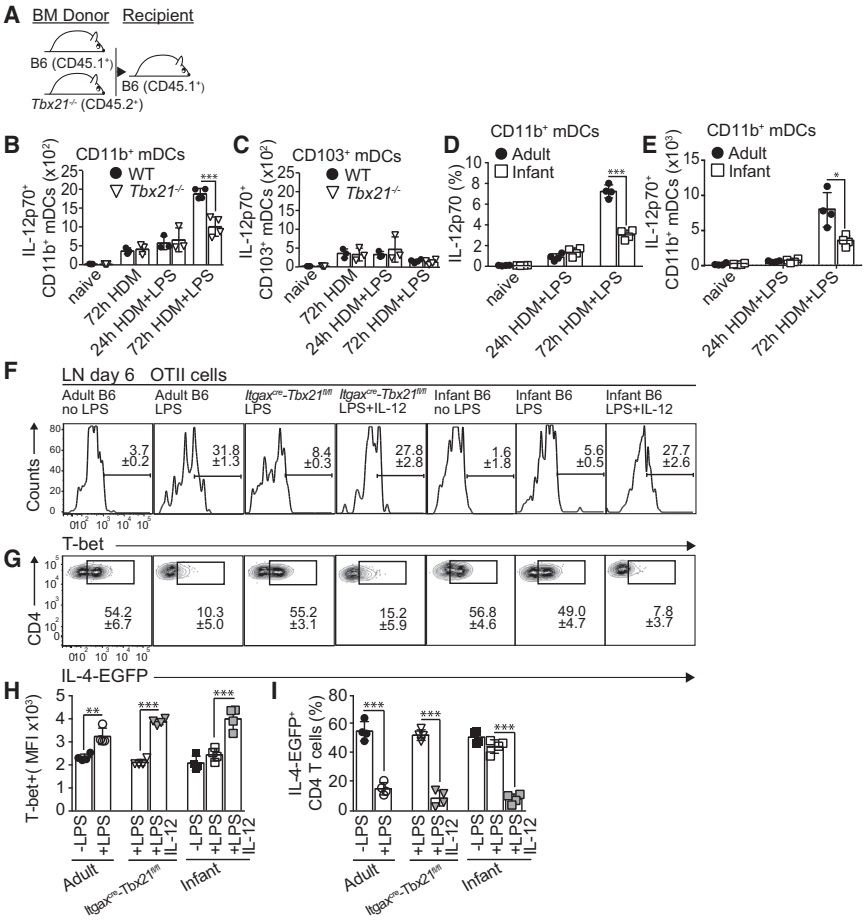


Figure 5. Infant and *Tbx21*$^{-/-}$ CD11b^{+} mDCs Are Intrinsically Impaired in Their Ability to Produce IL-12 and to Prevent Allergen-Specific Th2 Cell Responses upon LPS^{lo} Exposure

(A–C) B6 (CD45.1^{+}) mice were irradiated and reconstituted with a 1:1 mixture of BM from B6 (CD45.1^{+}) and *Tbx21*$^{-/-}$ (CD45.2^{+}) donors (A). Shown are numbers of IL-12p70^{+} WT and *Tbx21*$^{-/-}$ CD11b^{+} mDCs (B) and CD103^{+} mDCs (C) in the mLN of HDM ± LPS-treated reconstituted chimeric mice.

(D and E) Frequencies (D) and numbers (E) of IL-12p70^{+} CD11b^{+} mDCs in the mLN of HDM ± LPS-treated adult and 18-day-old infant B6 mice.

(F–I) Adult and 18-day-old infant B6 mice or *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ mice were transferred with CD45.1^{+} OT-II.4get cells and i.n. treated with HDM + OVA ± LPS ± 150 ng rIL-12p70 for 3 days. Shown are frequencies of T-bet expression (F and H) and EGFP^{+} cells (G and I) in donor OT-II cells from mLN on day 6.

*p < 0.05, ***p < 0.001 (unpaired Student's t test). Data are representative of two independent experiments (mean and SD of three to six mice per group). Please also see Figure S5.

T-bet is dispensable for the development of CD11b^{+} mDCs, it is intrinsically required for the capacity of these cells to produce sustained IL-12 in response to LPS. Corresponding with this, compared with those from adult mice, CD11b^{+} mDCs from HDM-sensitized infant mice failed to upregulate IL-12p70 production 3 days after LPS^{lo} administration (Figures 5D, 5E, and S5D). Although our data suggest that CD11b^{+} mDCs upregulate T-bet-dependent IL-12 production and suppress Th2 cell priming after HDM + LPS sensitization, a published report has suggested that CD103^{+} mDCs have the ability to restrain Th2 cell responses to HDMs through the production of IL-12 (Conejero et al., 2017). Thus, to test the role of CD103^{+} mDCs during LPS^{lo} sensitization, we analyzed *Batf3*$^{-/-}$ mice that selectively lack the CD103^{+}CD11b^{-} DC subset (Edelson et al., 2010). We transferred 4get.OT-II CD4^{+} T cells into either WT or *Batf3*$^{-/-}$ recipients, sensitized them, and analyzed them on day 6 (Figure S5E). LPS^{lo} sensitization abrogated GFP^{+} by donor OTII cells in both WT and *Batf3*$^{-/-}$ recipients (Figures S5F and S5G), suggesting that CD103^{+} mDCs are not necessary for suppressing Th2 cell priming in response to low-dose endotoxin sensitization.

Given that IL-12 induces T-bet expression in T cells (Zhu et al., 2012), we next tested whether recombinant IL-12 (rIL-12) administration during HDM + LPS^{lo} sensitization restores the capacity of low-LPS treatment to abrogate Th2 cell development in infant and *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ mice. Thus, we transferred 4get.OT-II CD4^{+} T cells into B6 adult, B6 infant, and *Itgax*^{cre}-*Tbx21t*$^{fl/fl}$ mice. Recipients were then sensitized with HDM + OVA ± LPS^{lo}, and PBS or rIL-12 was i.n. co-administered (Figure S5H). Expression of T-bet (Figures 5F and 5H) and GFP (Figures 5G and 5I) by donor 4get.OT-II cells was assessed in the mLN. As expected, sensitization in the presence of LPS^{lo} promoted T-bet upregulation (Figures 5F and 5H), thereby preventing GFP expression (Figures 5G and 5I) in B6 adults, but not in infant or *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ mice. Importantly, however, rIL-12 administration restored T-bet expression in infant and *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ mice sensitized in the presence of LPS^{lo} (Figures 5F and 5H). As a consequence, the frequency of donor GFP^{+} OTII cells was similar in HDM + LPS^{lo}-sensitized B6 adult and rIL-12-treated infant and *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ mice (Figures 5G and 5I). Administration of rIL-12, however, did not appear to affect T-bet or IL-12p70 expression by CD11b^{+} mDCs (Figures S5I–S5L). Collectively, our data indicate that adult CD11b^{+} mDCs upregulate T-bet after HDM + LPS^{lo} sensitization, which promotes IL-12 production, thus preventing Th2 cell differentiation. In contrast, infant CD11b^{+} mDCs fail to upregulate T-bet, which prevents optimal IL-12 production and subsequent Th2 cell inhibition.

TNF-α-Driven Activation of CD11b^{+} mDCs Is Required for Preventing Th2 Cell Responses to HDMs

We next tested whether direct activation of CD11b^{+} mDCs by LPS is required for inducing T-bet expression after HDM + LPS^{lo} sensitization. Thus, we made B6(CD45.1^{+}):*Tlr4*$^{-/-}$(CD45.2^{+}) mixed BM chimeras. Chimeras were sensitized, and the expression of T-bet in mLN CD11b^{+} mDCs was determined. As

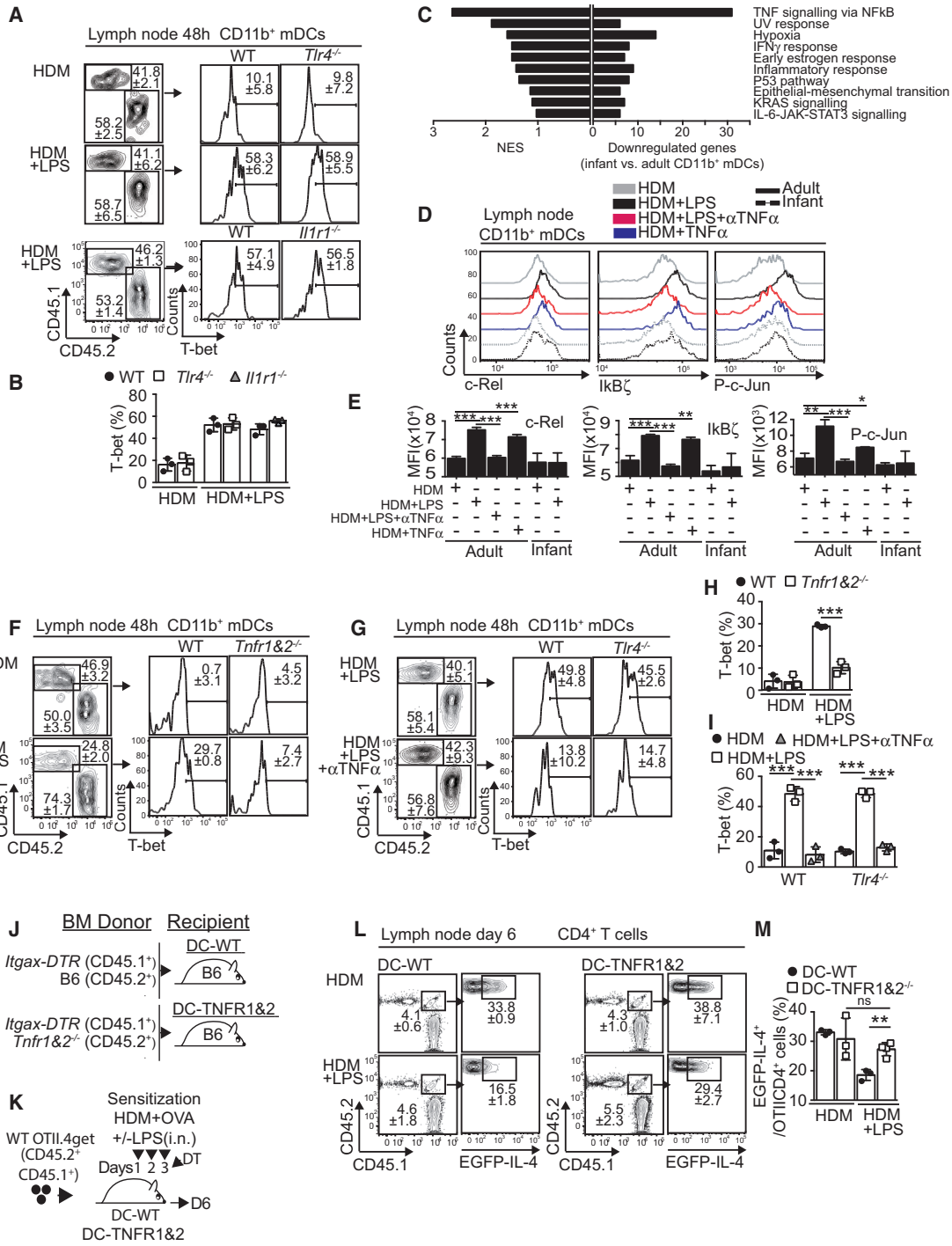


Figure 6. TNF-α Signaling Is Required for Upregulating T-bet in mCD11b⁺ DCs and Preventing Allergen-Specific Th2 Cell Responses

(A and B) B6 (CD45.1⁺) mice were irradiated and reconstituted with a 1:1 mixture of BM from B6 (CD45.1⁺) and *Tlr4*$^{-/-}$ (CD45.2⁺) donors or from B6 (CD45.1⁺) and *Il1r1*$^{-/-}$ (CD45.2⁺) donors. Shown are (A) fluorescence-activated cell-sorting (FACS) plots and (B) a graph of the frequencies of T-bet⁺ WT, *Tlr4*$^{-/-}$, and *Il1r1*$^{-/-}$ CD11b⁺ mDCs in the mLN of HDM ± LPS-treated reconstituted chimeric mice on day 2.

(C) mLN CD11b⁺ mDCs from B6 adults and 18-day-old infants were sorted 24 hr after being i.n. treated with HDM + 5 μg LPS, and RNA-seq was performed. Shown are the NES and number of downregulated genes in infant CD11b⁺ mDCs for each of the top ten hallmark signaling pathways resulting from GSEA (Broad institute; adjusted p value < 0.05, $\log_2$ fold change > 0.58).

(D and E) B6 adults and 18-day-old infants were i.n. treated with HDM ± 5 μg LPS or HDM ± 30 μg TNF. Some mice also received 250 μg anti-TNF-α intraperitoneally (i.p.). Shown are (D) FACS plots and (E) graphs of the expression of c-Rel, IKBz, and P-c-Jun in mLN CD11b⁺ mDCs on day 1.

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expected, CD11b$^+$ mDCs from the WT compartment upregulated T-bet expression after HDM + LPS^{lo} sensitization (Figures 6A and 6B). Likewise, CD11b$^+$ mDCs from the *Tlr4*$^{-/-}$ compartment also upregulated T-bet expression (Figures 6A and 6B), suggesting that LPS did not directly activate CD11b$^+$ mDCs to promote T-bet expression. LPS induces IL-1, which in turn activates IL-1R1−TLR4 signaling (Verstrepen et al., 2008); therefore, we next tested whether indirect activation of CD11b$^+$ mDCs through the IL-1R1 is required for inducing T-bet expression after HDM + LPS^{lo} sensitization. We analyzed B6(CD45.1$^+$):*Il1r1*$^{-/-}$(CD45.2$^+$) mixed BM chimeras and found that IL-1R1 signaling did not mediate T-bet upregulation in CD11b$^+$ mDCs (Figures 6A and 6B). To gain insight into the mechanisms underlying T-bet regulation in adult and infant CD11b$^+$ mDCs, we sorted CD11b$^+$ mDCs from the mLN of adult and infant mice 24 hr after HDM + LPS^{lo} sensitization and performed RNA sequencing (RNA-seq) and gene-set enrichment analysis (GSEA) to identify hallmark signaling pathways that were differentially regulated between adult and infant CD11b$^+$ mDCs. As a result, we identified 173 differentially expressed genes, 110 of which were downregulated and 63 of which were upregulated in infant CD11b$^+$ mDCs (Table S1). Notably, the pathway regulated by nuclear factor kappa B (NF-κB) in response to TNF-α had the highest normalized enrichment score (NES = 2.64, FDR q-value = 1.28e^{-42}) and contained the largest number of genes downregulated in infant CD11b$^+$ mDCs (Figures 6C, S6A, and S6B; Tables S2 and S3). TNF-α activates the NF-κB and activator protein-1 (AP-1) transcription factors (Verstrepen et al., 2008). Expression of genes encoding NF-κB-c-rel and the AP-1 family members c-Jun, JunB, c-Fos, and FosB was decreased in infant CD11b$^+$ mDCs after HDM + LPS^{lo} sensitization (Table S1). We next evaluated c-Rel and P-c-Jun expression in adult and infant mLN CD11b$^+$ mDCs. Adult CD11b$^+$ mDCs, but not infant CD11b$^+$ mDCs, induced c-Rel and P-c-Jun in response to HDM + LPS^{lo} sensitization (Figures S6C–S6E). We next tested whether LPS-induced TNF-α plays a role in the activation of NF-κB and AP-1 in CD11b$^+$ mDCs. Therefore, we evaluated the expression of NF-κB-c-Rel, the NF-κB early response protein IkBζ, and P-c-Jun-AP-1 in adult mLN CD11b$^+$ mDCs after sensitization in the presence or absence of TNF-α-neutralizing monoclonal antibody (mAb) or TNF-α cytokine. As a control, we also included the analysis of 18-day-old infant CD11b$^+$ mDCs. As expected, adult CD11b$^+$ mDCs, but not infant CD11b$^+$ mDCs, induced c-Rel, IkBζ, and P-c-Jun in response to HDM + LPS^{lo} sensitization (Figures 6D and 6E). However, anti-TNF-α treatment prevented these effects (Figures 6D and 6E). Additionally, compared with HDM sensitization alone, HDM sensitization in the presence of TNF-α cytokine was able to induce c-Rel, IkBζ, and P-c-Jun expression in CD11b$^+$ mDCs (Figures 6D and 6E). These data suggest that the activation of NF-κB and AP-1 in CD11b$^+$ mDCs after HDM + LPS^{lo} sensitization is mediated by TNF-α signaling.

We next examined whether the activation of CD11b$^+$ mDCs by TNF-α signaling through TNF receptors (TNFR1 and TNFR2) is required for inducing T-bet expression after HDM + LPS^{lo} sensitization. As above, 50% CD45.1$^+$WT and 50% CD45.2$^+$ *Tnfr1*$^{-/-}$*Tnfr2*$^{-/-}$ mixed BM chimeras were generated and sensitized, and the expression of T-bet in mLN CD11b$^+$ mDCs was determined. Whereas CD11b$^+$ mDCs from the WT compartment upregulated T-bet expression in HDM + LPS^{lo}-sensitized chimeras, CD11b$^+$ mDCs from the *Tnfr1*$^{-/-}$*Tnfr2*$^{-/-}$ compartment failed to upregulate T-bet (Figures 6F and 6H), suggesting that TNFR signaling in CD11b$^+$ mDCs is required for the upregulation of T-bet after HDM + LPS^{lo} sensitization. To confirm these data, we further determined the effect of TNF-α neutralization after sensitization. Thus, we administered anti-TNF-α mAb to 50% CD45.1$^+$WT and 50% CD45.2$^+$*Tlr4*$^{-/-}$ mixed BM chimeras at the time of HDM + LPS^{lo} sensitization. Anti-TNF-α treatment prevented T-bet upregulation in CD11b$^+$ mDCs from both WT and *Tlr4*$^{-/-}$ compartments (Figures 6G and 6I). Together, these data suggest that in adults, HDM + LPS^{lo} sensitization induces activation of NF-κB and AP-1 and T-bet upregulation in CD11b$^+$ mDCs by LPS-induced TNF-α production and TNF-α-mediated signaling.

To test whether TNF-α-mediated signaling in DCs is required for suppressing Th2 cell priming in response to low-dose endotoxin sensitization, we generated BM chimeras that expressed TNFR normally (DC-WT) or that were selectively TNFR deficient in CD11c$^+$ DCs after diphtheria toxin (DT) administration (DC-TNFR1&2) (Figure 6J) (León et al., 2012). After reconstitution, we transferred 4get.OT-II CD4$^+$ T cells into the chimeras, sensitized and treated them with DT to ablate CD11c$^+$ cells derived from the *Itgax*-DTR BM, and analyzed GFP expression by donor 4get.OT-II cells in the mLN (Figure 6K). Sensitization in the presence of LPS^{lo} prevented GFP in DC-WT chimeras, but not in DC-TNFR1&2 chimeras (Figures 6L and 6M). Thus, TNFR is required on CD11c$^+$ DCs for suppressing Th2 cell priming after HDM + LPS^{lo} sensitization.

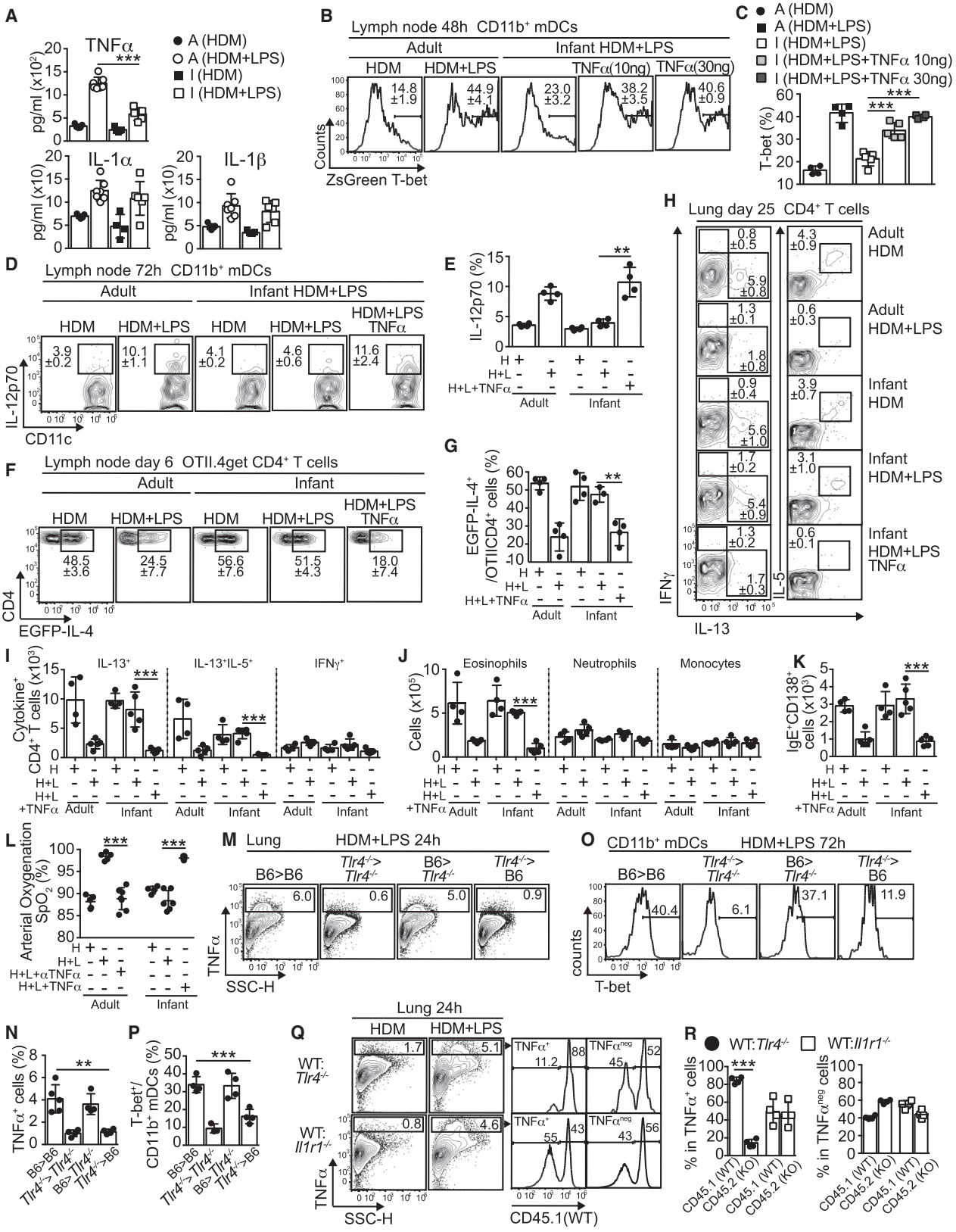
Impaired TNF-α-Driven Activation Precludes T-bet Expression in Infant CD11b$^+$ mDCs and Their Capability to Suppress Th2 Cell Priming

Our previous data show that in infants, HDM + LPS^{lo} sensitization induces poor NF-κB and AP-1 activation and T-bet upregulation in CD11b$^+$ mDCs, suggesting defective TNF-α-TNFR-mediated signaling. Thus, we next determined cytokine responses in bronchoalveolar lavage (BAL) fluid from adults and 18-day-old infants. HDM + LPS^{lo} sensitization caused a great increase in concentrations of TNF-α in the BAL fluid of adult mice

(F–I) CD45.1$^+$ mice were irradiated and reconstituted with 1:1 BM from B6 (CD45.1$^+$) and *Tnfr1&2*$^{-/-}$ (CD45.2$^+$) donors (F and H) or from B6 (CD45.1$^+$) and *Tlr4*$^{-/-}$ (CD45.2$^+$) donors (G and I). Shown are frequencies of T-bet$^+$ WT, *Tnfr1&2*$^{-/-}$ (F and H), and *Tlr4*$^{-/-}$ (G and I) CD11b$^+$ mDCs in the mLN of HDM ± LPS ± anti-TNF-α-treated reconstituted mice on day 2.

(J–M) Irradiated B6 mice were reconstituted with an 80:20 BM mix of *Itgax*-DTR and B6 donors (DC-WT chimeras) or *Itgax*-DTR and *Tnfr1&2*$^{-/-}$ donors (DC-TNFR1&2 chimeras) (J). Chimeras were transferred with CD45.1$^+$CD45.2$^+$ OT-II.4get cells, i.n. treated with HDM + OVA ± 5 μg LPS, and i.p. treated with 60 ng DT (K). Shown are frequencies of mLN donor EGFP$^+$ OT-II cells on day 6 (L and M).

*p < 0.05, **p < 0.01, ***p < 0.001 (unpaired Student's t test). Data are representative of two independent experiments (mean and SD of three to seven mice per group). Please also see Figure S6 and Tables S1–S3.



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but only a moderate increase in infants (Figure 7A). These differences were also observed between adults and 7- to 14-day-old mice (data not shown). No differences between adults and infants were found in the expression of other cytokines such as IL-1α, IL-1β (Figure 7A), and IL-6 (data not shown). These data indicate that infants have defective production of TNF-α after HDM + LPS^{lo} sensitization. To test whether defective T-bet upregulation in infant CD11b$^+$ mDCs after HDM + LPS^{lo} sensitization is due to defective TNF-α production, we sensitized 18-day-old T-bet-ZsGreen mice with HDM + LPS^{lo} in the presence of different concentrations of TNF-α and determined the expression of T-bet in mLN CD11b$^+$ mDCs. As expected, HDM + LPS^{lo} sensitization did not induce significant upregulation of T-bet in CD11b$^+$ mDCs from infant mice (Figures 7B and 7C). However, co-administration of TNF-α induced T-bet upregulation in infant CD11b$^+$ mDCs to amounts similar to those found in CD11b$^+$ mDCs from HDM + LPS^{lo}-sensitized adults (Figures 7B and 7C). Similar upregulation of T-bet in CD11b$^+$ mDCs was seen from 7-, 14-, and 18-day-old mice after exogenous administration of TNF-α (Figures S7A and S7B). Thus, our data indicate that defective TNF-α production in infants prevents T-bet upregulation in CD11b$^+$ mDCs after HDM + LPS^{lo} sensitization. In addition, administration of TNF-α during HDM + LPS^{lo} sensitization recovers the capacity of infant mLN CD11b$^+$ mDCs to produce IL-12 in amounts similar to those of adult CD11b$^+$ mDCs (Figures 7D and 7E).

Finally, we tested whether administration of TNF-α during HDM + LPS^{lo} sensitization restores the capacity of low-LPS treatment to abrogate Th2 cell development in infant mice. We transferred 4get.OT-II CD4$^+$ T cells into B6 adults and 18-day-old infants, sensitized them in the presence of TNF-α, and assessed expression of GFP by donor 4get.OT-II cells (Figures 7F and 7G). TNF-α administration restored the capacity of LPS^{lo} to prevent GFP expression in infant mice (Figures 7F and 7G). We next sensitized adult and 18-day-old infant B6 mice as above, challenged them with HDMs, and quantified IL-13$^+$, IL-5$^+$, and IFN-γ$^+$ CD4$^+$ T cells (Figures 7H and 7I). As expected, HDM + LPS^{lo} sensitization prevented the accumulation of IL-13$^+$ and IL-13$^+$IL-5$^+$ CD4$^+$ T cells in the lungs of adults, but not in infants (Figures 7H and 7I). Importantly, sensitization in the presence of TNF-α restored the capacity of LPS^{lo} to prevent the accumulation of IL-13$^+$ and IL-13$^+$IL-5$^+$ CD4$^+$ T cells in infants (Figures 7H and 7I). Likewise, HDM + LPS^{lo} sensitization in the presence of TNF-α also prevented the accumulation of lung eosinophils (Figures 7J, S7C, and S7D) and mLN IgE$^+$ ASCs (Figures 7K and S7E), prevented the induction of goblet cell hyperplasia (Figure S7F), and improved SpO2 (Figure 7L) in infant mice. The same results were seen when 10-day-old mice were analyzed (data not shown). In contrast, TNF-α neutralization after HDM + LPS^{lo} sensitization in adult mice abolished the suppressive effect of LPS on asthma, including the accumulation of IL-13$^+$ CD4$^+$ T cells (Figures S7I and S7J) and eosinophils (Figures S7G and S7H) in the lungs, the goblet cell hyperplasia (Figure S7F), and the beneficial effect on SpO2 (Figure 7L). Thus, our data indicate that defective TNF-α production prevents the suppression of Th2 cell responses to HDMs in the presence of low doses of endotoxin.

To assess the cellular source of TNF-α after HDM + LPS^{lo} sensitization, we first evaluated whether LPS-driven TNF-α production is mediated by radioresistant (including lung epithelium) or hematopoietic cells in the lungs. Therefore, B6 (WT) or *Tlr4*$^{-/-}$ recipients were irradiated and reconstituted with BM from WT or *Tlr4*$^{-/-}$ mice. After reconstitution, TNF-α production in the lungs was determined after HDM + LPS^{lo} sensitization. Importantly, chimeric *Tlr4*$^{-/-}$ mice transplanted with WT BM, but not chimeric mice transplanted with *Tlr4*$^{-/-}$ BM, were able to produce WT amounts of TNF-α$^+$ cells (Figures 7M and 7N), suggesting that radiosensitive (hematopoietic) cells contain the principal source of TNF$^+$ cells. Likewise, only mLN CD11b$^+$ mDCs of HDM + LPS^{lo}-sensitized chimeric mice transplanted with WT BM, but not with *Tlr4*$^{-/-}$ BM, were able to upregulate T-bet (Figures 7O and 7P). Next, we evaluated whether hematopoietic cells directly respond to LPS to produce TNF-α. Thus, WT:*Tlr4*$^{-/-}$ BM chimeras were sensitized, and TNF-α production in the lungs was determined. As expected, HDM + LPS^{lo} sensitization induced TNF-α production (Figure 7Q), but importantly, TNF-α$^+$ cells were principally restricted to the WT phenotype (Figures 7Q and 7R), suggesting that TNF-α was produced by lung hematopoietic cells after direct recognition of LPS by TLR4. Conversely, the analysis of WT:*Il1r1*$^{-/-}$ BM chimeras showed that lung cells did not require expression of IL-1R to produce TNF-α in response to LPS (Figures 7Q and 7R). These data suggest that

Figure 7. Defective TNF-α Signaling Prevents T-bet Upregulation in mCD11b$^+$ DCs from Infants and Contributes toward the Development of Allergen-Specific Th2 Cell Responses

(A) Cytokines in BAL of day-1 HDM ± LPS-treated B6 adults and 18-day-old infants.

(B and C) T-bet-ZsGreen reporter adults and 18-day-old infants were i.n. treated with HDM ± 5 μg LPS ± 10 or 30 ng TNF-α. Shown are (B) FACS plots and (C) graphs of the frequencies of ZsG$^+$ mLN CD11b$^+$ mDCs on day 2.

(D and E) B6 adults and 18-day-old infants were i.n. treated with HDM ± 5 μg LPS ± 30 ng TNF-α. Shown are (D) FACS plots and (E) graphs of the frequencies of IL-12p70$^+$ mLN CD11b$^+$ mDCs on day 3.

(F and G) B6 adults and 18-day-old infants were transferred with CD45.1$^+$ OT-II.4get cells and i.n. treated with HDM + OVA ± 5 μg LPS. PBS or 30 ng TNF-α was co-administered on day 1. Shown are (F) FACS plots and (G) graphs of the frequencies of mLN EGFP$^+$ donor OT-II cells on day 6.

(H–L) B6 adults and 18-day-old infants were i.n. treated with HDM ± 5 μg LPS ± 30 μg TNF-α. Some adults also received 250 μg anti-TNF-α i.p. On day 20, mice were i.n. challenged with HDMs and analyzed on day 25. Shown are frequencies (H) and numbers (I) of IL-13$^+$, IL-13-IL-5$^+$, and IFN-γ$^+$ CD4$^+$ T cells in the lungs. Numbers of lung eosinophils, neutrophils, and monocytes are shown in (J). Numbers of mLN IgE$^+$ ASCs are shown in (K). SpO2 was determined by pulse oximetry (L).

(M–P) B6 (WT) and *Tlr4*$^{-/-}$ recipient mice were lethally irradiated and reconstituted with WT and *Tlr4*$^{-/-}$ BM. Chimeras were i.n. treated with HDM ± 5 μg LPS. Shown are frequencies of lung TNF-α$^+$ cells at 24 hr (M and N) and T-bet expression in mLN CD11b$^+$ mDCs at 72 hr (O and P).

(Q and R) 1:1 B6 (CD45.1$^+$):*Tlr4*$^{-/-}$ (CD45.2$^+$) and 1:1 B6 (CD45.1$^+$):*Il1r1*$^{-/-}$ (CD45.2$^+$) mixed BM chimeras were i.n. treated with HDM ± 5 μg LPS. Also shown is TNF-α expression 24 hr later in lung suspensions (Q) and frequencies of WT (CD45.1$^+$) and knockout (CD45.2$^+$) cells within the TNF-α$^+$ and TNF-α$^-$ gates (R). $^{**}p < 0.01$, $^{***}p < 0.001$ (unpaired Student's t test). Data are representative of two independent experiments (mean and SD of three to five mice per group). Please also see Figure S7.

TNF-α-dependent upregulation of T-bet in CD11b$^+$ mDCs is driven by hematopoietic cells that produce TNF-α in response to LPS, but not by epithelial cells.

Collectively, our data indicate that adult CD11b$^+$ mDCs upregulate T-bet in response to TNF-α produced after HDM + LPS^{lo} sensitization, which in turn promotes IL-12 production, thereby preventing Th2 cell differentiation by inducing T-bet expression in responding CD4$^+$ T cells. In contrast, in early life and during a time window from day 7 to day 20 after birth, the CD11b$^+$ mDCs fail to upregulate T-bet in response to low doses of LPS as a result of impaired production of TNF-α, which prevents optimal IL-12 production and subsequent Th2 cell inhibition despite the presence of LPS.

DISCUSSION

Previous studies in mice have shown that during a time window from day 7 to day 20 after birth, the immune system is prone to allergic sensitization and Th2-driven allergic responses (de Kleer et al., 2016; Gollwitzer et al., 2014; Steer et al., 2017), suggesting that a "window of opportunity" in the postnatal period influences the immune responses to allergens. Comparative immunological evaluations have demonstrated that the immunity in mice at 7 days is comparable to that of term human neonates, whereas mice < 20 days old resemble infants (Siegrist, 2001). Thus, 1- to 3-week-old mice have yielded observations very similar to those of human neonates (0–2 months old) and infants (under 1–2 years old) (Siegrist, 2001). In correlation with animal studies, primary allergen sensitizations to environmental Ags gradually develop and peak in children 2–3 years of age (Holt, 1996) and are associated with later development of asthma and allergic disease (Lodge et al., 2011). Therefore, both in humans and in mice, a susceptibility window for inhalant-allergen sensitization covers the neonatal and infancy periods. Several studies in mice have evaluated this propensity to develop allergic sensitization during infancy (de Kleer et al., 2016; Steer et al., 2017). However, the understanding of the allergic sensitization process in the context of exposure to environmental endotoxins during infancy is still not clear. Previous studies have shown that LPS exposure prevents the development of allergic Th2 cell responses (Daan de Boer et al., 2013; Gereda et al., 2000; Schuijs et al., 2015; Stein et al., 2016; Zhu et al., 2010). Our data demonstrate, however, that adults and infants respond to LPS with different thresholds. As a result, whereas relatively low-dose LPS exposure normally prevents adults from developing allergic Th2 cell responses, infant mice require a relatively high-LPS environment to achieve the same prophylactic effect. The inability of low-LPS exposure to prevent Th2 cell differentiation in infants is attributed to the inability of responding infant CD4$^+$ T cells to upregulate T-bet, which we found was required for preventing Th2 cell development in response to HDMs. Impaired T-bet expression, however, was not due to an intrinsic defect in the infant CD4$^+$ T cells. Instead, we found that CD11b$^+$ mDCs from HDM + LPS^{lo}-sensitized infants poorly produced IL-12 and thereby prevented IL-12-dependent T-bet upregulation in responding CD4$^+$ T cells. Mechanistically, we demonstrated that T-bet upregulation in CD11b$^+$ mDCs is required for normal IL-12 production. However, whereas adult CD11b$^+$ mDCs normally upregulated T-bet after HDM + LPS^{lo} sensitization, infant CD11b$^+$ mDCs failed to express T-bet, which prevented CD11b$^+$ mDCs from secreting IL-12. Thus, the differential capacity to upregulate T-bet in infant and adult CD11b$^+$ mDCs provides the underlying mechanism for how LPS prevents allergic Th2 cell responses in adults and infants with different thresholds. Our data indicate that in addition to controlling important fate decisions in T cells (Szabo et al., 2000; Zhu et al., 2012), T-bet also critically regulates DC activity.

Importantly, our data show that T-bet upregulation in CD11b$^+$ mDCs does not require direct recognition of LPS, but instead it requires LPS-induced TNF-α production and TNF-α-TNFR-triggered signaling in CD11b$^+$ mDCs. Our data further show the requirement for crosstalk between TLR4$^+$ accessory hematopoietic cells producing TNF-α and TNFR$^+$CD11b$^+$ mDCs. Whereas adult hematopoietic cells produce large amounts of TNF-α in response to HDM + LPS sensitization, infants have impaired TNF-α production, which prevents TNF-α-TNFR signaling and T-bet upregulation in CD11b$^+$ mDCs. Therefore, the capacity of LPS to prevent allergic Th2 cell responses relies on the capacity to induce increased amounts of TNF-α. Our data reveal a largely unexplored role of TNF-α in mediating *in vivo* DC activation, in addition to its well-defined role in apoptosis and inflammation. In this way, TNF-α acts as a rheostat that can tune the activation of CD11b$^+$ mDCs and the subsequent balance of the T cell responses to inhaled Ags. Importantly, the regulatory activity of this rheostat is modulated by age.

Previous studies have suggested that CD4$^+$ T cells primed by CD103$^+$ mDCs preferentially differentiate into Th1 cells, whereas CD11b$^+$ mDCs promote Th2 cell differentiation (Conejero et al., 2017; Gao et al., 2013; Kumamoto et al., 2013; León, 2017; Plantinga et al., 2013). As such, CD103$^+$ mDCs produce IL-12p40 after HDM sensitization, thereby preventing Th2 cell differentiation (Conejero et al., 2017). Here, we found that LPS prevented Th2 cell differentiation in HDM-sensitized mice by an IL-12-dependent mechanism. We found, however, that CD103$^+$ mDCs were quickly depleted from the lung after HDM + LPS sensitization, possibly as a result of increased LPS-driven epithelial damage (Sung et al., 2006). In contrast, the number of CD11b$^+$ mDCs and their capacity to secrete IL-12 dramatically increased after HDM + LPS sensitization, allowing them to become the dominant IL-12$^+$ DC subset in the mLN. In addition, CD11b$^+$ mDCs are the principal DC subset that captures and transports HDM-derived Ags from the lung into the mLN. Thus, given that the numbers of IL-12$^+$CD11b$^+$ mDCs largely increased in HDM + LPS-sensitized adult mice and that IL-12 prevented Th2 cell differentiation, we conclude that IL-12$^+$CD11b$^+$ mDCs and not CD103$^+$ mDCs are the main drivers preventing allergic Th2 cell responses in adult HDM + LPS-sensitized mice. Supporting this conclusion, LPS normally prevented Th2 cell differentiation in *Batf3*$^{-/-}$ mice despite the absence of CD103$^+$ mDCs. In contrast, LPS failed to suppress allergic Th2 cell responses when CD11b$^+$ mDCs were unable to normally produce IL-12, such as in *Itgax*^{Cre}-*Tbx21*$^{fl/fl}$ or infant mice. Collectively, our results demonstrate that CD11b$^+$ mDCs have the ability to either promote or prevent Th2 cell responses depending on the amount of LPS. Therefore, the contribution of CD11b$^+$ mDCs relative to CD103$^+$ mDCs to the development of allergic Th2 cell responses is more complex than initially expected and is highly influenced by the environmental number of microbial products rather than being predetermined by their

intrinsic nature alone. Importantly, our data show that whereas IL-12 production by CD11b$^+$ mDCs requires the upregulation of T-bet, IL-12 production by CD103$^+$ mDCs is T-bet independent. However, the molecular mechanisms by which T-bet is required in only CD11b$^+$ mDCs is unclear. Various transcription factors, including IRF family members, have been implicated in the regulation of IL-12 production by DC subsets (Gao et al., 2013; Takaoka et al., 2005; Taki et al., 1997; Williams et al., 2013). Here, we found no differences in the expression of IRF1 or IRF5 between CD103$^+$ and CD11b$^+$ mDCs from infants and adults. Importantly, the transcription factor IRF4 is highly expressed in CD11b$^+$, but not in CD103$^+$, mDCs and essentially contributes to the differentiation and function of CD11b$^+$ mDCs. For example, IRF4 drives CD11b$^+$ mDCs to promote Th2 cell differentiation (Gao et al., 2013; Kumamoto et al., 2013; Williams et al., 2013), inhibits the capacity of DCs and macrophages to produce IL-12 (Akbari et al., 2014; Honma et al., 2005), and competes with IRF5 to inhibit IRF5-dependent upregulation of pro-inflammatory cytokines, such as IL-12 and IL-6 (Negishi et al., 2005). These data suggest a role for IRF4 in the negative-feedback regulation of IL-12 production, and because IRF4 is restricted to CD11b$^+$ mDCs, IRF4 might selectively control IL-12 regulation specifically in CD11b$^+$ mDCs. Therefore, one could speculate that T-bet upregulation, specifically in CD11b$^+$ mDCs, is important for overcoming the suppressive function of IRF4 in IL-12 production.

In summary, our comparative analysis between adult and neonatal-infant mice offers mechanistic insights into how the priming of allergen-specific Th2 cell responses is differentially regulated during childhood and adulthood, suggests a perspective for the biological mechanism underlying the hygiene hypothesis, and provides a plausible mechanism to explain higher susceptibility to allergic airway inflammation observed in children than in adults.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and three tables and can be found with this article online at https://doi.org/10.1016/j.immuni.2018.11.012.

ACKNOWLEDGMENTS

We thank Dr. J. Zhu (National Institute of Allergy and Infectious Diseases, NIH, Bethesda) for providing TBGR mice and Becca Burnham, Uma Mudunuru, and Thomas S. Simpler for animal husbandry. The work was supported by University of Alabama at Birmingham and NIH grant R01 AI116584 to B.L., grant U01 ES026458 to S.M., and grant R01 AI110480 to A.B.T.

AUTHOR CONTRIBUTIONS

B.L. designed the study and wrote the manuscript. H.B., K.K., A.P., Z.Y., S.M., and B.L. performed the experiments and analyzed the data. T.T.M. analyzed RNA-seq data. A.B.T. provided expertise and reagents.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: August 8, 2017
Revised: August 16, 2018
Accepted: November 5, 2018
Published: January 8, 2019

REFERENCES

Akbari, M., Honma, K., Kimura, D., Miyakoda, M., Kimura, K., Matsuyama, T., and Yui, K. (2014). IRF4 in dendritic cells inhibits IL-12 production and controls Th1 immune responses against Leishmania major. J. Immunol. *192*, 2271–2279.

Ballesteros-Tato, A., León, B., Lund, F.E., and Randall, T.D. (2010). Temporal changes in dendritic cell subsets, cross-priming and costimulation via CD70 control CD8(+) T cell responses to influenza. Nat. Immunol. *11*, 216–224.

Ballesteros-Tato, A., Randall, T.D., Lund, F.E., Spolski, R., Leonard, W.J., and León, B. (2016). T follicular helper cell plasticity shapes pathogenic T helper 2 cell-mediated immunity to inhaled house dust mite. Immunity *44*, 259–273.

Bar-Joseph, Z., Gifford, D.K., and Jaakkola, T.S. (2001). Fast optimal leaf ordering for hierarchical clustering. Bioinformatics *17* (*Suppl 1*), S22–S29.

Conejero, L., Khouili, S.C., Martínez-Cano, S., Izquierdo, H.M., Brandi, P., and Sancho, D. (2017). Lung CD103+ dendritic cells restrain allergic airway inflammation through IL-12 production. JCI Insight *2*, 2.

Daan de Boer, J., Roelofs, J.J., de Vos, A.F., de Beer, R., Schouten, M., Hommes, T.J., Hoogendijk, A.J., de Boer, O.J., Stroo, I., van der Zee, J.S., et al. (2013). Lipopolysaccharide inhibits Th2 lung inflammation induced by house dust mite allergens in mice. Am. J. Respir. Cell Mol. Biol. *48*, 382–389.

de Kleer, I.M., Kool, M., de Bruijn, M.J., Willart, M., van Moorleghem, J., Schuijs, M.J., Plantinga, M., Beyaert, R., Hams, E., Fallon, P.G., et al. (2016). Perinatal activation of the interleukin-33 pathway promotes type 2 immunity in the developing lung. Immunity *45*, 1285–1298.

Edelson, B.T., Kc, W., Juang, R., Kohyama, M., Benoit, L.A., Klekotka, P.A., Moon, C., Albring, J.C., Ise, W., Michael, D.G., et al. (2010). Peripheral CD103+ dendritic cells form a unified subset developmentally related to CD8alpha+ conventional dendritic cells. J. Exp. Med. *207*, 823–836.

Eisen, M.B., Spellman, P.T., Brown, P.O., and Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. USA *95*, 14863–14868.

Gao, Y., Nish, S.A., Jiang, R., Hou, L., Licona-Limón, P., Weinstein, J.S., Zhao, H., and Medzhitov, R. (2013). Control of T helper 2 responses by transcription factor IRF4-dependent dendritic cells. Immunity *39*, 722–732.

Gereda, J.E., Leung, D.Y., Thatayatikom, A., Streib, J.E., Price, M.R., Klinnert, M.D., and Liu, A.H. (2000). Relation between house-dust endotoxin exposure, type 1 T-cell development, and allergen sensitisation in infants at high risk of asthma. Lancet *355*, 1680–1683.

Gollwitzer, E.S., Saglani, S., Trompette, A., Yadava, K., Sherburn, R., McCoy, K.D., Nicod, L.P., Lloyd, C.M., and Marsland, B.J. (2014). Lung microbiota promotes tolerance to allergens in neonates via PD-L1. Nat. Med. *20*, 642–647.

Guilliams, M., Ginhoux, F., Jakubzick, C., Naik, S.H., Onai, N., Schraml, B.U., Segura, E., Tussiwand, R., and Yona, S. (2014). Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. Nat. Rev. Immunol. *14*, 571–578.

Holt, P.G. (1996). Primary allergic sensitization to environmental antigens: perinatal T cell priming as a determinant of responder phenotype in adulthood. J. Exp. Med. *183*, 1297–1301.

Honma, K., Udono, H., Kohno, T., Yamamoto, K., Ogawa, A., Takemori, T., Kumatori, A., Suzuki, S., Matsuyama, T., and Yui, K. (2005). Interferon regulatory factor 4 negatively regulates the production of proinflammatory cytokines by macrophages in response to LPS. Proc. Natl. Acad. Sci. USA *102*, 16001–16006.

Intlekofer, A.M., Banerjee, A., Takemoto, N., Gordon, S.M., Dejong, C.S., Shin, H., Hunter, C.A., Wherry, E.J., Lindsten, T., and Reiner, S.L. (2008). Anomalous type 17 response to viral infection by CD8+ T cells lacking T-bet and eomesodermin. Science *321*, 408–411.

Kumamoto, Y., Linehan, M., Weinstein, J.S., Laidlaw, B.J., Craft, J.E., and Iwasaki, A. (2013). CD301b$^+$ dermal dendritic cells drive T helper 2 cell-mediated immunity. Immunity *39*, 733–743.

León, B. (2017). T cells in allergic asthma: key players beyond the Th2 pathway. Curr. Allergy Asthma Rep. *17*, 43.

León, B., Ballesteros-Tato, A., Browning, J.L., Dunn, R., Randall, T.D., and Lund, F.E. (2012). Regulation of T(H)2 development by CXCR5+ dendritic cells and lymphotoxin-expressing B cells. Nat. Immunol. *13*, 681–690.

Lipscomb, M.W., Chen, L., Taylor, J.L., Goldbach, C., Watkins, S.C., Kalinski, P., Butterfield, L.H., Wesa, A.K., and Storkus, W.J. (2009). Ectopic T-bet expression licenses dendritic cells for IL-12-independent priming of type 1 T cells in vitro. J. Immunol. *183*, 7250–7258.

Lodge, C.J., Lowe, A.J., Gurrin, L.C., Hill, D.J., Hosking, C.S., Khalafzai, R.U., Hopper, J.L., Matheson, M.C., Abramson, M.J., Allen, K.J., and Dharmage, S.C. (2011). House dust mite sensitization in toddlers predicts current wheeze at age 12 years. J. Allergy Clin. Immunol. *128*, 782–788.e9.

Lugo-Villarino, G., Maldonado-Lopez, R., Possemato, R., Penaranda, C., and Glimcher, L.H. (2003). T-bet is required for optimal production of IFN-gamma and antigen-specific T cell activation by dendritic cells. Proc. Natl. Acad. Sci. USA *100*, 7749–7754.

Masoli, M., Fabian, D., Holt, S., and Beasley, R.; Global Initiative for Asthma (GINA) Program (2004). The global burden of asthma: executive summary of the GINA Dissemination Committee report. Allergy *59*, 469–478.

Negishi, H., Ohba, Y., Yanai, H., Takaoka, A., Honma, K., Yui, K., Matsuyama, T., Taniguchi, T., and Honda, K. (2005). Negative regulation of Toll-like-receptor signaling by IRF-4. Proc. Natl. Acad. Sci. USA *102*, 15989–15994.

Plantinga, M., Guilliams, M., Vanheerswynghels, M., Deswarte, K., Branco-Madeira, F., Toussaint, W., Vanhoutte, L., Neyt, K., Killeen, N., Malissen, B., et al. (2013). Conventional and monocyte-derived CD11b(+) dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. Immunity *38*, 322–335.

Reynolds, L.A., and Finlay, B.B. (2017). Early life factors that affect allergy development. Nat. Rev. Immunol. *17*, 518–528.

Schuijs, M.J., Willart, M.A., Vergote, K., Gras, D., Deswarte, K., Ege, M.J., Madeira, F.B., Beyaert, R., van Loo, G., Bracher, F., et al. (2015). Farm dust and endotoxin protect against allergy through A20 induction in lung epithelial cells. Science *349*, 1106–1110.

Siegrist, C.A. (2001). Neonatal and early life vaccinology. Vaccine *19*, 3331–3346.

Song, W., Wei, S., Liu, G., Yu, Z., Estell, K., Yadav, A.K., Schwiebert, L.M., and Matalon, S. (2011). Postexposure administration of a beta2-agonist decreases chlorine-induced airway hyperreactivity in mice. Am. J. Respir. Cell Mol. Biol. *45*, 88–94.

Steer, C.A., Martinez-Gonzalez, I., Ghaedi, M., Allinger, P., Mathä, L., and Takei, F. (2017). Group 2 innate lymphoid cell activation in the neonatal lung drives type 2 immunity and allergen sensitization. J. Allergy Clin. Immunol. *140*, 593–595.e3.

Stein, M.M., Hrusch, C.L., Gozdz, J., Igartua, C., Pivniouk, V., Murray, S.E., Ledford, J.G., Marques Dos Santos, M., Anderson, R.L., Metwali, N., et al. (2016). Innate immunity and asthma risk in amish and hutterite farm children. N. Engl. J. Med. *375*, 411–421.

Stranges, P.B., Watson, J., Cooper, C.J., Choisy-Rossi, C.M., Stonebraker, A.C., Beighton, R.A., Hartig, H., Sundberg, J.P., Servick, S., Kaufmann, G., et al. (2007). Elimination of antigen-presenting cells and autoreactive T cells by Fas contributes to prevention of autoimmunity. Immunity *26*, 629–641.

Sung, S.S., Fu, S.M., Rose, C.E., Jr., Gaskin, F., Ju, S.T., and Beaty, S.R. (2006). A major lung CD103 (alphaE)-beta7 integrin-positive epithelial dendritic cell population expressing Langerin and tight junction proteins. J. Immunol. *176*, 2161–2172.

Szabo, S.J., Kim, S.T., Costa, G.L., Zhang, X., Fathman, C.G., and Glimcher, L.H. (2000). A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell *100*, 655–669.

Takaoka, A., Yanai, H., Kondo, S., Duncan, G., Negishi, H., Mizutani, T., Kano, S., Honda, K., Ohba, Y., Mak, T.W., and Taniguchi, T. (2005). Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. Nature *434*, 243–249.

Taki, S., Sato, T., Ogasawara, K., Fukuda, T., Sato, M., Hida, S., Suzuki, G., Mitsuyama, M., Shin, E.H., Kojima, S., et al. (1997). Multistage regulation of Th1-type immune responses by the transcription factor IRF-1. Immunity *6*, 673–679.

Trinchieri, G. (2003). Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat. Rev. Immunol. *3*, 133–146.

Verstrepen, L., Bekaert, T., Chau, T.L., Tavernier, J., Chariot, A., and Beyaert, R. (2008). TLR-4, IL-1R and TNF-R signaling to NF-kappaB: variations on a common theme. Cell. Mol. Life Sci. *65*, 2964–2978.

Williams, J.W., Tjota, M.Y., Clay, B.S., Vander Lugt, B., Bandukwala, H.S., Hrusch, C.L., Decker, D.C., Blaine, K.M., Fixsen, B.R., Singh, H., et al. (2013). Transcription factor IRF4 drives dendritic cells to promote Th2 differentiation. Nat. Commun. *4*, 2990.

Zhu, J., Jankovic, D., Oler, A.J., Wei, G., Sharma, S., Hu, G., Guo, L., Yagi, R., Yamane, H., Punkosdy, G., et al. (2012). The transcription factor T-bet is induced by multiple pathways and prevents an endogenous Th2 cell program during Th1 cell responses. Immunity *37*, 660–673.

Zhu, Z., Oh, S.Y., Zheng, T., and Kim, Y.K. (2010). Immunomodulating effects of endotoxin in mouse models of allergic asthma. Clin Exp Allergy. *40*, 536–546.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-B220 Clone RA3-6B2	BD Biosciences	Cat# 552094, RRID: AB_394335
Anti-CD4 Clone GK1.5	BD Biosciences	Cat# 563050, RRID: AB_2737973
Anti-CD11b Clone M1/70	BD Biosciences	Cat# 562127, RRID: AB_10893815
Anti-CD11c Clone HL3	BD Biosciences	Cat# 558079, RRID: AB_647251
Anti-CD44 Clone IM7	BD Biosciences	Cat# 560780, RRID: AB_1937316
Anti-CD45.1 Clone A20	BD Biosciences	Cat# 558701, RRID: AB_1645214
Anti-CD45.2 Clone 104	BD Biosciences	Cat# 560695, RRID: AB_1727493
Anti-CD69 Clone H1-2F3	BD Biosciences	Cat# 560689, RRID: AB_1727506
Anti-CD86 Clone GL1	BD Biosciences	Cat# 557426, RRID: AB_396698
Anti-CD103 Clone M290	BD Biosciences	Cat# 557495, RRID: AB_396732
Anti-CD138 Clone 281-2	BD Biosciences	Cat# 558626, RRID: AB_1645216
Anti-IgE Clone R35-72	BD Biosciences	Cat# 553414, RRID: AB_394847
Anti-Ly6c Clone AL-21	BD Biosciences	Cat# 560596, RRID: AB_1727555
Anti-Siglec-F Clone E50-2440	BD Biosciences	Cat# 565183, RRID: AB_2739097
Anti-Siglec-H Clone 440c	BD Biosciences	Cat# 747671, RRID: AB_2744232
Anti-CD64 Clone X54-5/7.1	Biolegend.	Cat# 139306, RRID: AB_11219391
Anti-MHC class II Clone M5/114.15.2	Biolegend.	Cat# 107620, RRID: AB_493527
Anti-IL-13 Clone 13A	eBiosciences	Cat# 12-7133-41, RRID: AB_10852712
Anti-IFNγ Clone XMG1.2	BD Biosciences	Cat# 557649, RRID: AB_396766
Anti-IL-5 Clone TRFK5	BioLegend	Cat# 504311, RRID: AB_2563161
Anti-IL-12 Clone C15.6	BD-Biosciences	Cat# 554479, RRID: AB_395420
Anti-TNF-α Clone MP6-XT22	BD-Biosciences	Cat# 554418, RRID: AB_395379
Anti-T-bet Clone 4B10	Biolegend	Cat# 644814, RRID: AB_10901173
Anti-GATA-3 Clone TWAJ	eBioscience	Cat# 25-9966-42, RRID: AB_2573568
Anti-Foxp3 Clone FJK-16 s	eBioscience	Cat# 25-5773-82, RRID: AB_891552
Anti-IRF1 Clone ab216114	Abcam	Cat# ab216114
Anti-IRF4 Clone IRF4.3E4	BioLegend	Cat# 646404, RRID: AB_2563005
Anti-IRF5 Clone ab212239	Abcam	Cat# ab212239
Anti-c-Rel Clone sc-6955	Santa Cruz	Cat# sc-6955 AF488, RRID: AB_670194
Anti-P-c-Jun Clone D47G9	Cell Signaling Technology	Cat# 8752S
Anti-IkBζ Clone LK2NAP	eBioscience	Cat# 46-6801-82, RRID: AB_11149496
Anti-CD16/32	BioXCell	Cat# CUS-HB-197, RRID: AB_2687830
Anti-TNF-α	BioXCell	Cat# BE0058, RRID: AB_1107764
Chemicals, Peptides, and Recombinant Proteins		
Recombinant mouse IL-12	PeproTech	Cat# 210-12
Recombinant mouse TNF-α	R&D Systems	Cat# 410-MT-010
EndoFit ovalbumin	InvivoGen	Cat# NC1005858
Diphtheria toxin	Sigma-Aldrich	Cat# D0564-1MG
PMA	Sigma-Aldrich	Cat# P8139-1MG
Calcimycin	Life Technologies	Cat# A1493
Brefeldin-A	Sigma-Aldrich	Cat# B7651-5MG
Methacholine	Sigma-Aldrich	Cat# 1396364

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical Commercial Assays		
Transcription factor staining buffer set	eBioscience	Cat# 00-5523-00
Anti-L3T4 (CD4) Microbeads, mouse	Miltenyi Biotec	Cat# 130-049-201, RRID: AB_2722753
CD11c MicroBeads UltraPure, mouse	Miltenyi Biotec	Cat# 130-108-338
CellTrace™ CFSE	ThermoFisher	Cat# C34554
AF647 labeling kit	Invitrogen	Cat# A20173
ProcartaPlex assay	Life Technologies	Cat# PPX-12
RNeasy Plus Micro Kit	QIAGEN	Cat# 74034
Deposited Data		
RNA-Seq. data. CD11b^{+} lung DC subset from adult (> 8-week-old) and infant (18-day-old) mice after HDM+LPS i.n. sensitization	This paper	GEO: GSE110046
Experimental Models: Organisms/Strains		
House-dust mite (Dermatophagoides pteronyssinus and Dermatophagoides farinae) extracts	Greer laboratories	N/A
Lipopolysaccharide from *Escherichia coli* 0111:B4	Sigma-Aldrich	Cat# L2630
Mouse: C57BL/6J (B6)	The Jackson Laboratory	Stock No: 000664
Mouse: B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ (CD45.1^{+} B6 congenic)	The Jackson Laboratory	Stock No: 002014
Mouse: C57BL/6-Tg(TcraTcrb)425Cbn/J (OT-II)	The Jackson Laboratory	Stock No: 004194
Mouse: B6.129-Il4^{tm1Lky}/J (B6.4get IL-4 reporter mice)	Laboratory of Dr. M. Mohrs (Trudeau Institute)	N/A
Mouse: B6.129S(C)-Batf3^{tm1Kmm}/J (*Baft3*$^{-/-}$)	The Jackson Laboratory	Stock No: 013755
Mouse: B6.129-Tbx21^{tm2Srnr}/J (*Tbx21*$^{fl/fl}$)	The Jackson Laboratory	Stock No: 022741
Mouse: C57BL/6J-Tg(Itgax-cre,-EGFP)4097Ach/J (*Itgax-cre,-EGFP*)	The Jackson Laboratory	Stock No: 007567
Mouse: B6.129S6-*Tbx21*^{tm1Glm}/J (*Tbx21*$^{-/-}$)	The Jackson Laboratory	Stock No: 004648
Mouse: B6(Cg)-*Tlr4*$^{tm1.2Karp}$/J (*Tlr4*$^{-/-}$)	The Jackson Laboratory	Stock No: 029015
Mouse: B6.129S7-*Il1r1*^{tm1Imx}/J (*Il1r1*$^{-/-}$)	The Jackson Laboratory	Stock No: 003245
Mouse: B6.129S-Tnfrsf1a^{tm1Imx} Tnfrsf1b^{tm1Imx}/J (*Tnfr1*$^{-/-}$*Tnfr2*$^{-/-}$)	The Jackson Laboratory	Stock No: 003243
Mouse: B6.FVB-Tg(Itgax-DTR/EGFP)^{57Lan}/J (*Itgax*-DTR,-EGFP)	The Jackson Laboratory	Stock No: 004512
Mouse: C57BL/6 T-bet-ZsGreen reporter (TBGR)	Laboratory of Dr. J. Zhu (NIAID, NIH)	N/A
Software and Algorithms		
Prism V7	GraphPad	https://www.graphpad.com/
FlowJo V9.9.6	TreeStar	https://www.flowjo.com
javaGSEA V3.0	Broad Institute	http://www.gsea-msigdb.org/gsea/index.jsp
R2017b V9.3	MATLAB	https://www.mathworks.com/products/matlab/whatsnew.html

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Beatriz León (bleon@uab.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

The mouse strains used in these experiments include: C57BL/6J (B6), B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ (CD45.1^{+} B6 congenic), C57BL/6-Tg(TcraTcrb)425Cbn/J (OT-II), B6.129-Il4^{tm1Lky}/J (B6.4get IL-4 reporter mice), B6.129S(C)-Batf3^{tm1Kmm}/J (*Baft3*$^{-/-}$), B6.129-Tbx21^{tm2Srnr}/J (*Tbx21*$^{fl/fl}$), C57BL/6J-Tg(Itgax-cre,-EGFP)4097Ach/J (*Itgax-cre,-EGFP*), B6.129S6-*Tbx21*^{tm1Glm}/J (*Tbx21*$^{-/-}$),

B6(Cg)-*Tlr4*$^{tm1.2Karp}$/J (*Tlr4*$^{-/-}$), B6.129S7-*Il1r1*^{tm1Imx}/J (*Il1r1*$^{-/-}$), B6.129S-Tnfrsf1a^{tm1Imx} Tnfrsf1b^{tm1Imx}/J (*Tnfr1*$^{-/-}$*Tnfr2*$^{-/-}$) B6.FVB-Tg(Itgax-DTR/EGFP)^{57Lan}/J (*Itgax*-DTR,-EGFP) and C57BL/6 T-bet-ZsGreen reporter (TBGR). B6.4get mice were originally obtained from Dr. M. Mohrs (Trudeau Institute). TBGR mice were developed and kindly provided by Dr. J. Zhu (NIAID, NIH). All other mice were originally obtained from Jackson Laboratory and were bred and housed in the University of Alabama at Birmingham animal facility under specific pathogen-free conditions. Experiments were equally performed with male and female mice. The University of Alabama at Birmingham Institutional Animal Care and Use Committee approved all procedures involving animals.

METHOD DETAILS

Immunizations

HDM (Dermatophagoides pteronyssinus and D. farinae) extracts were obtained from Greer laboratories (< 12 EU/mg endotoxin). Mice were administered (i.n. or i.t.) with 50 μg of HDM extract ± 5 μg of LPS-free EndoFit OVA (InvivoGen) +/− LPS from *Escherichia coli* 0111:B4 (Sigma-Aldrich) +/− 150 ng of recombinant IL-12 (PeproTech) +/− 10-30 ng of recombinant TNFα (R&D Systems) daily for 1-3 days and challenged (i.n.) with 50 μg of HDM ± 5 μg of LPS-free EndoFit OVA. i.n. and i.t. administrations were given in 50 μL of PBS for adults and 18-20d-old infant mice and in 10 μL of PBS for 7d-old, 10d-old and 14d-old infant mice. In some experiments, mice were intraperitoneally administered (i.p.) with 250 μg of anti-TNFα mAb (BioXCell) at the time of initial sensitization. To deplete CD11c$^+$ cells, *Itgax*-DTR BM chimeras were i.p. treated with 60 ng DT (Sigma-Aldrich) on day 3 after sensitization. In some experiments HDM extract was labeled with AF647 labeling kit (Invitrogen) prior to administration to mice.

BM chimeras

Recipient mice were irradiated with 950 Rads from a high-energy X-rays source delivered in a split dose and reconstituted with 10^7 total BM cells. Mice were allowed to reconstitute for at least 8-12 weeks before HDM treatment.

Cell preparation and flow cytometry

Lungs were isolated, cut into small fragments and digested for 45 min at 37°C with 0.6 mg/mL collagenase A (Sigma) and 30 μg/mL DNase I (Sigma) in RPMI-1640 medium (GIBCO). Digested lungs, mLN or spleens were mechanically disrupted by passage through a wire mesh. Red blood cells were lysed with 150 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA. Fc receptors were blocked with anti-mouse CD16/32 (5 μg/mL; BioXCell5), followed by staining with fluorochrome-conjugated Ab. Fluorochrome-labeled anti-B220 (RA3-6B2), anti-CD4 (GK1.5), anti-CD11b (M1/70), anti-CD11c (HL3), anti-CD44 (IM7), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD69 (H1-2F3), anti-CD86 (GL1), anti-CD103 (M290), anti-CD138 (281-2), anti-IgE (R35-72y), anti-Ly6c (AL-21), anti-Siglec-F (E50-2440) and anti-Siglec-H (440c) were from BD Biosciences. Fluorochrome-labeled anti-CD64 (X54-5/7.1) and anti-MHC class II (M5/114.15.2) were from Biolegend.

For intracellular cytokine staining of T cells, cell suspensions were stimulated with PMA (20 ng/mL) plus Calcimycin (1 μg/mL) in the presence of Brefeldin-A (BFA, 10 μg/mL) for 4h. Restimulated cells were surface stained, fixed in 4% paraformaldehyde, permeabilized with 0.1% saponin, and stained with Abs against IL-13 (13A; eBiosciences), IFNγ (XMG1.2; BD-Biosciences) and IL-5 (TRFK5; BioLegend). For cytokine staining of DCs, cell suspensions were surface stained, fixed, permeabilized, and stained with anti-IL-12 (C15.6; BD-Biosciences). For TNFα staining, lung cell suspensions were incubated in the presence of BFA for 4h, fixed, permeabilized, and stained with anti-TNFα (MP6-XT22; BD-Biosciences). T-bet, GATA-3, Foxp3, IRFs, c-Rel, P-c-Jun and IkBζ intracellular staining was performed using the Mouse regulatory T cell staining kit (eBioscience) and Abs against T-bet (4B10; biolegend), GATA-3 (TWAJ; eBioscience), Foxp3 (.FJK-16 s; eBioscience), IRF1 (ab216114; Abcam), IRF4 (IRF4.3E4; BioLegend), IRF5 (ab212239; Abcam), c-Rel (sc-6955; Santa Cruz), P-c-Jun (D47G9; Cell Signaling Technology) and IkBζ (LK2NAP; eBioscience). Flow cytometry was performed on Attune NxT and FACSCanto II (BD-Biosciences) instruments.

Cell purifications, cell transfers and *in vitro* cultures

CD4$^+$ T cells were isolated by MACs (Miltenyi Biotec) from the spleens of naive OTII mice. CD11c$^+$ cells were isolated by MACs from pooled lungs or mLN of day 3 HDM+/−OVA+/−LPS-treated mice. All T and CD11c$^+$ cell preparations were more than 95% pure. HDM+/−LPS sensitization greatly reduced the proportion of CD11c$^+$ alveolar macrophages in the lungs (data not shown) and purification of CD11c$^+$ cells from the lungs of sensitized mice resulted in more than 80%–90% enrichment of CD11c$^+$ Siglec-F$^-$ MHC^{hi} lung DCs. Equivalent numbers (25×10^3) of naive OTII cells were transferred (i.v.) into naive congenic recipients. Equivalent numbers (1×10^6) of CD11c$^+$ lung cells were transferred (i.n.) into naive congenic infant recipients daily for 3 days. For *in vitro* cultures, OTII cells were labeled for 10 min at 37°C with CellTrace CFSE (ThermoFisher). $2x10^4$ DCs and $1x10^5$ CFSE-labeled OTII cells were cultured in 200 μL of complete medium in round-bottomed 96-well plates for 72 h at 37°C. Complete medium included RPMI 1640 supplemented with sodium pyruvate, HEPES, non-essential amino acids, penicillin, streptomycin, 2-mercaptoethanol and 10% heat-inactivated FBS from Fisher Scientific.

BAL collection and measurement of cytokines

BAL was collected using 0.5-1 mL sterilized PBS per mouse. The BAL fluids were centrifuged at 5,000 × g for 10 min and the supernatants were frozen at −80°C. Cytokine measurements were performed using ProcartaPlex assay (Life Technologies) and Luminex xMAP technology as per manufacturer's instructions.

Lung histology

The lungs were fixed in buffered formalin (10%). Microtome sections of paraffin-embedded lungs were stained with periodic acid-Schiff (PAS) for visualization of goblet cells.

Pulmonary function assessment

To measure airway responsiveness, mice on day 5 post-challenge were mechanically ventilated and challenged with increasing concentrations of methacholine, as described previously (Song et al., 2011). Briefly, mice were anesthetized, intubated, connected to a ventilator (FlexiVent; SCIREQ, Montreal, PQ, Canada), and ventilated at a rate of 160 breaths per minute. Airway responsiveness was recorded at baseline and after a linear dose response with methacholine challenge (10–75 mg/mL; Sigma-Aldrich). Data were normalized to basal airway resistance and expressed as changes induced by methacholine. To measure arterial O_2 saturation (SpO2), the MouseOx Pulse-oximeter (Starr Life Sciences, Oakmont PA) was used on conscious mice 5 days-post-challenged, in accordance with manufacturer's instructions. Mice were shaved and the pulse-oximeter collar was placed behind the ears. To ensure accurate measurement, SpO2 data points were excluded from analysis if received an error code during measurement.

RNA-sequencing (RNA-seq). mCD11b$^+$DCs (B220$^-$CD64$^-$CD103$^-$CD11c$^+$MHCII^{hi}CD11b$^+$) from adult (7wks-old) and infant (2-3wks-old) mice were sorted from the mLN 24h after HDM+LPS sensitization using a FACSAria (BD Biosciences) after positive selection with anti-CD11c MACS beads (Miltenyi Biotec). RNA was isolated from the sorted cells using the RNeasy Plus Micro RNA purification kit (QIAGEN). Three or four replicates from three or four independent experiments for each condition were analyzed with RNA-seq. Library preparation and RNA sequencing was conducted through Genewiz. Libraries were sequenced using a 1Å~50-bp single end rapid run on the HiSeq2500 platform. Sequence reads were trimmed to remove possible adaptor sequences and nucleotides with poor quality (error rate < 0.05) at the end. After trimming, sequence reads shorter than 30 nucleotides were discarded. Remaining sequence reads were mapped to the *Mus musculus* mm10 reference genome using CLC genomics workbench v. 9.0.1. Differential gene expression was determined using DESeq2. Genes with an adjusted P value < 0.05 and an absolute log1.5-fold change (ratio ± 0.58) were considered significantly differentially expressed genes between adult and infant mCD11b$^+$DCs.

GSEA, hierarchical clustering and visualization of RNA sequencing results. GSEA was performed using the Molecular Signatures Database on the publically available MIT BROAD Institute server. We ranked the 173 genes obtained from RNA sequencing (Genewiz) according to a logarithmic transformation of each gene's P value multiplied by the sign of the corresponding logarithmic fold change, and subsequently used these rank lists to perform a gene set enrichment analysis (Broad Institute's GSEA Java app, version 3.0). Given gene sets can include both activated and repressed genes, we additionally performed an extended enrichment assessment of all identified gene sets. Separately, we performed hierarchical clustering analysis (Bar-Joseph et al., 2001; Eisen et al., 1998) of TNFα induced genes that are differentially expressed in adult versus infant mice, using MATLAB (version R2017b). Differential clusters are presented in form of an annotated heatmap based on standardized expression values, along with the resulting hierarchical clustering dendogram.

QUANTIFICATION AND STATISTICAL ANALYSIS

All plots and histograms were plotted in FlowJo v.9 software (Treestar). GraphPad Prism software (Version 7) was used for data and statistical analysis. Data were analyzed using the unpaired Student's t test. Values of $p < 0.05$ were considered significant ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

DATA AND SOFTWARE AVAILABILITY

The accession number for the raw data files for the RNA-Seq analyses reported in this paper is GEO: GSE110046.

Immunity, Volume *50*

Supplemental Information

Impaired Tumor-Necrosis-Factor-α-driven Dendritic Cell Activation Limits Lipopolysaccharide-Induced Protection from Allergic Inflammation in Infants

Holly Bachus, Kamaljeet Kaur, Amber M. Papillion, Tatiana T. Marquez-Lago, Zhihong Yu, André Ballesteros-Tato, Sadis Matalon, and Beatriz León

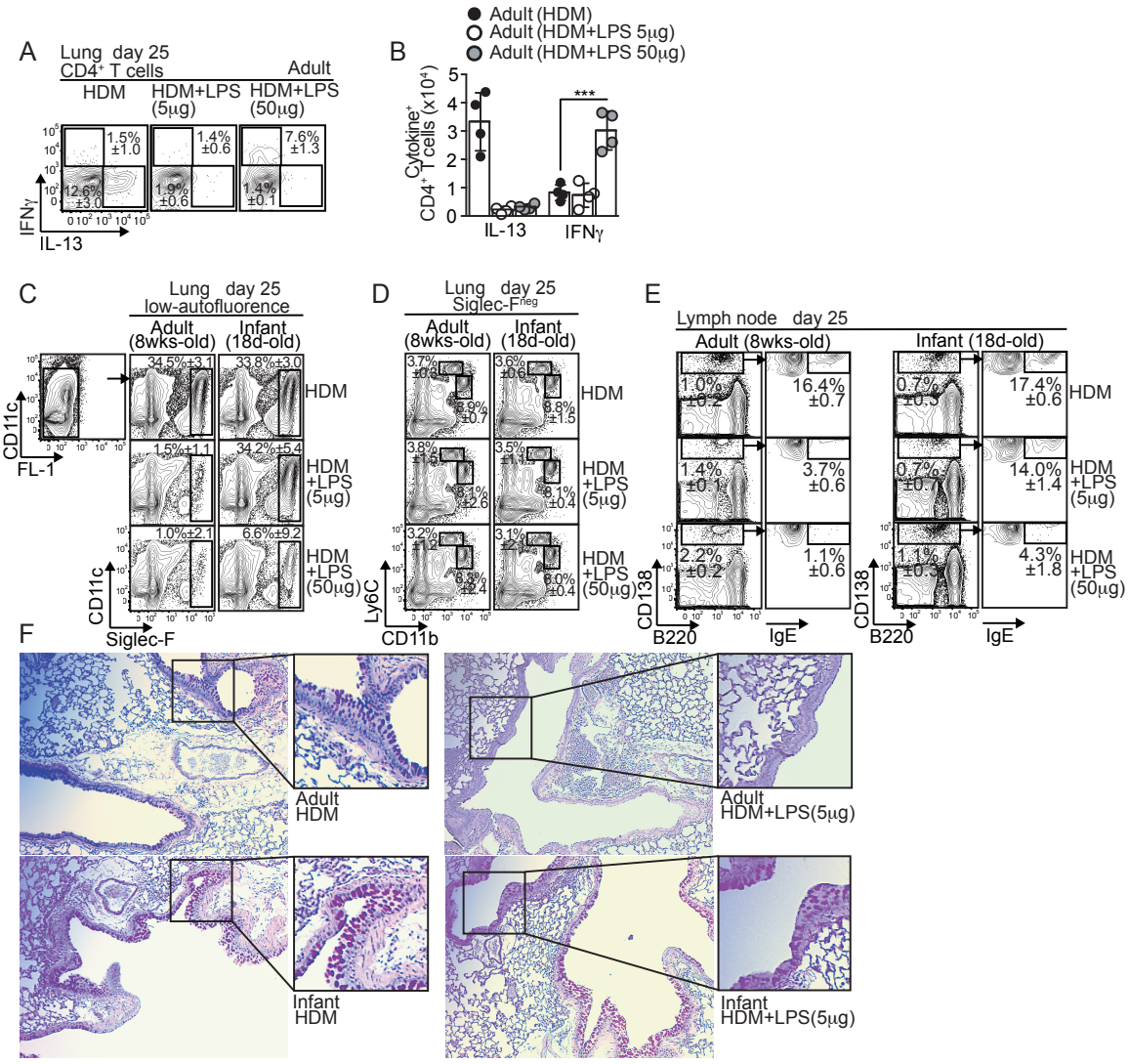


Figure S1. Related to Figure 1. Low-dose LPS sensitization in 18d-old infants does not prevent HDM-induced asthma features in adult life. B6 adult and 18d-old infant mice were i.n treated with 50μg of HDM plus different concentrations of LPS for 3 consecutive days starting on day 1. On day 20, mice were i.n challenged with 50μg of HDM daily for 3 days and analyzed on day 25. (**A-B**) Frequencies (**A**) and numbers (**B**) of IL-13- and IFNγ-expressing $CD4^+$ T cells in the lungs determined by ICS after restimulation for 4 h. (**C-D**) Frequencies of eosinophils (autofluorescence^{lo} $CD11c^{lo}$ Siglec-F^+) (**C**), neutrophils (Siglec-F^-CD11b^{hi}-$Ly6C^{int}$) and monocytes (Siglec-$F^-CD11b^{int}Ly6C^{hi}$) (**D**) in the lungs. (**E**) Frequencies of IgE^+ ACS in the mLN. (**F**) Lung sections stained with Periodic Acid-Schiff (PAS) to identify globlet cells. *P < 0.05 **P < 0.01 ***P < 0.001 (unpaired Student's t test). Data are representative of at least two independent experiments (mean and S.D. of 4-5 mice per group).

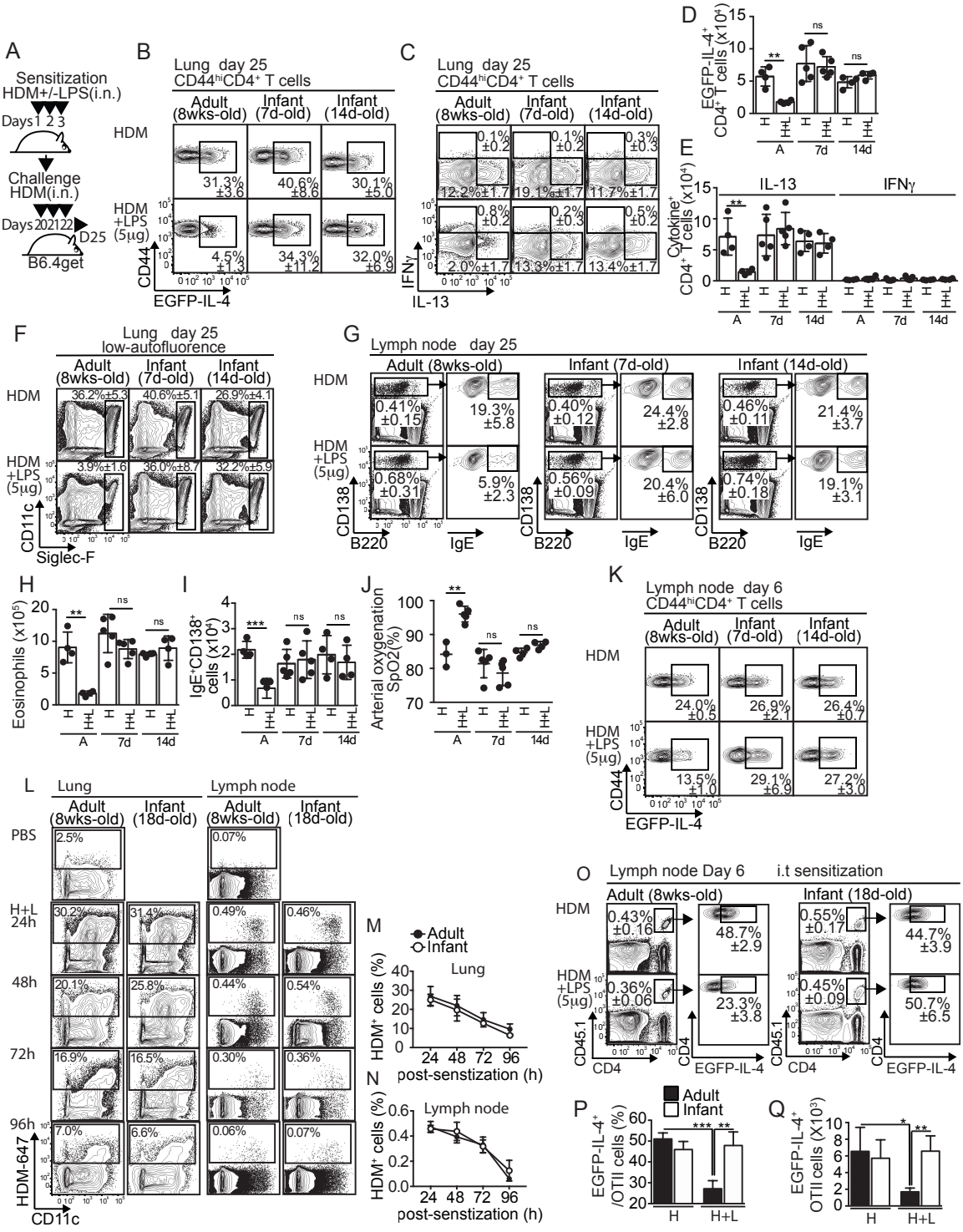


Figure S2. Related to Figure 1. Low-dose LPS sensitization in 7 to 18d-old infants does not prevent HDM-induced Th2 priming and development of Th2 cell-mediated asthmatic features later in life. (**A-J**) B6.4get adults, 7d-old neonates and 14d-old infants were i.n treated with 50µg of HDM+/-5µg of LPS for 3 consecutive days starting on day 1. On day 20, mice were i.n challenged with 50µg of HDM daily for 3 days and analyzed on day 25. Frequencies (**B-C**) and numbers (**D-E**) of IL-4-expressing (EGFP$^+$) CD4$^+$ T cells (**B, D**) and IL-13- and IFNγ-expressing CD4$^+$ T cells (**C, E**) in the lungs. Frequencies (**F**) and numbers (**H**) of eosinophils in the lungs. Frequencies (**G**) and numbers (**I**) of IgE$^+$ ACS in the mLN. SpO2 as determined by pulse-oximetry (**J**). (**K**) B6.4get adults, 7d-old neonates and 14d-old infants were i.n treated with 50µg of HDM+/-5µg of LPS for 3 consecutive days starting on day 1 and analyzed on day 6. Frequencies of IL-4-expressing (EGFP$^+$) CD4$^+$T cells in the mLN. (**L-N**) B6 adult and 18d-old infant mice were i.n treated with Alexa Fluor 647-labeled HDM+5µg of LPS on day 1 and analyzed at different time points. Frequencies of HDM-bearing cells in the lungs (**L-M**) and mLNs (**L, N**). (**O-Q**) Adult and 18d-old infant naïve B6 mice were adoptively transferred with 25x10^3 CD45.1$^+$ OTII.4get cells at day 0, i.t treated with 50µg of HDM+5µg of OVA+/-5µg of LPS for 3 consecutive days starting on day 1 and analyzed by flow cytometry on day 6. Frequencies (**O-P**) and numbers (**Q**) of donor IL-4-expressing (EGFP$^+$) OTII T cells in the mLN of recipient mice. *P < 0.05 **P < 0.01 ***P < 0.001 (unpaired Student's t test). Data are representative of two independent experiments (mean and S.D. of 3-5 mice per group).



Figure S3. Related to Figure 2. Kinetic analysis of specific CD4+ T cells and mDCs in the mLN after low-dose LPS sensitization. (**A-D**) Adult and 18d-old infant naïve B6 mice were adoptively transferred with 25x10³ CD45.1+ OTII.4get cells at day 0, i.n treated with 50μg of HDM+5μg of OVA+/-5μg of LPS for 3 consecutive days starting on day 1 and analyzed by flow cytometry on days 4,5 and 6. Frequencies of donor OTII T cells (**A**) and frequencies of GATA-3 and T-bet-expressing cells (**B**), MFI of GATA-3 expression (**C**) and MFI of T-bet expression (**D**) in donor OTII T cells from mLN of recipient mice. (**E-K**) B6 adult and 18d-old infant mice were i.n treated with HDM+/-5μg of LPS (**E-G**) or with Alexa Fluor 647-labeled HDM+/-5μg of LPS (**H-K**) for 3 consecutive days and analyzed at different time points. Frequencies and numbers of CD103+ mDCs (**E-F**), CD11b+ mDCs (**E,G**), HDM-bearing mDCs (**H-I**), HDM-bearing CD103+ mDCs (**H,J**) and HDM-bearing CD11b+ mDCs (**H,K**). Data are representative of three independent experiments (mean ± SD of 4 mice per time point).

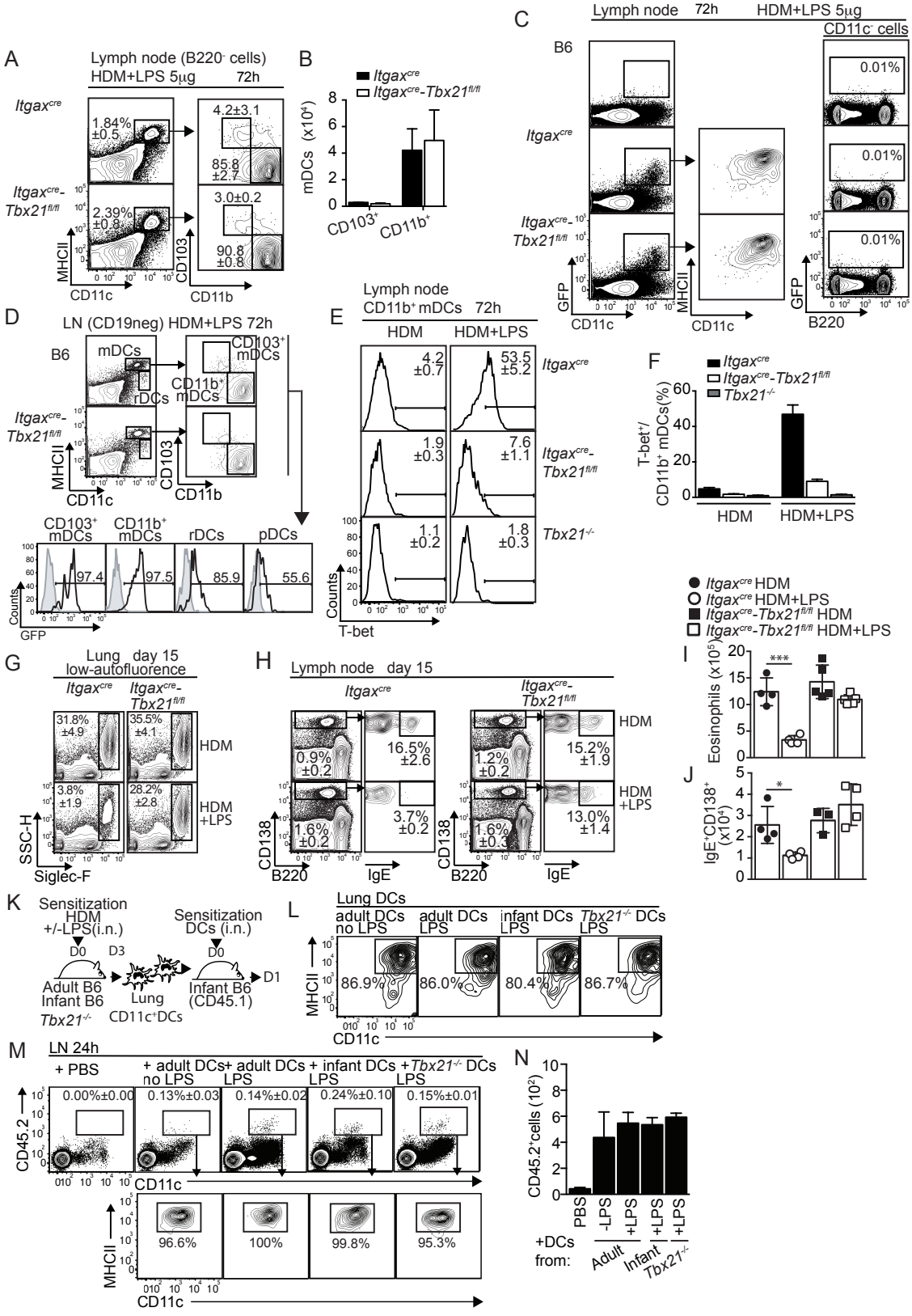


Figure S4. Related to Figure 4. Analysis of DCs in *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ mice and analysis of lung mDCs in recipient mice after i.n. transference. (**A-F**) *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ and control mice were i.n. HDM or HDM+5µg of LPS sensitized on day 1 and analyzed on day 4. Frequencies (**A**) and numbers (**B**) of CD103$^+$ mDCs and CD11b$^+$ mDCs in the mLN. Expression of GFP in total mLN cells and in CD11c$^-$ mLN cells and expression of CD11c and MHCII in GFP$^+$ cells from mLN (**C**). Expression of GFP in gated CD103$^+$ mDCs, CD11b$^+$ mDCs, rDCs and pDCs (Siglec-H+, CD11cl^o, B220$^+$ CD19$^-$). Gray filled overlays indicate control staining from B6 mice (**D**). Frequencies of T-bet expression in CD11b$^+$ mDCs from mLN (**E-F**). (**G-J**) *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ and control mice were i.n treated with 50µg of HDM+/-5µg of LPS for 3 consecutive days starting on day 1. On day 10, mice were i.n challenged with 50µg of HDM daily for 3 days and analyzed on day 15. Frequencies (**G**) and numbers (**I**) of eosinophils in the lungs. Frequencies (**H**) and numbers (**J**) of IgE$^+$ ACS in the mLN. (**K-N**) Day 3 CD11c$^+$ lung mDCs from HDM/OVA+/-LPS-treated adult and infant B6 mice or *Tbx21*$^{-/-}$ mice were purified and i.n. transferred into B6 (CD45.1$^+$) infant mice (K). Frequencies of CD11c$^+$MHCII^{hi} lung mDCs in purified lung preparations before transference (**L**). Frequencies (**M**) and numbers (**N**) of donor mDCs in the mLN 24h after transference. *P < 0.05 ***P < 0.001 (unpaired Student's t test). Data are representative of at least two independent experiments (mean and S.D. of 3-5 mice per group).

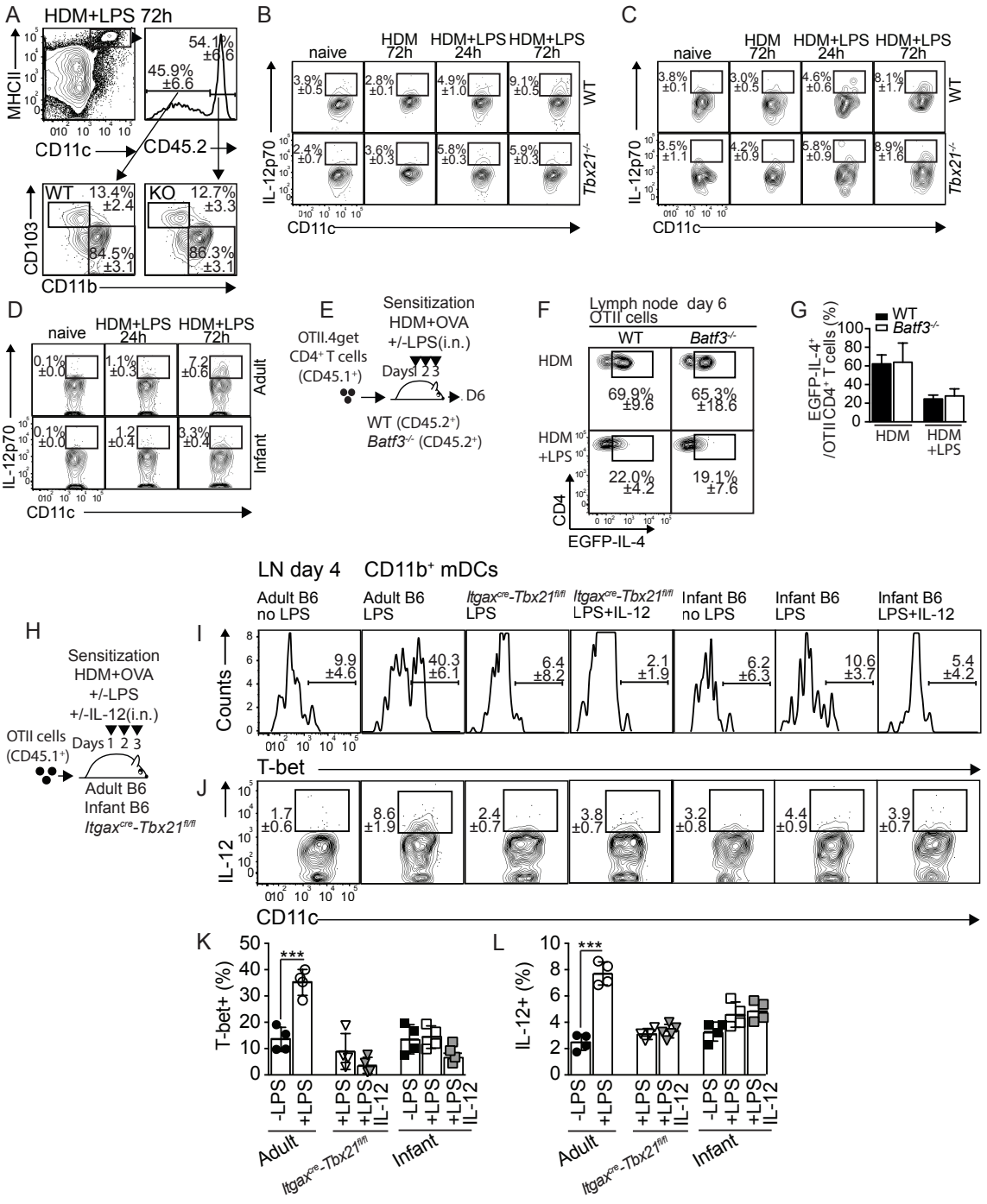


Figure S5. Related to Figure 5. *Tbx21*$^{-/-}$ and infant CD11b^{+} mDCs are intrinsically impaired in their ability to produce IL-12 after low-dose LPS exposure. (**A-C**) B6 (CD45.1^{+}) mice were irradiated and reconstituted with 1:1 mixture of BM from B6 (CD45.1^{+}) and *Tbx21*$^{-/-}$ (CD45.2^{+}) donors. Frequency of WT and *Tbx21*$^{-/-}$ CD11b^{+} and CD103^{+} mDCs in the mLN of day 3-HDM+LPS-treated reconstituted chimeric mice (**A**). Frequencies of IL-12p70-expressing WT and *Tbx21*$^{-/-}$ CD11b^{+} mDCs (**B**) and CD103^{+} mDCs (**C**) in the mLN of HDM+/-LPS-treated reconstituted chimeric mice. (**D**) Frequency of IL-12p70-expressing CD11b^{+} mDCs in the mLN of HDM+/-LPS-treated adult and infant B6 mice. (**E-G**) *Batf3*$^{-/-}$ and control mice were adoptively transferred with 25x10^{3} CD45.1^{+} OTII.4get cells at day 0 and i.n treated with 50µg of HDM+5µg of OVA+/-5µg of LPS for 3 consecutive days starting on day 1 (**E**). Frequencies of IL-4-expressing (EGFP^{+}) cells in donor OTII T cells from the mLN on day 6 (**F-G**). (**H-L**) Adult and 18d-old infant B6 mice or *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ mice were i.n treated with 50µg of HDM+5µg of OVA+/-5µg of LPS+/-150ng of rIL-12p70 for 3 consecutive days starting on day 1 (**H**). Frequencies of T-bet (**I,K**) and IL-12p70 (**J,L**) expression in CD11b^{+} mDCs from mLN on day 4. ***P < 0.001 (unpaired Student's t test). Data are representative of two independent experiments (mean and S.D. of 3-4 mice per group).

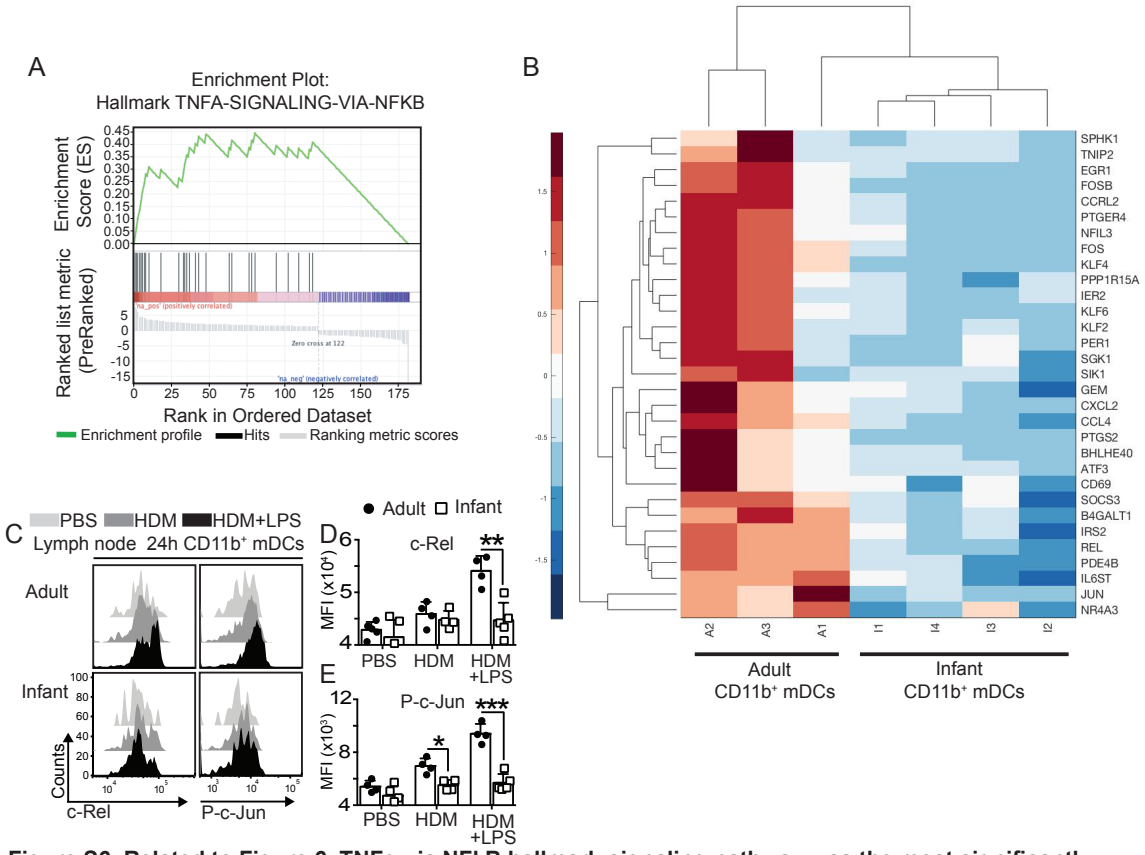


Figure S6. Related to Figure 6. TNFα-via NFkB hallmark-signaling pathway was the most significantly down-regulated pathway in infant CD11b⁺ mDCs after low-dose LPS sensitization. (**A-B**) CD11b⁺ mDCs were sorted from the mLN of adult and 18d-old infant mice 24 after HDM+LPS^{lo} sensitization and RNA-seq was performed. (**A**) GSEA showing enrichment in the TNF-α via NFkB hallmark-signaling pathway in adults vs. infant CD11b⁺ DCs (adjusted p-value <0.05). (**B**) Heat map displaying the expression of the TNFα-via NFkB hallmark signaling pathway genes that are differentially expressed in adult CD11b⁺ mDCs relative to infant CD11b⁺ mDCs. Replicates for each cell type were obtained from three or four independent experiments. (**C-E**) B6 adult and 18d-old infant mice were i.n treated with HDM+/-5µg of LPS. Expression c-Rel (**C,D**) and P-c-Jun (**C,E**) in CD11b⁺ mDCs from the mLN on day 1. *P < 0.05 **P < 0.01 ***P < 0.001 (unpaired Student's t test). Data are representative of two independent experiments (mean and S.D. of 4-5 mice per group).

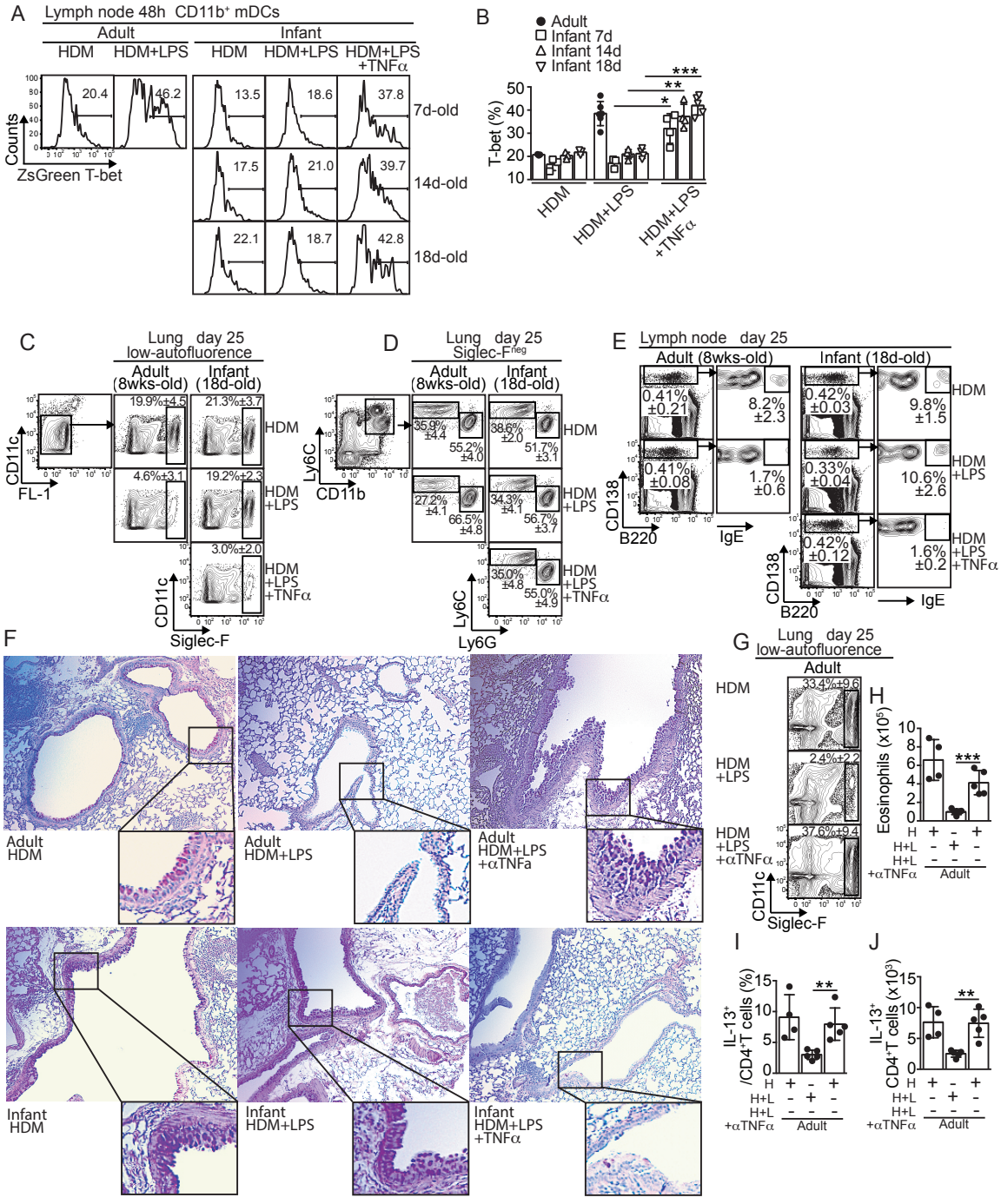


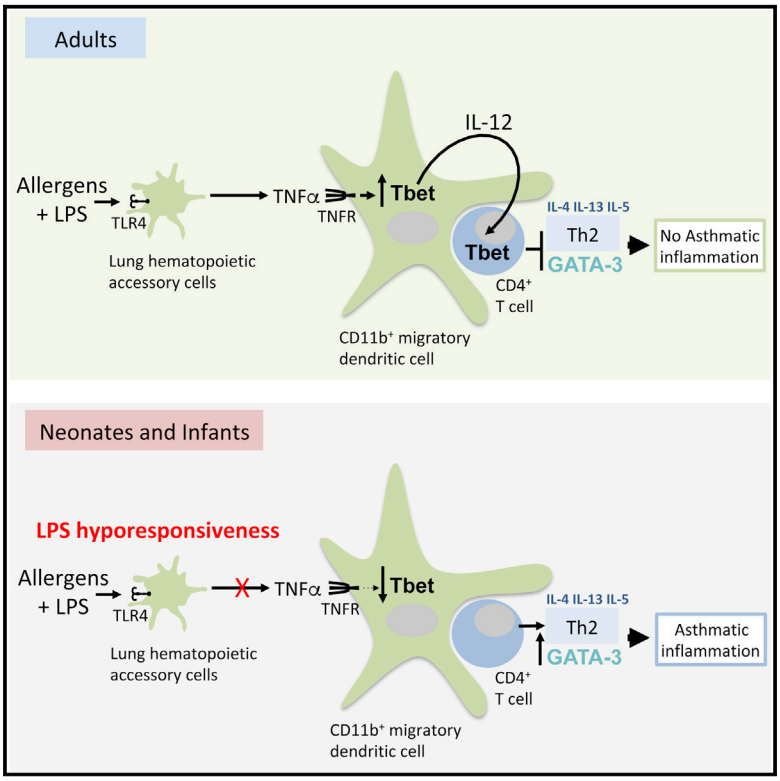
Figure S7. Related to Figure 7. Asthma features in the presence and absence of TNF-α. (**A-B**) T-bet-ZsGreen reporter adults, 7d-old neonates, 14d-old infants and 18d-old infants were i.n treated with HDM+/-5μg of LPS+/-30ng of TNF-α. Frequencies of T-bet-expressing (ZsG+) mLN CD11b$^+$ mDCs on day2. (**C-J**) B6 adult and 18d-old infant mice were i.n treated with HDM+/-5μg of LPS+/-30μg of TNF-α. Some adult mice also receive 250μg of anti-TNFα (i.p.). On day 20, mice were i.n challenged with HDM and analyzed on day 25. Frequencies of eosinophils (autofluorescencelo CD11c^{lo} Siglec-F$^+$) (**C, G**), neutrophils (Siglec-F$^-$CD11b^{hi}Ly6C^{int}) and monocytes (Siglec-F$^-$CD11b^{int}Ly6C^{hi}) (**D**) and numbers of eosinophils (**H**) in the lungs. Frequencies of IgE$^+$ ACS (**E**) in the mLN. Lung sections stained with PAS to identify globlet cells (**F**). Frequencies (**I**) and numbers (**J**) of IL-13-expressing CD4$^+$ T cells in the lungs determined by ICS after restimulation for 4 h. *P < 0.05 **P < 0.01 ***P < 0.001 (unpaired Student's t test). Data are representative of two independent experiments (mean and S.D. of 3-5 mice per group).

Article

Immunity

Impaired Tumor-Necrosis-Factor-α-driven Dendritic Cell Activation Limits Lipopolysaccharide-Induced Protection from Allergic Inflammation in Infants

Graphical Abstract



Highlights

- Infant mice require high doses of LPS to suppress Th2-driven allergic asthma to HDMs
- T-bet^{+} lung CD11b^{+} DCs inhibit Th2 cell development after HDM + LPS sensitization
- T-bet expression on CD11b^{+} DCs depends on TNF-α production and TNFR signaling
- Infants have an impaired ability to induce TNF-α and T-bet^{+}CD11b^{+} DCs in response to LPS

Authors

Holly Bachus, Kamaljeet Kaur, Amber M. Papillion, ..., André Ballesteros-Tato, Sadis Matalon, Beatriz León

Correspondence

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In Brief

Children have a higher risk of developing asthma in low-LPS "clean" environments than adults. Bachus et al. demonstrate that infant mice require higher doses of LPS than adult mice to prevent Th2-dependent allergic responses because of the decreased ability to induce LPS-driven TNF-α production and TNFR-mediated CD11b^{+} dendritic cell activation for Th2 cell suppression.

Bachus et al., 2019, Immunity *50*, 225–240
January 15, 2019 Published by Elsevier Inc.
https://doi.org/10.1016/j.immuni.2018.11.012

CellPress

Immunity
Article

Impaired Tumor-Necrosis-Factor-α-driven Dendritic Cell Activation Limits Lipopolysaccharide-Induced Protection from Allergic Inflammation in Infants

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https://doi.org/10.1016/j.immuni.2018.11.012

SUMMARY

Infants have a higher risk of developing allergic asthma than adults. However, the underlying mechanism remains unknown. We show here that sensitization of mice with house-dust mites (HDMs) in the presence of low-dose lipopolysaccharide (LPS) prevented T helper 2 (Th2) cell allergic responses in adult, but not infant, mice. Mechanistically, adult CD11b$^+$ migratory dendritic cells (mDCs) upregulated the transcription factor T-bet in response to tumor necrosis factor-α (TNF-α), which was rapidly induced after HDM + LPS sensitization. Consequently, adult CD11b$^+$ mDCs produced interleukin-12 (IL-12), which prevented Th2 cell development by promoting T-bet upregulation in responding T cells. Conversely, infants failed to induce TNF-α after HDM + LPS sensitization. Therefore, CD11b$^+$ mDCs failed to upregulate T-bet and did not secrete IL-12 and Th2 cell responses normally developed in infant mice. Thus, the availability of TNF-α dictates the ability of CD11b$^+$ mDCs to suppress allergic Th2-cell responses upon dose-dependent endotoxin sensitization and is a key mediator governing susceptibility to allergic airway inflammation in infant mice.

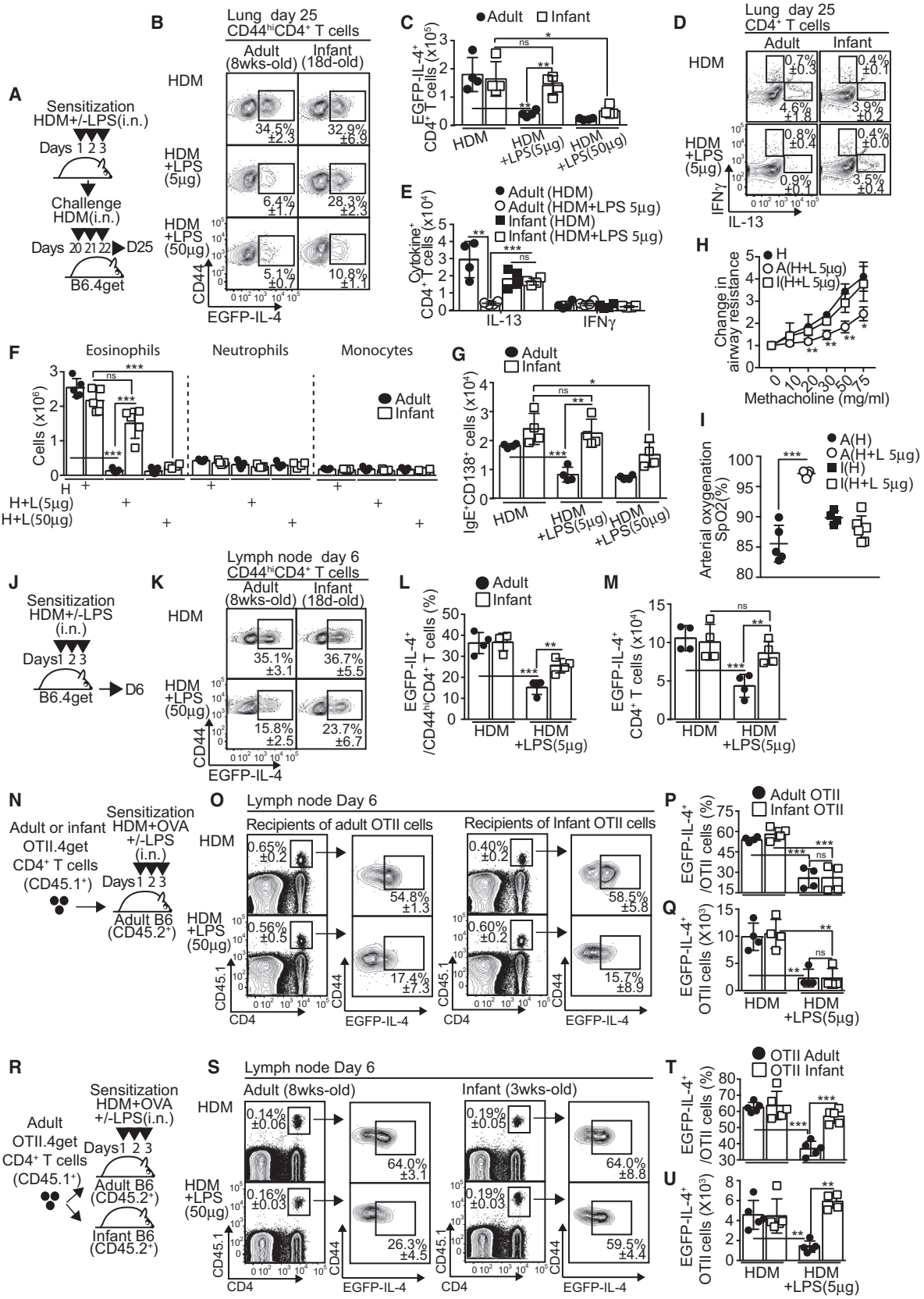
INTRODUCTION

Asthma is the most common chronic disease of childhood and currently affects ~10% of school-aged children in the US (Masoli et al., 2004). About 95% of those children begin developing asthma in very early childhood, normally before they turn 5 years of age (Masoli et al., 2004). Most cases of pediatric asthma are triggered by early sensitization to common environmental allergens, which ultimately leads to the activation of T helper 2 (Th2) cells and the development of chronic Th2-cell-driven lung inflammation (Reynolds and Finlay, 2017). Despite the epidemiological and mechanistic studies, however, the underlying mechanisms for the high susceptibility to airway allergic inflammation and asthma development in infants remain elusive.

The incidence of allergic airway disease has increased over the past decades in industrialized countries (Masoli et al., 2004). Although numerous environmental factors associated with this change of lifestyle might have contributed to this rise, the hygiene hypothesis proposes that the decreased exposure to microbial products is one of the main drivers of allergic airway disease. Supporting this idea, exposure to microbial products, such as lipopolysaccharide (LPS), protects from the development of experimental-allergen-induced asthma (Daan de Boer et al., 2013; Schuijs et al., 2015). In addition, infants sensitized to common circulating allergens in a "clean" environment are at a higher risk of developing asthma later in life than infants exposed to high levels of enviromental endotoxin (Gereda et al., 2000; Reynolds and Finlay, 2017; Schuijs et al., 2015; Stein et al., 2016; Zhu et al., 2010), thus suggesting a particular requirement for high endotoxin exposure during infancy for allergic asthma protection, although this requirement is not fully understood.

Dendritic cells (DCs) are the main antigen-presenting cells for T cell activation and polarization. In lymph nodes (LNs), DCs can be broadly divided into LN-resident DCs (rDCs) and migratory DCs (mDCs), which differentiate in peripheral tissues and migrate constantly through afferent lymphatics to the draining LN. Both rDCs and mDCs contain two major subsets: IRF8-dependent CD103$^+$ DCs (recently named cDC1s) and IRF4-dependent CD11b$^+$ DCs (recently named cDC2s) (Guilliams et al., 2014). Although the roles of the various DC subsets are not fully understood, CD11b$^+$ mDCs have been shown to be critical for the development of Th2 cell responses to common allergens (León, 2017; Plantinga et al., 2013). In contrast, CD103$^+$ mDCs are likely to prevent the development of allergic inflammation (Conejero et al., 2017). Whether alterations in individual DC subsets during infancy contribute to asthma susceptibility remains unexplored.

Here, we show that CD11b$^+$ mDCs from adult mice upregulated the transcription factor T-bet and produced interleukin-12 (IL-12) after house-dust-mite (HDM) sensitization in the presence of LPS. As a consequence, CD4$^+$ T cells interacting with CD11b$^+$



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mDCs in adult mice upregulated T-bet, which precluded Th2 cell differentiation and subsequent pathogenic allergic responses to HDMs. We found that the upregulation of T-bet on $CD11b^+$ mDCs was dependent on TNF-α production and TNFR signaling. Adults rapidly produced TNF-α in response to low-dose LPS sensitization; however, infant mice had an impaired ability to produce TNF-α in response to LPS. As a result, $CD11b^+$ mDCs from infant mice failed to upregulate T-bet and IL-12 in low-LPS conditions. Thus, $CD4^+$ T cells interacting with $CD11b^+$ mDCs in infants failed to receive IL-12 signals, did not upregulate T-bet, and fully differentiated into pathogenic Th2 cells. Thus, whereas low-LPS exposure during HDM sensitization protected adult mice from developing Th2-driven allergic asthma, infant mice required higher doses of LPS. Collectively, our data demonstrate that LPS prevents Th2-dependent allergic responses with different thresholds in adults and infants and provide a plausible mechanism underlying the higher susceptibility to allergic airway inflammation observed in children.

RESULTS

LPS Inhibits Th2-Cell-Mediated Immunity to Inhaled HDMs with Different Thresholds in Infant and Adult Mice

To test whether LPS influenced Th2 cell response to HDM sensitization in infancy and adulthood, we intranasally (i.n.) sensitized adult ($\geq$8-week-old) and infant (18-day-old) IL-4 reporter B6.4get mice with HDMs in the presence or absence of different amounts of LPS (sensitization phase; Figure 1A). We then challenged them i.n. with HDMs (challenge phase; Figure 1A) and determined the frequency (Figure 1B) and number (Figure 1C) of $GFP^+CD44^{hi}CD4^+$ T cells in the lungs. GFP^+CD4^+ T cells similarly accumulated in the lungs of adult and infant mice that had been sensitized with HDMs (Figures 1B and 1C) but failed to accumulate in adult mice that had been sensitized with HDMs in the presence of either low (5 μg; LPS^{lo}) or high (50 μg; LPS^{hi}) doses of LPS (Figures 1B and 1C). Similarly, LPS^{hi} significantly prevented the accumulation of GFP^+CD4^+ T cells in infant mice (Figures 1B and 1C). Importantly, however, LPS^{lo} exposure failed to prevent the expansion of GFP^+CD4^+ T cells in infant mice (Figures 1B and 1C). As a result, GFP^+CD4^+ T cells largely accumulated in the lungs of HDM + LPS^{lo}-sensitized infants relative to adult counterparts (Figure 1C). We next sensitized and challenged adult and infant C57BL/6J (B6) mice as described in Figure 1A and quantified IL-13^+ and IFN$^+$ $CD4^+$ T cells in the lungs. As predicted, IL-13^+CD4^+ T cells were less abundant in adults that had been initially sensitized with HDM + LPS^{lo} than in those initially sensitized with HDMs only (Figures 1D and 1E). Conversely, this effect was not observed in infants (Figures 1D and 1E). LPS^{lo} sensitization did not induce IFN-γ^+CD4^+ T cells in the lungs (Figures 1D and 1E), but inhalation of LPS^{hi} resulted in IFN-γ^+CD4^+ T cells (Figures S1A and S1B). These data show that relatively higher amounts of endotoxin exposure are necessary for preventing the accumulation of Th2 cells in infants. In agreement with these results, whereas LPS^{lo} administration at the time of initial sensitization prevented eosinophilic airway inflammation (Figures 1F, S1C, and S1D), airway goblet cell hyperplasia (Figure S1F) and accumulation of IgE^+CD138^+ antibody-secreting cells (IgE^+ ASCs) in the lung-draining, mediastinal lymph node (mLN) (Figures 1G and S1E) in adult challenged mice, it failed to do so in infants. Moreover, LPS^{lo} sensitization in adult, but not in infant, mice reduced airway responsiveness to methacholine (Figure 1H) and improved peripheral-blood arterial oxygen saturation (SpO2) (Figure 1I). Similar differences from adults were observed in neonatal (7-day-old), early infant (14-day-old), and infant (18-day-old) mice (Figures S2A–S2J). Altogether, these data indicate that exposure to relatively low endotoxin amounts, in a defined time window from the neonatal to infant period, fails to prevent the development of Th2 cell responses and allergic inflammation to inhaled HDMs.

The Environment of Infants Is More Resistant to LPS and More Prone to Prime HDM-Specific Th2 Cell Responses Than That of Adults

The development of allergen-specific Th2 effector cells that migrate to the lungs requires T cell priming in the mLN, which occurs after initial sensitization (Ballesteros-Tato et al., 2016). Therefore, we next enumerated $GFP^+CD44^{hi}CD4^+$ T cells in the mLN of adult and 18-day-old infant B6.4get mice during the sensitization phase (Figure 1J). The frequency (Figures 1K and 1L) and number (Figure 1M) of GFP^+CD4^+ T cells were significantly lower in HDM + LPS^{lo}-sensitized adult mice than in HDM-sensitized adult mice. In contrast, LPS^{lo} did not prevent the accumulation of GFP^+CD4^+ T cells in infants (Figures 1K–1M). Similar results were found in 7- and 14-day-old mice (Figure S2K). Lung neutrophilia were not examined because they are unlikely to significantly affect Th2 cell priming. These results suggest that low-dose LPS prevents Th2 cell priming in the mLN after HDM sensitization in adult, but not in neonatal or infant, mice. Next, we tested whether the differential capacity of low-dose LPS to prevent Th2 cell priming in adults and infants is

Figure 1. LPS^{lo} Sensitization in Infants Does Not Prevent HDM-Induced Th2 Cell Priming or Development of Lung Th2 Cell Responses in Adult Life

(A–I) B6.4get (A–C) or B6 (D–I) adults (>8 weeks old) and infants (18 days old) were i.n. treated with 50 μg HDMs plus different concentrations of LPS for 3 days. On day 20, mice were i.n. challenged with 50 μg HDMs daily for 3 days and analyzed on day 25 (A). Shown are frequencies and numbers of IL-4^+ ($EGFP^+$) (B and C) and IL-13^+ and IFN-γ^+ (D and E) $CD4^+$ T cells in the lungs. Numbers of eosinophils, neutrophils, and monocytes in the lungs are shown in (F). Numbers of IgE^+ ASCs in the mLN are shown in (G). Changes in lung resistance (cm H_2O/mL/s) from baseline were analyzed via mechanical ventilation with the flexiVent system (H). Blood oxygen saturation (SpO2) was determined by pulse oximetry (I).

(J–M) B6.4get adults and 18-day-old infants were i.n. treated with 50 μg HDM ± 5 μg LPS for 3 days and analyzed on day 6 (J). Shown are frequencies (K and L) and numbers (M) of $EGFP^+CD4^+$ T cells in the mLN.

(N–U) Adult B6 mice were adoptively transferred with 25 × 10^3 $CD45.1^+$ OT-II.4get cells from adult or infant donor mice (N–Q), or adult and 18-day-old infant B6 mice were adoptively transferred with 25 × 10^3 $CD45.1^+$ OT-II.4get cells from naive adult donor mice (R–U). Recipients were i.n. treated with 50 μg HDM + 5 μg OVA ± 5 μg LPS for 3 days and analyzed on day 6. Shown are frequencies (O, P, S, and T) and numbers (Q and U) of donor $EGFP^+$ OT-II T cells in the mLN.

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (unpaired Student's t test). Data are representative of at least two independent experiments (mean and SD of four or five mice per group). Please also see Figures S1 and S2.

dictated by intrinsic differences in the CD4$^+$ T cell compartment (Figures 1N–1Q). We transferred 4get.OT-II TCR-transgenic CD4$^+$ T cells from adult or infant mice into congenic adult recipients (Figure 1N). Recipients were then i.n. sensitized with HDM + endotoxin-free ovalbumin (HDM + OVA) ± LPS^{lo}, and donor 4get.OT-II (CD45.1$^+$) cells were analyzed for the expression of GFP-IL-4 in the mLN (Figures 1O–1Q). Data showed that the differential capacity of LPS^{lo} to prevent Th2 cell development in adults and infants was not due to intrinsic differences in the CD4$^+$ T cells. We next transferred 4get.OT-II cells from adult mice into adult or infant recipients (Figure 1R). Those were then sensitized with HDM + OVA ± LPS^{lo}, and donor-derived GFP$^+$ cells were analyzed (Figures 1S–1U). LPS^{lo} failed to prevent the accumulation of donor-derived GFP$^+$ 4get.OT-II cells when developing in infant, but not in adult, recipients (Figures 1S–1U). i.n. HDM + LPS delivery was similarly effective in adults and infants (Figures S2L–S2N). Similar results were obtained when sensitization was given intratracheally (i.t.) rather than i.n. (Figures S2O–S2Q). Thus, whereas low-LPS prevents Th2 cell differentiation in adult mice after HDM sensitization, the environment of the infants is more resilient to LPS; hence, infants are at higher risk for priming allergen-specific Th2 cell responses in the presence of low doses of endotoxin.

Infants Do Not Induce T-bet Expression in T Cells under LPS^{lo} Sensitization

T-bet suppresses the Th2-cell-associated program (Zhu et al., 2012). To test whether CD4$^+$ T cells developing in an infant or adult environment differently upregulate T-bet, we transferred 4get.OT-II cells from adult mice into either adult or infant recipients, sensitized them, and analyzed the dynamics of T-bet expression (Figures 2A–2C and S3A). As expected, LPS^{lo} treatment prevented the accumulation of 4get.OT-II GFP$^+$ cells in adult mice, but not infant mice, from day 5 (Figure 2B). Importantly, 4get.OT-II cells expressed higher amounts of T-bet when developing in adults sensitized with HDM + OVA + LPS^{lo} than when developing in non-LPS-sensitized recipients (Figure 2C). Further, this upregulation of T-bet occurred concomitantly with the downregulation of GATA-3 (Figures S3B–S3D). In contrast, 4get.OT-II cells failed to upregulate T-bet in HDM + OVA + LPS^{lo}-sensitized infants (Figure 2C). These results indicate that, although LPS^{lo} promotes T-bet expression in CD4$^+$ T cells differentiating in adult mice, CD4$^+$ T cells fail to upregulate T-bet when developing in infants. We next investigated whether T-bet upregulation to LPS is necessary for preventing Th2 cell differentiation. Thus, we co-transferred wild-type (WT) (CD45.2$^+$CD45.1$^+$) and *Tbx21*$^{-/-}$ (CD45.2$^+$) 4get.OT-II cells into CD45.1$^+$ adult recipients, sensitized them, and analyzed the progeny from the WT and *Tbx21*$^{-/-}$ donors (Figure 2D). As expected, the frequency of GFP$^+$ cells within the WT 4get.OT-II cells was lower in the mLN of HDM + OVA + LPS^{lo}-sensitized mice than in HDM + OVA-sensitized mice (Figures 2E and 2F). In contrast, the frequency of GFP$^+$ cells within the *Tbx21*$^{-/-}$ 4get.OT-II cells was equivalent in both groups (Figures 2E and 2F). We next analyzed the frequency of IL-13$^+$ cells within the donor WT and *Tbx21*$^{-/-}$ OT-II cells in the lung after the challenge phase (Figure 2G). As predicted, the frequency of IL-13$^+$ WT OT-II cells was lower in mice that had been initially sensitized with HDM + OVA + LPS^{lo} than in those that had been sensitized with HDM + OVA (Figures 2H and 2I). In contrast, the frequency of IL-13$^+$*Tbx21*$^{-/-}$ OT-II cells was equivalent in both groups (Figures 2H and 2I). Thus, expression of T-bet by CD4$^+$ T cells in response to low-dose endotoxin is important for suppressing the endogenous Th2 cell differentiation program in response to HDMs.

Lung mDCs from Adult and Infant Mice Differ in Their Ability to Induce T-bet in Responding T Cells

Lung mDCs are essential for priming Th2 cells to HDMs (León, 2017). Thus, we tested whether LPS^{lo} administration differentially affects adult and infant mDCs. Given that expression of T-bet by CD4$^+$ T cells was important to suppressing Th2 cell development after HDM + LPS^{lo} sensitization (Figures 2A–2I), we first tested the ability of infant and adult DCs to activate naive CD4$^+$ T cells and promote T-bet expression. mLN CD11c$^+$ DCs were co-cultured with CFSE-labeled naive OT-II cells (Figure 2J). O-TII proliferated equally when primed with CD11c$^+$ cells from adults or infants (Figure 2K). OT-II cells primed by CD11c$^+$ cells from HDM + OVA + LPS^{lo}-sensitized adults upregulated T-bet in relation to OT-II cells primed by CD11c$^+$ cells from HDM + OVA-sensitized adults (Figures 2L and 2M). In contrast, OT-II cells primed by CD11c$^+$ cells from HDM + OVA + LPS^{lo}-sensitized infants failed to upregulate T-bet (Figures 2L and 2M). These results indicate that DCs from infant and adult mice differ in their ability to induce T-bet expression in T cells after HDM + LPS^{lo} sensitization. We next characterized mLN mDC subsets in adult and infant mice as previously described (Ballesteros-Tato et al., 2010). Frequency (Figure S3E) and number (Figure S3F) of CD103$^+$mDCs decreased after HDM + LPS^{lo} sensitization in adult mice. In contrast, CD11b$^+$ mDCs largely accumulated (Figures S3E and S3G). Similar results were obtained in infants (Figures S3E–S3G). We next assessed the capacity of DC subsets to capture and transport HDM-derived antigens (Ags) into the mLN. Thus, we i.n. sensitized adults and infants with Alexa-647-labeled HDM + LPS^{lo} and analyzed Alexa-647$^+$CD11c$^+$ DCs in the mLN (Figures S3H–S3K). Although some CD103$^+$ mDCs were Alexa-647$^+$, the vast majority of HDM-bearing, Alexa-647$^+$CD11c$^+$ DCs were CD11b$^+$ mDCs in both adults and infants (Figures S3H–S3K). Moreover, the frequency (Figure S3H) and number (Figures S3I–S3K) of HDM-bearing, Alexa-647$^+$ CD11b mDCs and CD103$^+$mDCs were identical in infants and adults. Thus, the differences in Th2 cell responses between adults and infants after HDM + LPS^{lo} sensitization were not due to differences in the number, or the capacity, of mDC subsets to capture and transport HDM-derived Ags into the mLN but to their functional capacity to induce T-bet expression in T cells.

T-bet Expression by CD11b$^+$mDCs Is Required for Preventing Th2 Cell Responses to HDMs

Few reports have suggested that intrinsic T-bet expression might fine-tune the capacity of DCs to differentially induce Th1 and Th2 cells (Lipscomb et al., 2009; Lugo-Villarino et al., 2003). Thus, we next used T-bet-ZsGreen reporter mice (Zhu et al., 2012) to investigate whether infant and adult mDCs upregulate T-bet in response to HDM + LPS sensitization (Figures 3A–3D). CD11b$^+$ mDCs from HDM + LPS^{lo}-sensitized adult mice upregulated T-bet expression after 48 hr in comparison with that of HDM-sensitized mice (Figures 3A and 3C). In

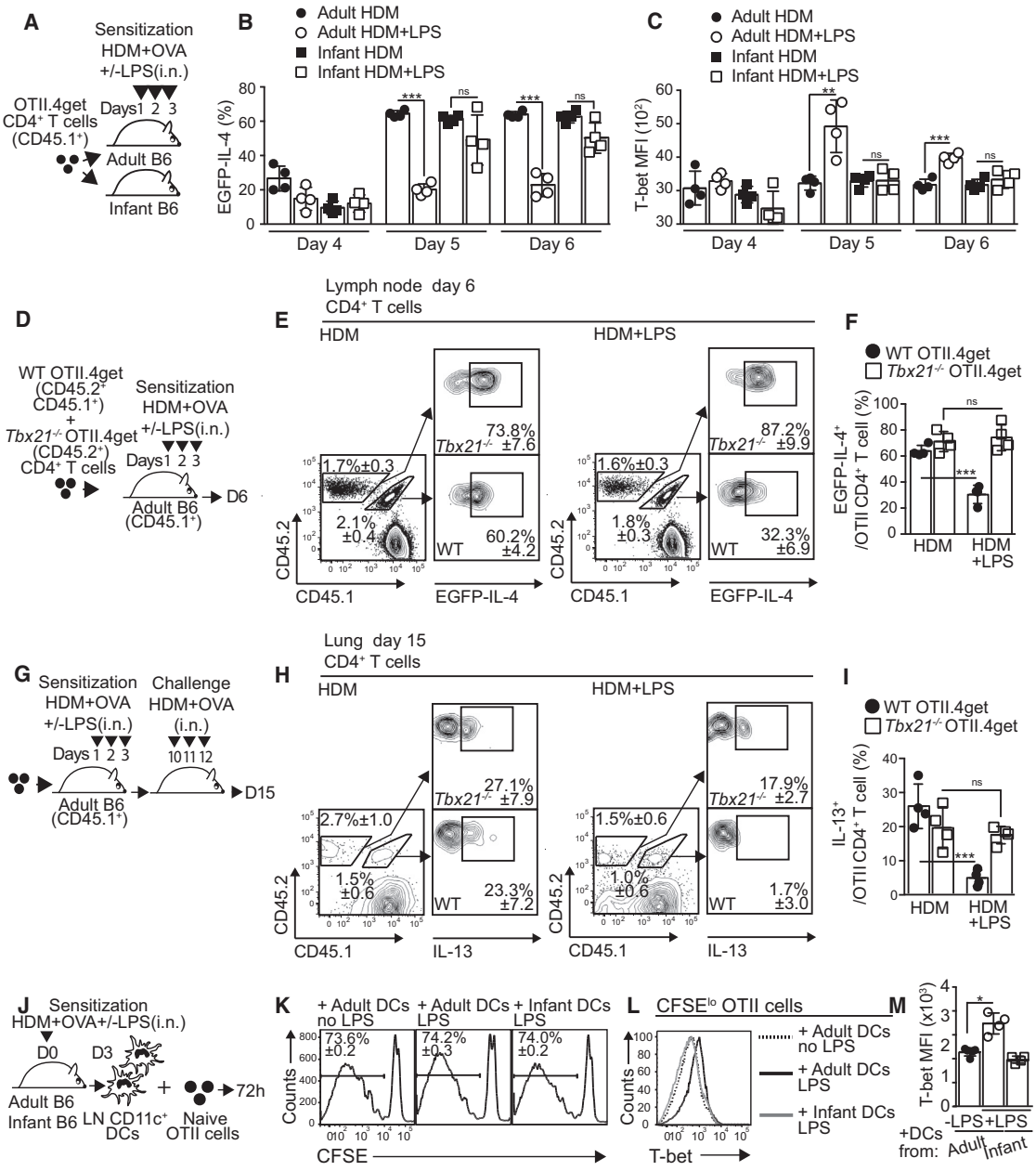


Figure 2. T-bet Induction by Responding T Cells Is Required for Suppressing the Endogenous Th2 Cell Differentiation Program

(A–C) Adult and 18-day-old infant B6 mice were transferred with 25 × 10^3 CD45.1$^+$ OT-II.4get cells at day 0, i.n. treated with 50 μg HDM + 5 μg OVA ± 5 μg LPS for 3 days starting on day 1 (A) and analyzed on days 4, 5, and 6. Shown are frequencies of EGFP$^+$ cells (B) and mean fluorescence intensity (MFI) of T-bet expression (C) in donor mLN OT-II cells.

(D–I) Adult CD45.1$^+$ B6 mice were co-transferred with 15 × 10^3 CD45.1$^+$CD45.2$^+$ OT-II.4get cells + 15 × 10^3 CD45.2$^+$*Tbx21*$^{-/-}$ OT-II.4get cells and treated as described above (D). Shown are frequencies of donor EGFP$^+$ WT and *Tbx21*$^{-/-}$ OT-II cells in the mLN at day 6 (E and F). On day 10, recipients were i.n. challenged with 50 μg HDM + 5 μg OVA for 3 days and analyzed on day 15 (G). Shown are frequencies of donor IL-13$^+$ WT and *Tbx21*$^{-/-}$ OT-II cells in the lungs (H and I).

(J–M) Day-3 CD11c$^+$ mLN DCs from HDM + OVA ± LPS-treated adults and 18-day-old infants were co-cultured for 3 days with CFSE-labeled OT-II cells (J). Shown are frequencies of CFSE^{lo} OT-II cells (K), a representative overlaid histogram of T-bet expression (L), and MFI of T-bet expression (M) in CFSE^{lo} OT-II cells. *p < 0.05, **p < 0.01, ***p < 0.001 (unpaired Student's t test). Data are representative of three independent experiments (mean and SD of four mice per group). Please also see Figure S3.

contrast, CD11b$^+$ mDCs from HDM + LPS^{lo}-sensitized infants only modestly upregulated T-bet expression (Figures 3A and 3C). Notably, CD103$^+$ mDCs (Figures 3B and 3D) failed to express T-bet. Similar results were obtained when T-bet expression was analyzed by intracellular staining (Figures 3E and 3F). Members of the IFN regulatory factor (IRF) family play essential roles in the functional activation of DCs (Gao et al., 2013; Takaoka et al., 2005; Taki et al., 1997; Williams et al., 2013).

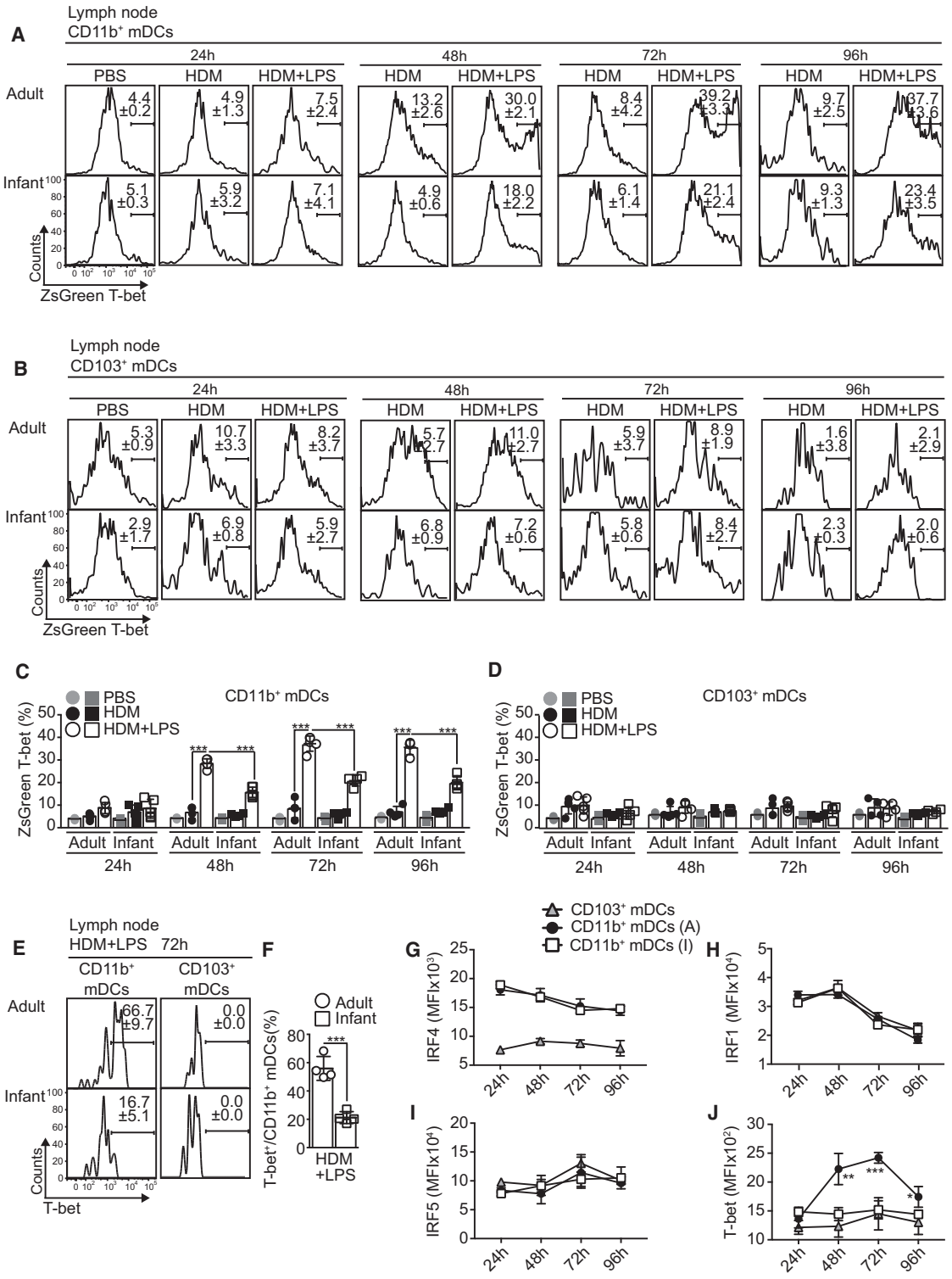


Figure 3. Infant CD11b$^+$ mDCs Fail to Upregulate T-bet Expression in Response to LPS^{lo} Sensitization

(A–D) T-bet-ZsGreen reporter adults and 18-day-old infants were i.n. treated with HDM ± 5 μg LPS. Shown are frequencies of T-bet-expressing (ZsG$^+$) CD11b$^+$ mDCs (A and C) and CD103$^+$ mDCs (B and D).

(E–J) B6 adult and 18-day-old infant mice were i.n. treated with HDM + 5 μg LPS. Shown are frequencies of T-bet expression at 72 hr (E and F) and kinetics of IRF4 (G), IRF1 (H), IFR5 (I), and T-bet (J) expression in mLN CD11b$^+$ and CD103$^+$ mDCs.

***p < 0.001 (unpaired Student's t test). Data are representative of at least two independent experiments (mean and SD of three or four mice per group).

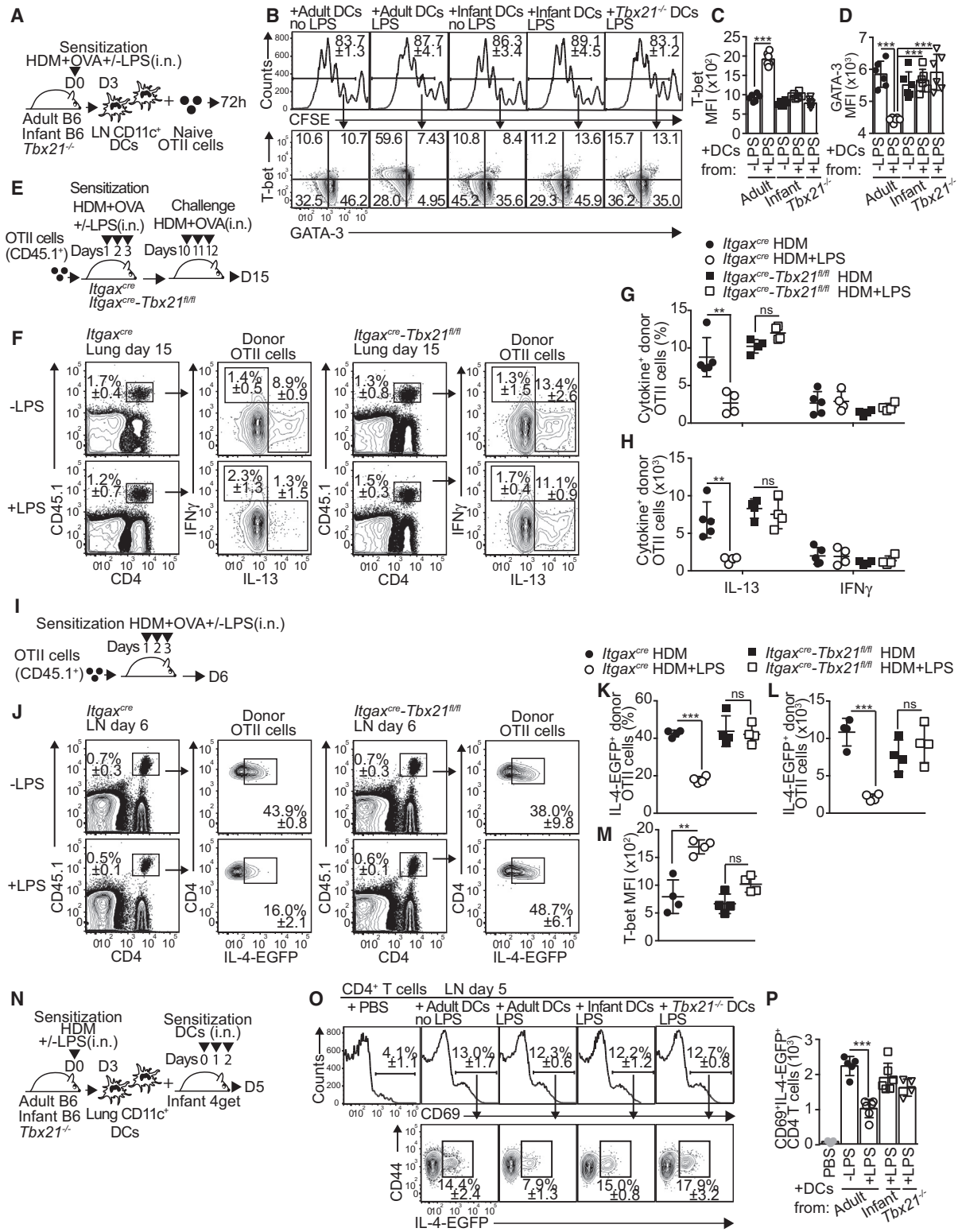


Figure 4. T-bet Expression by DCs Is Required for Preventing Allergen-Specific Th2 Cell Responses upon LPS^{lo} Exposure

(A–D) Day-3 CD11c$^+$ mLN DCs from HDM + OVA ± LPS-treated adult and 18-day-old infant B6 mice or *Tbx21*$^{-/-}$ mice were co-cultured for 3 days with CFSE-labeled OT-II cells (A). Shown are frequencies of CFSE^{lo}OT-II cells (B), frequencies of T-bet and GATA-3 expression (B), and MFI of T-bet (C) and GATA-3 (D) expression in CFSE^{lo}OT-II cells.

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Thus, we also investigated whether HDM + LPS^{lo} sensitization differentially affects the expression of IRF members in infant and adult mDCs. Although CD11b^{+} mDCs from HDM + LPS^{lo}-sensitized adult and infant mice differentially upregulated T-bet expression (Figure 3J), no differences were observed in the expression of IRF4 (Figure 3G), IRF1 (Figure 3H), or IRF5 (Figure 3I). Together, these data indicate that CD11b^{+} mDCs from infant mice fail to upregulate T-bet expression in response to HDM + LPS^{lo} sensitization. To address whether T-bet expression by CD11b^{+} mDCs is required for inducing T-bet expression in T cells, we used CD11c^{+} cells from the mLN of HDM + OVA ± LPS^{lo}-sensitized *Tbx21*$^{-/-}$ mice or adult and infant B6 mice to prime OT-II cells *in vitro* (Figure 4A). OT-II cells proliferated equally well in all conditions (Figure 4B). Compared with non-LPS-sensitized adult-derived DCs, however, only CD11c^{+} cells from the mLN of HDM + LPS^{lo}-sensitized B6 adults, but not B6 infants or *Tbx21*$^{-/-}$ mice, induced significant T-bet expression (Figures 4B and 4C). Correlating with this, OT-II cells activated in the presence of CD11c^{+} cells from HDM + LPS^{lo}-sensitized B6 adults expressed low amounts of GATA-3 (Figures 4B and 4D). These data suggest that T-bet expression by DCs is required for inducing T-bet and preventing GATA-3 expression in responding T cells. To address whether T-bet expression by DCs is required for preventing allergen-specific Th2 cell responses *in vivo*, we crossed *Itgax-cre-EGFP* (Stranges et al., 2007) mice to *Tbx21*$^{fl/fl}$ mice (Intlekofer et al., 2008) to generate mice in which T-bet is conditionally deleted in CD11c^{+} cells (*Itgax*^{cre}-*Tbx21*$^{fl/fl}$). Conditional deletion of T-bet in CD11c^{+} cells did not lead to defects in mDC-subset development (Figures S4A and S4B). Importantly, MHCII^{hi}CD11c^{+} DCs from the mLN of *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ mice, but not CD11c^{-} cells, expressed high amounts of GFP (Figure S4C). All CD11c^{hi} DCs, including CD103^{+} mDCs, CD11b^{+} mDCs, and rDCs, expressed GFP (Figure S4D) and were expected to have equimolar expression with Cre (Stranges et al., 2007). Plasmacytoid DCs expressed lower amounts of GFP than other DC subpopulations (Figure S4D) (Stranges et al., 2007). As predicted, mLN CD11b^{+} mDCs of HDM + LPS^{lo}-sensitized *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ mice failed to express T-bet (Figures S4E and S4F). Thus, we next transferred OT-II cells into either *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ or control mice. Recipients were then sensitized and challenged, and donor OT-II cells were analyzed in the lungs (Figure 4E). As expected, the frequency (Figures 4F and 4G) and number (Figure 4H) of IL-13^{+}OTII cells were reduced in control LPS^{lo}-sensitized mice compared with non-LPS-sensitized mice. Conversely, LPS^{lo} sensitization failed to prevent the accumulation of IL-13^{+} OT-II cells in the lungs of *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ mice (Figures 4F–4H). As a control, LPS^{lo} sensitization did not induce IFN-γ^{+}CD4^{+} T cells (Figures 4F–4H). Likewise, HDM + LPS^{lo} sensitization failed to prevent lung eosinophilia (Figures S4G and S4I) and accumulation of mLN IgE^{+} ASCs (Figures S4H and S4J) in *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ mice. Finally, 4get.OT-II cells were transferred into control or *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ mice, and the frequency of donor GFP^{+}OTII was calculated in the mLN after sensitization (Figure 4I). LPS^{lo} sensitization prevented the accumulation of GFP^{+} OT-II cells in the control recipients, but it failed to do so in *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ mice (Figures 4J–4L). Correlating with these results, LPS^{lo} sensitization induced T-bet upregulation in control, but not *Itgax*^{cre}-*Tbx21*$^{fl/fl}$, mice (Figure 4M). These results indicate that T-bet-expressing DCs are required for the suppression of Th2 cell responses to HDM in the presence of low doses of endotoxin. To further confirm these results, we used CD11c^{+} cells from the lungs of HDM ± LPS^{lo} -sensitized adult B6 and *Tbx21*$^{-/-}$ mice to i.n. sensitize naive infant B6.4get mice (Figure 4N). The frequency (Figure 4O) and number (Figure 4P) of GFP^{+}CD69^{+-} CD44^{hi}CD4^{+} responding T cells were determined in the mLN. As a control, some mice were also sensitized with infant DCs. Purified lung CD11c^{+} cells were enriched over 80% in mDCs (Figure S4L) and were recovered in similar numbers in the mLN of all groups (Figures S4M and S4N). Importantly, from the different donor mDC types, only the donor mDCs from HDM + LPS–-sensitized adult mice were able to prevent the accumulation of activated GFP^{+}CD4^{+} T cells in the infant recipients (Figures 4O and 4P). Collectively, these data suggest that upregulation of T-bet in lung mDCs is required for preventing Th2 cell responses to low-dose endotoxin and HDM sensitization.

CD11b^{+} mDCs from *Tbx21*$^{-/-}$ and Infant Mice Are Defective at Producing IL-12 upon Low-Dose Endotoxin Sensitization

We next made B6:*Tbx21*$^{-/-}$ mixed bone marrow (BM) chimeras to investigate the role of T-bet in DC function. In brief, irradiated B6 (CD45.1^{+}) recipient mice were reconstituted with a 50:50 combination of BM from B6 WT (CD45.1^{+}) and *Tbx21*$^{-/-}$ (CD45.2^{+}) donor mice (Figure 5A). WT:*Tbx21*$^{-/-}$ mice were sensitized with HDM ± LPS^{lo}, and mLN mDCs were analyzed. mDC subsets derived equally from the WT and *Tbx21*$^{-/-}$ progenitors (Figure S5A). Given than IL-12 production is upregulated in DCs after LPS activation (Trinchieri, 2003), we next assessed IL-12 production by mDC subsets. WT CD11b^{+} mDCs largely produced IL-12p70 in day-3 HDM + LPS^{lo}-sensitized mice relative to HDM-sensitized or day-1 HDM + LPS^{lo}-sensitized mice (Figures 5C and S5B). In contrast, sensitization in the presence of LPS^{lo} failed to promote IL-12p70 production by *Tbx21*$^{-/-}$ CD11b^{+} mDCs (Figures 5C and S5B). Conversely, equivalent low numbers of IL-12^{+} WT and *Tbx21*$^{-/-}$ CD103^{+} mDCs were detected in HDM- and HDM + LPS^{lo}-sensitized mice (Figures 5C and S5C). These results indicate that, although

(E–H) *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ and control mice were transferred with CD45.1^{+} OT-II.4get cells and i.n. treated with HDM + OVA ± 5 μg LPS. On day 10, mice were i.n. challenged with HDM + OVA and analyzed on day 15 (E). Shown are frequencies of donor OT-II cells (F) and frequencies (F and G) and numbers (H) of IL-13^{+} and IFN-γ^{+} OTII cells from the lungs.

(I–M) Mice sensitized as described above were analyzed at day 6 (I). Shown are frequencies of donor OT-II cells (J), frequencies (J and K) and numbers (L) of EGFP^{+} cells, and MFI of T-bet expression (M) in donor OT-II cells from the mLN.

(N–P) Day-3 CD11c^{+} lung mDCs from HDM + OVA ± LPS-treated adult and 18-day-old infant B6 mice or *Tbx21*$^{-/-}$ mice were i.n. transferred into B6.4get infant mice on days 0, 1, and 2. Recipient mice were analyzed on day 5 (N). Shown are frequencies (O) and numbers (P) of EGFP^{+} cells in CD69^{+}CD4^{+} T cells from the mLN.

$^{**}p < 0.01$, $^{***}p < 0.001$ (unpaired Student's t test). Data are representative of three independent experiments (mean and SD of four to six mice per group). Please also see Figure S4.

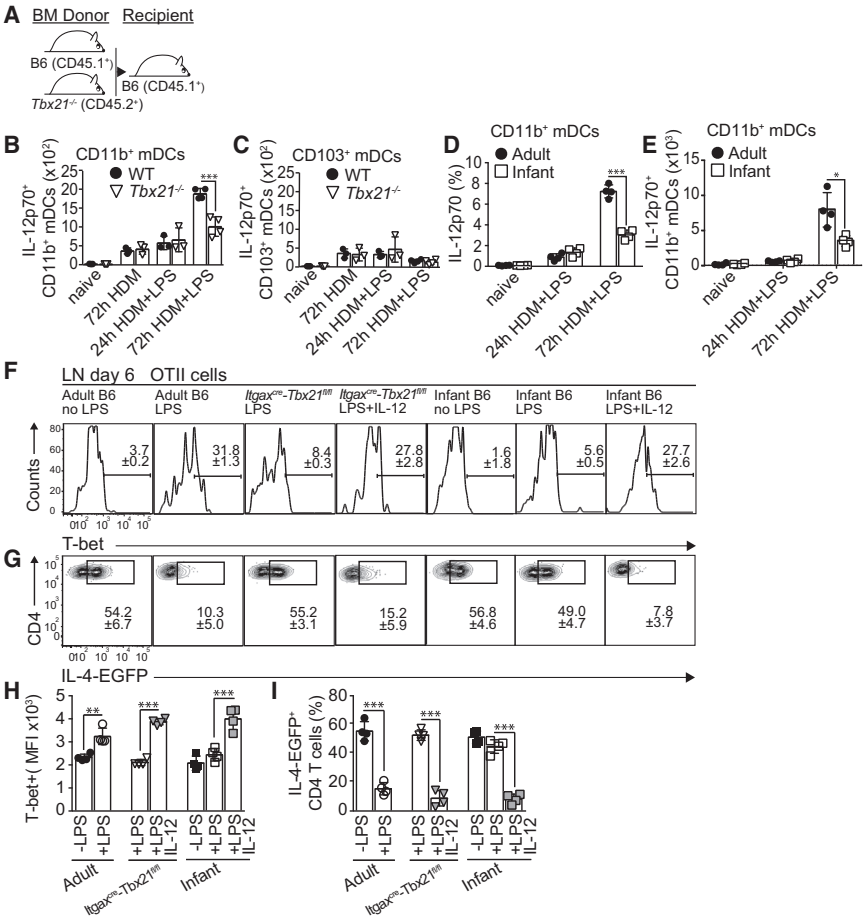


Figure 5. Infant and *Tbx21*$^{-/-}$ CD11b^{+} mDCs Are Intrinsically Impaired in Their Ability to Produce IL-12 and to Prevent Allergen-Specific Th2 Cell Responses upon LPS^{lo} Exposure

(A–C) B6 (CD45.1^{+}) mice were irradiated and reconstituted with a 1:1 mixture of BM from B6 (CD45.1^{+}) and *Tbx21*$^{-/-}$ (CD45.2^{+}) donors (A). Shown are numbers of IL-12p70^{+} WT and *Tbx21*$^{-/-}$ CD11b^{+} mDCs (B) and CD103^{+} mDCs (C) in the mLN of HDM ± LPS-treated reconstituted chimeric mice.

(D and E) Frequencies (D) and numbers (E) of IL-12p70^{+} CD11b^{+} mDCs in the mLN of HDM ± LPS-treated adult and 18-day-old infant B6 mice.

(F–I) Adult and 18-day-old infant B6 mice or *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ mice were transferred with CD45.1^{+} OT-II.4get cells and i.n. treated with HDM + OVA ± LPS ± 150 ng rIL-12p70 for 3 days. Shown are frequencies of T-bet expression (F and H) and EGFP^{+} cells (G and I) in donor OT-II cells from mLN on day 6.

*p < 0.05, ***p < 0.001 (unpaired Student's t test). Data are representative of two independent experiments (mean and SD of three to six mice per group). Please also see Figure S5.

T-bet is dispensable for the development of CD11b^{+} mDCs, it is intrinsically required for the capacity of these cells to produce sustained IL-12 in response to LPS. Corresponding with this, compared with those from adult mice, CD11b^{+} mDCs from HDM-sensitized infant mice failed to upregulate IL-12p70 production 3 days after LPS^{lo} administration (Figures 5D, 5E, and S5D). Although our data suggest that CD11b^{+} mDCs upregulate T-bet-dependent IL-12 production and suppress Th2 cell priming after HDM + LPS sensitization, a published report has suggested that CD103^{+} mDCs have the ability to restrain Th2 cell responses to HDMs through the production of IL-12 (Conejero et al., 2017). Thus, to test the role of CD103^{+} mDCs during LPS^{lo} sensitization, we analyzed *Batf3*$^{-/-}$ mice that selectively lack the CD103^{+}CD11b^{-} DC subset (Edelson et al., 2010). We transferred 4get.OT-II CD4^{+} T cells into either WT or *Batf3*$^{-/-}$ recipients, sensitized them, and analyzed them on day 6 (Figure S5E). LPS^{lo} sensitization abrogated GFP^{+} by donor OTII cells in both WT and *Batf3*$^{-/-}$ recipients (Figures S5F and S5G), suggesting that CD103^{+} mDCs are not necessary for suppressing Th2 cell priming in response to low-dose endotoxin sensitization.

Given that IL-12 induces T-bet expression in T cells (Zhu et al., 2012), we next tested whether recombinant IL-12 (rIL-12) administration during HDM + LPS^{lo} sensitization restores the capacity of low-LPS treatment to abrogate Th2 cell development in infant and *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ mice. Thus, we transferred 4get.OT-II CD4^{+} T cells into B6 adult, B6 infant, and *Itgax*^{cre}-*Tbx21t*$^{fl/fl}$ mice. Recipients were then sensitized with HDM + OVA ± LPS^{lo}, and PBS or rIL-12 was i.n. co-administered (Figure S5H). Expression of T-bet (Figures 5F and 5H) and GFP (Figures 5G and 5I) by donor 4get.OT-II cells was assessed in the mLN. As expected, sensitization in the presence of LPS^{lo} promoted T-bet upregulation (Figures 5F and 5H), thereby preventing GFP expression (Figures 5G and 5I) in B6 adults, but not in infant or *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ mice. Importantly, however, rIL-12 administration restored T-bet expression in infant and *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ mice sensitized in the presence of LPS^{lo} (Figures 5F and 5H). As a consequence, the frequency of donor GFP^{+} OTII cells was similar in HDM + LPS^{lo}-sensitized B6 adult and rIL-12-treated infant and *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ mice (Figures 5G and 5I). Administration of rIL-12, however, did not appear to affect T-bet or IL-12p70 expression by CD11b^{+} mDCs (Figures S5I–S5L). Collectively, our data indicate that adult CD11b^{+} mDCs upregulate T-bet after HDM + LPS^{lo} sensitization, which promotes IL-12 production, thus preventing Th2 cell differentiation. In contrast, infant CD11b^{+} mDCs fail to upregulate T-bet, which prevents optimal IL-12 production and subsequent Th2 cell inhibition.

TNF-α-Driven Activation of CD11b^{+} mDCs Is Required for Preventing Th2 Cell Responses to HDMs

We next tested whether direct activation of CD11b^{+} mDCs by LPS is required for inducing T-bet expression after HDM + LPS^{lo} sensitization. Thus, we made B6(CD45.1^{+}):*Tlr4*$^{-/-}$(CD45.2^{+}) mixed BM chimeras. Chimeras were sensitized, and the expression of T-bet in mLN CD11b^{+} mDCs was determined. As

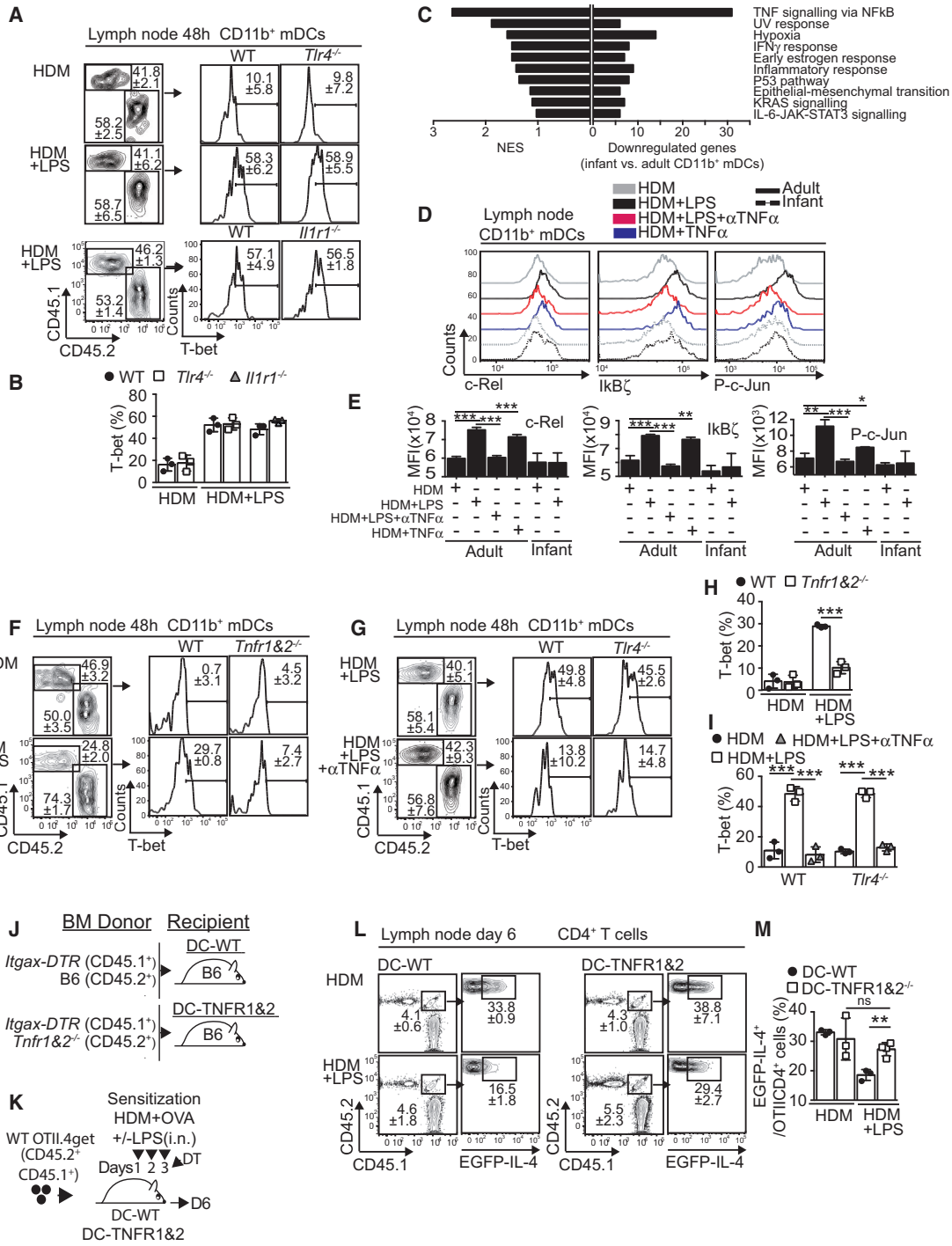


Figure 6. TNF-α Signaling Is Required for Upregulating T-bet in mCD11b⁺ DCs and Preventing Allergen-Specific Th2 Cell Responses

(A and B) B6 (CD45.1⁺) mice were irradiated and reconstituted with a 1:1 mixture of BM from B6 (CD45.1⁺) and *Tlr4*$^{-/-}$ (CD45.2⁺) donors or from B6 (CD45.1⁺) and *Il1r1*$^{-/-}$ (CD45.2⁺) donors. Shown are (A) fluorescence-activated cell-sorting (FACS) plots and (B) a graph of the frequencies of T-bet⁺ WT, *Tlr4*$^{-/-}$, and *Il1r1*$^{-/-}$ CD11b⁺ mDCs in the mLN of HDM ± LPS-treated reconstituted chimeric mice on day 2.

(C) mLN CD11b⁺ mDCs from B6 adults and 18-day-old infants were sorted 24 hr after being i.n. treated with HDM + 5 μg LPS, and RNA-seq was performed. Shown are the NES and number of downregulated genes in infant CD11b⁺ mDCs for each of the top ten hallmark signaling pathways resulting from GSEA (Broad institute; adjusted p value < 0.05, $\log_2$ fold change > 0.58).

(D and E) B6 adults and 18-day-old infants were i.n. treated with HDM ± 5 μg LPS or HDM ± 30 μg TNF. Some mice also received 250 μg anti-TNF-α intraperitoneally (i.p.). Shown are (D) FACS plots and (E) graphs of the expression of c-Rel, IKBz, and P-c-Jun in mLN CD11b⁺ mDCs on day 1.

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expected, CD11b$^+$ mDCs from the WT compartment upregulated T-bet expression after HDM + LPS^{lo} sensitization (Figures 6A and 6B). Likewise, CD11b$^+$ mDCs from the *Tlr4*$^{-/-}$ compartment also upregulated T-bet expression (Figures 6A and 6B), suggesting that LPS did not directly activate CD11b$^+$ mDCs to promote T-bet expression. LPS induces IL-1, which in turn activates IL-1R1−TLR4 signaling (Verstrepen et al., 2008); therefore, we next tested whether indirect activation of CD11b$^+$ mDCs through the IL-1R1 is required for inducing T-bet expression after HDM + LPS^{lo} sensitization. We analyzed B6(CD45.1$^+$):*Il1r1*$^{-/-}$(CD45.2$^+$) mixed BM chimeras and found that IL-1R1 signaling did not mediate T-bet upregulation in CD11b$^+$ mDCs (Figures 6A and 6B). To gain insight into the mechanisms underlying T-bet regulation in adult and infant CD11b$^+$ mDCs, we sorted CD11b$^+$ mDCs from the mLN of adult and infant mice 24 hr after HDM + LPS^{lo} sensitization and performed RNA sequencing (RNA-seq) and gene-set enrichment analysis (GSEA) to identify hallmark signaling pathways that were differentially regulated between adult and infant CD11b$^+$ mDCs. As a result, we identified 173 differentially expressed genes, 110 of which were downregulated and 63 of which were upregulated in infant CD11b$^+$ mDCs (Table S1). Notably, the pathway regulated by nuclear factor kappa B (NF-κB) in response to TNF-α had the highest normalized enrichment score (NES = 2.64, FDR q-value = 1.28e^{-42}) and contained the largest number of genes downregulated in infant CD11b$^+$ mDCs (Figures 6C, S6A, and S6B; Tables S2 and S3). TNF-α activates the NF-κB and activator protein-1 (AP-1) transcription factors (Verstrepen et al., 2008). Expression of genes encoding NF-κB-c-rel and the AP-1 family members c-Jun, JunB, c-Fos, and FosB was decreased in infant CD11b$^+$ mDCs after HDM + LPS^{lo} sensitization (Table S1). We next evaluated c-Rel and P-c-Jun expression in adult and infant mLN CD11b$^+$ mDCs. Adult CD11b$^+$ mDCs, but not infant CD11b$^+$ mDCs, induced c-Rel and P-c-Jun in response to HDM + LPS^{lo} sensitization (Figures S6C–S6E). We next tested whether LPS-induced TNF-α plays a role in the activation of NF-κB and AP-1 in CD11b$^+$ mDCs. Therefore, we evaluated the expression of NF-κB-c-Rel, the NF-κB early response protein IkBζ, and P-c-Jun-AP-1 in adult mLN CD11b$^+$ mDCs after sensitization in the presence or absence of TNF-α-neutralizing monoclonal antibody (mAb) or TNF-α cytokine. As a control, we also included the analysis of 18-day-old infant CD11b$^+$ mDCs. As expected, adult CD11b$^+$ mDCs, but not infant CD11b$^+$ mDCs, induced c-Rel, IkBζ, and P-c-Jun in response to HDM + LPS^{lo} sensitization (Figures 6D and 6E). However, anti-TNF-α treatment prevented these effects (Figures 6D and 6E). Additionally, compared with HDM sensitization alone, HDM sensitization in the presence of TNF-α cytokine was able to induce c-Rel, IkBζ, and P-c-Jun expression in CD11b$^+$ mDCs (Figures 6D and 6E). These data suggest that the activation of NF-κB and AP-1 in CD11b$^+$ mDCs after HDM + LPS^{lo} sensitization is mediated by TNF-α signaling.

We next examined whether the activation of CD11b$^+$ mDCs by TNF-α signaling through TNF receptors (TNFR1 and TNFR2) is required for inducing T-bet expression after HDM + LPS^{lo} sensitization. As above, 50% CD45.1$^+$WT and 50% CD45.2$^+$ *Tnfr1*$^{-/-}$*Tnfr2*$^{-/-}$ mixed BM chimeras were generated and sensitized, and the expression of T-bet in mLN CD11b$^+$ mDCs was determined. Whereas CD11b$^+$ mDCs from the WT compartment upregulated T-bet expression in HDM + LPS^{lo}-sensitized chimeras, CD11b$^+$ mDCs from the *Tnfr1*$^{-/-}$*Tnfr2*$^{-/-}$ compartment failed to upregulate T-bet (Figures 6F and 6H), suggesting that TNFR signaling in CD11b$^+$ mDCs is required for the upregulation of T-bet after HDM + LPS^{lo} sensitization. To confirm these data, we further determined the effect of TNF-α neutralization after sensitization. Thus, we administered anti-TNF-α mAb to 50% CD45.1$^+$WT and 50% CD45.2$^+$*Tlr4*$^{-/-}$ mixed BM chimeras at the time of HDM + LPS^{lo} sensitization. Anti-TNF-α treatment prevented T-bet upregulation in CD11b$^+$ mDCs from both WT and *Tlr4*$^{-/-}$ compartments (Figures 6G and 6I). Together, these data suggest that in adults, HDM + LPS^{lo} sensitization induces activation of NF-κB and AP-1 and T-bet upregulation in CD11b$^+$ mDCs by LPS-induced TNF-α production and TNF-α-mediated signaling.

To test whether TNF-α-mediated signaling in DCs is required for suppressing Th2 cell priming in response to low-dose endotoxin sensitization, we generated BM chimeras that expressed TNFR normally (DC-WT) or that were selectively TNFR deficient in CD11c$^+$ DCs after diphtheria toxin (DT) administration (DC-TNFR1&2) (Figure 6J) (León et al., 2012). After reconstitution, we transferred 4get.OT-II CD4$^+$ T cells into the chimeras, sensitized and treated them with DT to ablate CD11c$^+$ cells derived from the *Itgax*-DTR BM, and analyzed GFP expression by donor 4get.OT-II cells in the mLN (Figure 6K). Sensitization in the presence of LPS^{lo} prevented GFP in DC-WT chimeras, but not in DC-TNFR1&2 chimeras (Figures 6L and 6M). Thus, TNFR is required on CD11c$^+$ DCs for suppressing Th2 cell priming after HDM + LPS^{lo} sensitization.

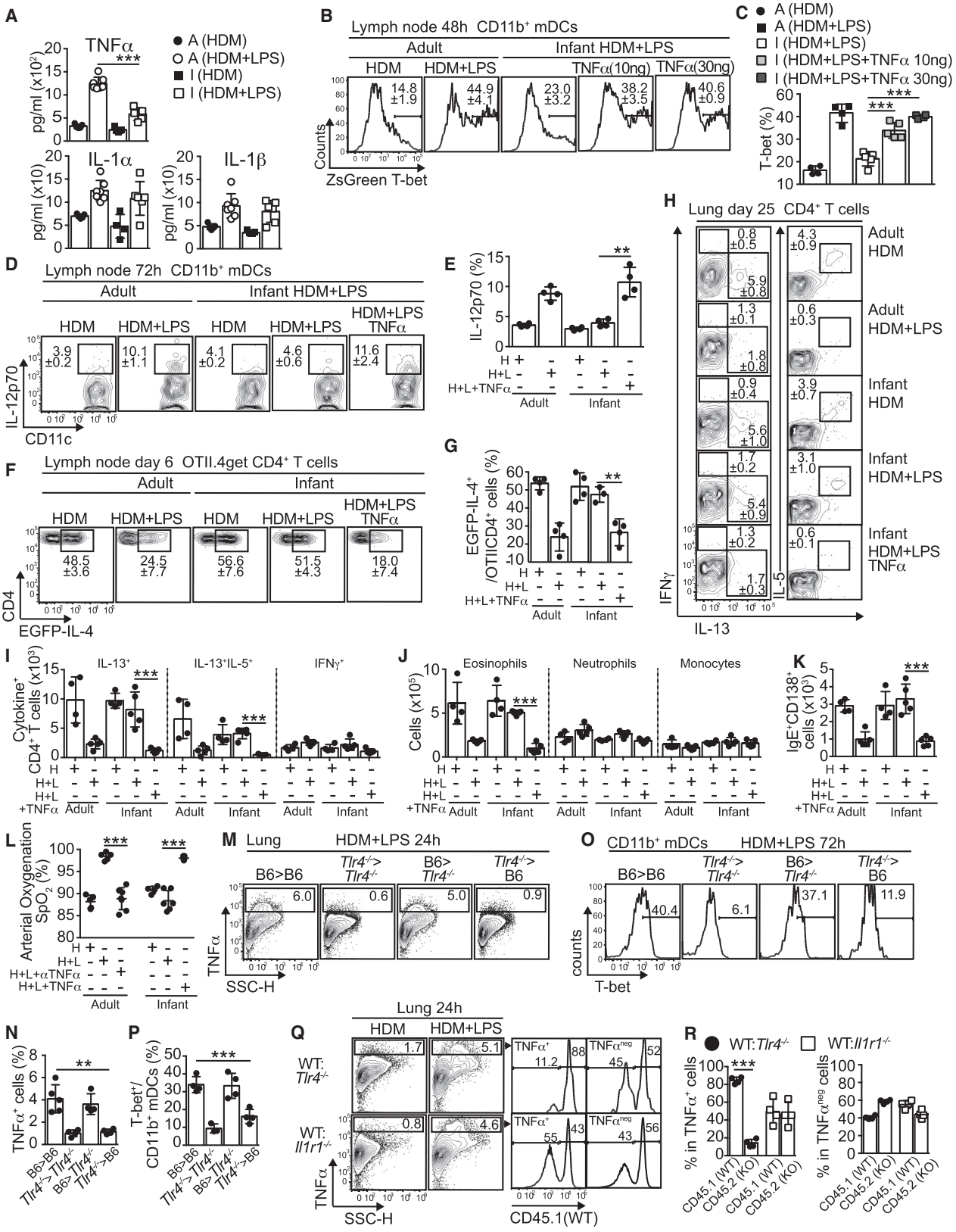
Impaired TNF-α-Driven Activation Precludes T-bet Expression in Infant CD11b$^+$ mDCs and Their Capability to Suppress Th2 Cell Priming

Our previous data show that in infants, HDM + LPS^{lo} sensitization induces poor NF-κB and AP-1 activation and T-bet upregulation in CD11b$^+$ mDCs, suggesting defective TNF-α-TNFR-mediated signaling. Thus, we next determined cytokine responses in bronchoalveolar lavage (BAL) fluid from adults and 18-day-old infants. HDM + LPS^{lo} sensitization caused a great increase in concentrations of TNF-α in the BAL fluid of adult mice

(F–I) CD45.1$^+$ mice were irradiated and reconstituted with 1:1 BM from B6 (CD45.1$^+$) and *Tnfr1&2*$^{-/-}$ (CD45.2$^+$) donors (F and H) or from B6 (CD45.1$^+$) and *Tlr4*$^{-/-}$ (CD45.2$^+$) donors (G and I). Shown are frequencies of T-bet$^+$ WT, *Tnfr1&2*$^{-/-}$ (F and H), and *Tlr4*$^{-/-}$ (G and I) CD11b$^+$ mDCs in the mLN of HDM ± LPS ± anti-TNF-α-treated reconstituted mice on day 2.

(J–M) Irradiated B6 mice were reconstituted with an 80:20 BM mix of *Itgax*-DTR and B6 donors (DC-WT chimeras) or *Itgax*-DTR and *Tnfr1&2*$^{-/-}$ donors (DC-TNFR1&2 chimeras) (J). Chimeras were transferred with CD45.1$^+$CD45.2$^+$ OT-II.4get cells, i.n. treated with HDM + OVA ± 5 μg LPS, and i.p. treated with 60 ng DT (K). Shown are frequencies of mLN donor EGFP$^+$ OT-II cells on day 6 (L and M).

*p < 0.05, **p < 0.01, ***p < 0.001 (unpaired Student's t test). Data are representative of two independent experiments (mean and SD of three to seven mice per group). Please also see Figure S6 and Tables S1–S3.



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but only a moderate increase in infants (Figure 7A). These differences were also observed between adults and 7- to 14-day-old mice (data not shown). No differences between adults and infants were found in the expression of other cytokines such as IL-1α, IL-1β (Figure 7A), and IL-6 (data not shown). These data indicate that infants have defective production of TNF-α after HDM + LPS^{lo} sensitization. To test whether defective T-bet upregulation in infant CD11b$^+$ mDCs after HDM + LPS^{lo} sensitization is due to defective TNF-α production, we sensitized 18-day-old T-bet-ZsGreen mice with HDM + LPS^{lo} in the presence of different concentrations of TNF-α and determined the expression of T-bet in mLN CD11b$^+$ mDCs. As expected, HDM + LPS^{lo} sensitization did not induce significant upregulation of T-bet in CD11b$^+$ mDCs from infant mice (Figures 7B and 7C). However, co-administration of TNF-α induced T-bet upregulation in infant CD11b$^+$ mDCs to amounts similar to those found in CD11b$^+$ mDCs from HDM + LPS^{lo}-sensitized adults (Figures 7B and 7C). Similar upregulation of T-bet in CD11b$^+$ mDCs was seen from 7-, 14-, and 18-day-old mice after exogenous administration of TNF-α (Figures S7A and S7B). Thus, our data indicate that defective TNF-α production in infants prevents T-bet upregulation in CD11b$^+$ mDCs after HDM + LPS^{lo} sensitization. In addition, administration of TNF-α during HDM + LPS^{lo} sensitization recovers the capacity of infant mLN CD11b$^+$ mDCs to produce IL-12 in amounts similar to those of adult CD11b$^+$ mDCs (Figures 7D and 7E).

Finally, we tested whether administration of TNF-α during HDM + LPS^{lo} sensitization restores the capacity of low-LPS treatment to abrogate Th2 cell development in infant mice. We transferred 4get.OT-II CD4$^+$ T cells into B6 adults and 18-day-old infants, sensitized them in the presence of TNF-α, and assessed expression of GFP by donor 4get.OT-II cells (Figures 7F and 7G). TNF-α administration restored the capacity of LPS^{lo} to prevent GFP expression in infant mice (Figures 7F and 7G). We next sensitized adult and 18-day-old infant B6 mice as above, challenged them with HDMs, and quantified IL-13$^+$, IL-5$^+$, and IFN-γ$^+$ CD4$^+$ T cells (Figures 7H and 7I). As expected, HDM + LPS^{lo} sensitization prevented the accumulation of IL-13$^+$ and IL-13$^+$IL-5$^+$ CD4$^+$ T cells in the lungs of adults, but not in infants (Figures 7H and 7I). Importantly, sensitization in the presence of TNF-α restored the capacity of LPS^{lo} to prevent the accumulation of IL-13$^+$ and IL-13$^+$IL-5$^+$ CD4$^+$ T cells in infants (Figures 7H and 7I). Likewise, HDM + LPS^{lo} sensitization in the presence of TNF-α also prevented the accumulation of lung eosinophils (Figures 7J, S7C, and S7D) and mLN IgE$^+$ ASCs (Figures 7K and S7E), prevented the induction of goblet cell hyperplasia (Figure S7F), and improved SpO2 (Figure 7L) in infant mice. The same results were seen when 10-day-old mice were analyzed (data not shown). In contrast, TNF-α neutralization after HDM + LPS^{lo} sensitization in adult mice abolished the suppressive effect of LPS on asthma, including the accumulation of IL-13$^+$ CD4$^+$ T cells (Figures S7I and S7J) and eosinophils (Figures S7G and S7H) in the lungs, the goblet cell hyperplasia (Figure S7F), and the beneficial effect on SpO2 (Figure 7L). Thus, our data indicate that defective TNF-α production prevents the suppression of Th2 cell responses to HDMs in the presence of low doses of endotoxin.

To assess the cellular source of TNF-α after HDM + LPS^{lo} sensitization, we first evaluated whether LPS-driven TNF-α production is mediated by radioresistant (including lung epithelium) or hematopoietic cells in the lungs. Therefore, B6 (WT) or *Tlr4*$^{-/-}$ recipients were irradiated and reconstituted with BM from WT or *Tlr4*$^{-/-}$ mice. After reconstitution, TNF-α production in the lungs was determined after HDM + LPS^{lo} sensitization. Importantly, chimeric *Tlr4*$^{-/-}$ mice transplanted with WT BM, but not chimeric mice transplanted with *Tlr4*$^{-/-}$ BM, were able to produce WT amounts of TNF-α$^+$ cells (Figures 7M and 7N), suggesting that radiosensitive (hematopoietic) cells contain the principal source of TNF$^+$ cells. Likewise, only mLN CD11b$^+$ mDCs of HDM + LPS^{lo}-sensitized chimeric mice transplanted with WT BM, but not with *Tlr4*$^{-/-}$ BM, were able to upregulate T-bet (Figures 7O and 7P). Next, we evaluated whether hematopoietic cells directly respond to LPS to produce TNF-α. Thus, WT:*Tlr4*$^{-/-}$ BM chimeras were sensitized, and TNF-α production in the lungs was determined. As expected, HDM + LPS^{lo} sensitization induced TNF-α production (Figure 7Q), but importantly, TNF-α$^+$ cells were principally restricted to the WT phenotype (Figures 7Q and 7R), suggesting that TNF-α was produced by lung hematopoietic cells after direct recognition of LPS by TLR4. Conversely, the analysis of WT:*Il1r1*$^{-/-}$ BM chimeras showed that lung cells did not require expression of IL-1R to produce TNF-α in response to LPS (Figures 7Q and 7R). These data suggest that

Figure 7. Defective TNF-α Signaling Prevents T-bet Upregulation in mCD11b$^+$ DCs from Infants and Contributes toward the Development of Allergen-Specific Th2 Cell Responses

(A) Cytokines in BAL of day-1 HDM ± LPS-treated B6 adults and 18-day-old infants.

(B and C) T-bet-ZsGreen reporter adults and 18-day-old infants were i.n. treated with HDM ± 5 μg LPS ± 10 or 30 ng TNF-α. Shown are (B) FACS plots and (C) graphs of the frequencies of ZsG$^+$ mLN CD11b$^+$ mDCs on day 2.

(D and E) B6 adults and 18-day-old infants were i.n. treated with HDM ± 5 μg LPS ± 30 ng TNF-α. Shown are (D) FACS plots and (E) graphs of the frequencies of IL-12p70$^+$ mLN CD11b$^+$ mDCs on day 3.

(F and G) B6 adults and 18-day-old infants were transferred with CD45.1$^+$ OT-II.4get cells and i.n. treated with HDM + OVA ± 5 μg LPS. PBS or 30 ng TNF-α was co-administered on day 1. Shown are (F) FACS plots and (G) graphs of the frequencies of mLN EGFP$^+$ donor OT-II cells on day 6.

(H–L) B6 adults and 18-day-old infants were i.n. treated with HDM ± 5 μg LPS ± 30 μg TNF-α. Some adults also received 250 μg anti-TNF-α i.p. On day 20, mice were i.n. challenged with HDMs and analyzed on day 25. Shown are frequencies (H) and numbers (I) of IL-13$^+$, IL-13-IL-5$^+$, and IFN-γ$^+$ CD4$^+$ T cells in the lungs. Numbers of lung eosinophils, neutrophils, and monocytes are shown in (J). Numbers of mLN IgE$^+$ ASCs are shown in (K). SpO2 was determined by pulse oximetry (L).

(M–P) B6 (WT) and *Tlr4*$^{-/-}$ recipient mice were lethally irradiated and reconstituted with WT and *Tlr4*$^{-/-}$ BM. Chimeras were i.n. treated with HDM ± 5 μg LPS. Shown are frequencies of lung TNF-α$^+$ cells at 24 hr (M and N) and T-bet expression in mLN CD11b$^+$ mDCs at 72 hr (O and P).

(Q and R) 1:1 B6 (CD45.1$^+$):*Tlr4*$^{-/-}$ (CD45.2$^+$) and 1:1 B6 (CD45.1$^+$):*Il1r1*$^{-/-}$ (CD45.2$^+$) mixed BM chimeras were i.n. treated with HDM ± 5 μg LPS. Also shown is TNF-α expression 24 hr later in lung suspensions (Q) and frequencies of WT (CD45.1$^+$) and knockout (CD45.2$^+$) cells within the TNF-α$^+$ and TNF-α$^-$ gates (R). $^{**}p < 0.01$, $^{***}p < 0.001$ (unpaired Student's t test). Data are representative of two independent experiments (mean and SD of three to five mice per group). Please also see Figure S7.

TNF-α-dependent upregulation of T-bet in CD11b$^+$ mDCs is driven by hematopoietic cells that produce TNF-α in response to LPS, but not by epithelial cells.

Collectively, our data indicate that adult CD11b$^+$ mDCs upregulate T-bet in response to TNF-α produced after HDM + LPS^{lo} sensitization, which in turn promotes IL-12 production, thereby preventing Th2 cell differentiation by inducing T-bet expression in responding CD4$^+$ T cells. In contrast, in early life and during a time window from day 7 to day 20 after birth, the CD11b$^+$ mDCs fail to upregulate T-bet in response to low doses of LPS as a result of impaired production of TNF-α, which prevents optimal IL-12 production and subsequent Th2 cell inhibition despite the presence of LPS.

DISCUSSION

Previous studies in mice have shown that during a time window from day 7 to day 20 after birth, the immune system is prone to allergic sensitization and Th2-driven allergic responses (de Kleer et al., 2016; Gollwitzer et al., 2014; Steer et al., 2017), suggesting that a "window of opportunity" in the postnatal period influences the immune responses to allergens. Comparative immunological evaluations have demonstrated that the immunity in mice at 7 days is comparable to that of term human neonates, whereas mice < 20 days old resemble infants (Siegrist, 2001). Thus, 1- to 3-week-old mice have yielded observations very similar to those of human neonates (0–2 months old) and infants (under 1–2 years old) (Siegrist, 2001). In correlation with animal studies, primary allergen sensitizations to environmental Ags gradually develop and peak in children 2–3 years of age (Holt, 1996) and are associated with later development of asthma and allergic disease (Lodge et al., 2011). Therefore, both in humans and in mice, a susceptibility window for inhalant-allergen sensitization covers the neonatal and infancy periods. Several studies in mice have evaluated this propensity to develop allergic sensitization during infancy (de Kleer et al., 2016; Steer et al., 2017). However, the understanding of the allergic sensitization process in the context of exposure to environmental endotoxins during infancy is still not clear. Previous studies have shown that LPS exposure prevents the development of allergic Th2 cell responses (Daan de Boer et al., 2013; Gereda et al., 2000; Schuijs et al., 2015; Stein et al., 2016; Zhu et al., 2010). Our data demonstrate, however, that adults and infants respond to LPS with different thresholds. As a result, whereas relatively low-dose LPS exposure normally prevents adults from developing allergic Th2 cell responses, infant mice require a relatively high-LPS environment to achieve the same prophylactic effect. The inability of low-LPS exposure to prevent Th2 cell differentiation in infants is attributed to the inability of responding infant CD4$^+$ T cells to upregulate T-bet, which we found was required for preventing Th2 cell development in response to HDMs. Impaired T-bet expression, however, was not due to an intrinsic defect in the infant CD4$^+$ T cells. Instead, we found that CD11b$^+$ mDCs from HDM + LPS^{lo}-sensitized infants poorly produced IL-12 and thereby prevented IL-12-dependent T-bet upregulation in responding CD4$^+$ T cells. Mechanistically, we demonstrated that T-bet upregulation in CD11b$^+$ mDCs is required for normal IL-12 production. However, whereas adult CD11b$^+$ mDCs normally upregulated T-bet after HDM + LPS^{lo} sensitization, infant CD11b$^+$ mDCs failed to express T-bet, which prevented CD11b$^+$ mDCs from secreting IL-12. Thus, the differential capacity to upregulate T-bet in infant and adult CD11b$^+$ mDCs provides the underlying mechanism for how LPS prevents allergic Th2 cell responses in adults and infants with different thresholds. Our data indicate that in addition to controlling important fate decisions in T cells (Szabo et al., 2000; Zhu et al., 2012), T-bet also critically regulates DC activity.

Importantly, our data show that T-bet upregulation in CD11b$^+$ mDCs does not require direct recognition of LPS, but instead it requires LPS-induced TNF-α production and TNF-α-TNFR-triggered signaling in CD11b$^+$ mDCs. Our data further show the requirement for crosstalk between TLR4$^+$ accessory hematopoietic cells producing TNF-α and TNFR$^+$CD11b$^+$ mDCs. Whereas adult hematopoietic cells produce large amounts of TNF-α in response to HDM + LPS sensitization, infants have impaired TNF-α production, which prevents TNF-α-TNFR signaling and T-bet upregulation in CD11b$^+$ mDCs. Therefore, the capacity of LPS to prevent allergic Th2 cell responses relies on the capacity to induce increased amounts of TNF-α. Our data reveal a largely unexplored role of TNF-α in mediating *in vivo* DC activation, in addition to its well-defined role in apoptosis and inflammation. In this way, TNF-α acts as a rheostat that can tune the activation of CD11b$^+$ mDCs and the subsequent balance of the T cell responses to inhaled Ags. Importantly, the regulatory activity of this rheostat is modulated by age.

Previous studies have suggested that CD4$^+$ T cells primed by CD103$^+$ mDCs preferentially differentiate into Th1 cells, whereas CD11b$^+$ mDCs promote Th2 cell differentiation (Conejero et al., 2017; Gao et al., 2013; Kumamoto et al., 2013; León, 2017; Plantinga et al., 2013). As such, CD103$^+$ mDCs produce IL-12p40 after HDM sensitization, thereby preventing Th2 cell differentiation (Conejero et al., 2017). Here, we found that LPS prevented Th2 cell differentiation in HDM-sensitized mice by an IL-12-dependent mechanism. We found, however, that CD103$^+$ mDCs were quickly depleted from the lung after HDM + LPS sensitization, possibly as a result of increased LPS-driven epithelial damage (Sung et al., 2006). In contrast, the number of CD11b$^+$ mDCs and their capacity to secrete IL-12 dramatically increased after HDM + LPS sensitization, allowing them to become the dominant IL-12$^+$ DC subset in the mLN. In addition, CD11b$^+$ mDCs are the principal DC subset that captures and transports HDM-derived Ags from the lung into the mLN. Thus, given that the numbers of IL-12$^+$CD11b$^+$ mDCs largely increased in HDM + LPS-sensitized adult mice and that IL-12 prevented Th2 cell differentiation, we conclude that IL-12$^+$CD11b$^+$ mDCs and not CD103$^+$ mDCs are the main drivers preventing allergic Th2 cell responses in adult HDM + LPS-sensitized mice. Supporting this conclusion, LPS normally prevented Th2 cell differentiation in *Batf3*$^{-/-}$ mice despite the absence of CD103$^+$ mDCs. In contrast, LPS failed to suppress allergic Th2 cell responses when CD11b$^+$ mDCs were unable to normally produce IL-12, such as in *Itgax*^{Cre}-*Tbx21*$^{fl/fl}$ or infant mice. Collectively, our results demonstrate that CD11b$^+$ mDCs have the ability to either promote or prevent Th2 cell responses depending on the amount of LPS. Therefore, the contribution of CD11b$^+$ mDCs relative to CD103$^+$ mDCs to the development of allergic Th2 cell responses is more complex than initially expected and is highly influenced by the environmental number of microbial products rather than being predetermined by their

intrinsic nature alone. Importantly, our data show that whereas IL-12 production by CD11b$^+$ mDCs requires the upregulation of T-bet, IL-12 production by CD103$^+$ mDCs is T-bet independent. However, the molecular mechanisms by which T-bet is required in only CD11b$^+$ mDCs is unclear. Various transcription factors, including IRF family members, have been implicated in the regulation of IL-12 production by DC subsets (Gao et al., 2013; Takaoka et al., 2005; Taki et al., 1997; Williams et al., 2013). Here, we found no differences in the expression of IRF1 or IRF5 between CD103$^+$ and CD11b$^+$ mDCs from infants and adults. Importantly, the transcription factor IRF4 is highly expressed in CD11b$^+$, but not in CD103$^+$, mDCs and essentially contributes to the differentiation and function of CD11b$^+$ mDCs. For example, IRF4 drives CD11b$^+$ mDCs to promote Th2 cell differentiation (Gao et al., 2013; Kumamoto et al., 2013; Williams et al., 2013), inhibits the capacity of DCs and macrophages to produce IL-12 (Akbari et al., 2014; Honma et al., 2005), and competes with IRF5 to inhibit IRF5-dependent upregulation of pro-inflammatory cytokines, such as IL-12 and IL-6 (Negishi et al., 2005). These data suggest a role for IRF4 in the negative-feedback regulation of IL-12 production, and because IRF4 is restricted to CD11b$^+$ mDCs, IRF4 might selectively control IL-12 regulation specifically in CD11b$^+$ mDCs. Therefore, one could speculate that T-bet upregulation, specifically in CD11b$^+$ mDCs, is important for overcoming the suppressive function of IRF4 in IL-12 production.

In summary, our comparative analysis between adult and neonatal-infant mice offers mechanistic insights into how the priming of allergen-specific Th2 cell responses is differentially regulated during childhood and adulthood, suggests a perspective for the biological mechanism underlying the hygiene hypothesis, and provides a plausible mechanism to explain higher susceptibility to allergic airway inflammation observed in children than in adults.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and three tables and can be found with this article online at https://doi.org/10.1016/j.immuni.2018.11.012.

ACKNOWLEDGMENTS

We thank Dr. J. Zhu (National Institute of Allergy and Infectious Diseases, NIH, Bethesda) for providing TBGR mice and Becca Burnham, Uma Mudunuru, and Thomas S. Simpler for animal husbandry. The work was supported by University of Alabama at Birmingham and NIH grant R01 AI116584 to B.L., grant U01 ES026458 to S.M., and grant R01 AI110480 to A.B.T.

AUTHOR CONTRIBUTIONS

B.L. designed the study and wrote the manuscript. H.B., K.K., A.P., Z.Y., S.M., and B.L. performed the experiments and analyzed the data. T.T.M. analyzed RNA-seq data. A.B.T. provided expertise and reagents.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: August 8, 2017
Revised: August 16, 2018
Accepted: November 5, 2018
Published: January 8, 2019

REFERENCES

Akbari, M., Honma, K., Kimura, D., Miyakoda, M., Kimura, K., Matsuyama, T., and Yui, K. (2014). IRF4 in dendritic cells inhibits IL-12 production and controls Th1 immune responses against Leishmania major. J. Immunol. *192*, 2271–2279.

Ballesteros-Tato, A., León, B., Lund, F.E., and Randall, T.D. (2010). Temporal changes in dendritic cell subsets, cross-priming and costimulation via CD70 control CD8(+) T cell responses to influenza. Nat. Immunol. *11*, 216–224.

Ballesteros-Tato, A., Randall, T.D., Lund, F.E., Spolski, R., Leonard, W.J., and León, B. (2016). T follicular helper cell plasticity shapes pathogenic T helper 2 cell-mediated immunity to inhaled house dust mite. Immunity *44*, 259–273.

Bar-Joseph, Z., Gifford, D.K., and Jaakkola, T.S. (2001). Fast optimal leaf ordering for hierarchical clustering. Bioinformatics *17* (*Suppl 1*), S22–S29.

Conejero, L., Khouili, S.C., Martínez-Cano, S., Izquierdo, H.M., Brandi, P., and Sancho, D. (2017). Lung CD103+ dendritic cells restrain allergic airway inflammation through IL-12 production. JCI Insight *2*, 2.

Daan de Boer, J., Roelofs, J.J., de Vos, A.F., de Beer, R., Schouten, M., Hommes, T.J., Hoogendijk, A.J., de Boer, O.J., Stroo, I., van der Zee, J.S., et al. (2013). Lipopolysaccharide inhibits Th2 lung inflammation induced by house dust mite allergens in mice. Am. J. Respir. Cell Mol. Biol. *48*, 382–389.

de Kleer, I.M., Kool, M., de Bruijn, M.J., Willart, M., van Moorleghem, J., Schuijs, M.J., Plantinga, M., Beyaert, R., Hams, E., Fallon, P.G., et al. (2016). Perinatal activation of the interleukin-33 pathway promotes type 2 immunity in the developing lung. Immunity *45*, 1285–1298.

Edelson, B.T., Kc, W., Juang, R., Kohyama, M., Benoit, L.A., Klekotka, P.A., Moon, C., Albring, J.C., Ise, W., Michael, D.G., et al. (2010). Peripheral CD103+ dendritic cells form a unified subset developmentally related to CD8alpha+ conventional dendritic cells. J. Exp. Med. *207*, 823–836.

Eisen, M.B., Spellman, P.T., Brown, P.O., and Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. USA *95*, 14863–14868.

Gao, Y., Nish, S.A., Jiang, R., Hou, L., Licona-Limón, P., Weinstein, J.S., Zhao, H., and Medzhitov, R. (2013). Control of T helper 2 responses by transcription factor IRF4-dependent dendritic cells. Immunity *39*, 722–732.

Gereda, J.E., Leung, D.Y., Thatayatikom, A., Streib, J.E., Price, M.R., Klinnert, M.D., and Liu, A.H. (2000). Relation between house-dust endotoxin exposure, type 1 T-cell development, and allergen sensitisation in infants at high risk of asthma. Lancet *355*, 1680–1683.

Gollwitzer, E.S., Saglani, S., Trompette, A., Yadava, K., Sherburn, R., McCoy, K.D., Nicod, L.P., Lloyd, C.M., and Marsland, B.J. (2014). Lung microbiota promotes tolerance to allergens in neonates via PD-L1. Nat. Med. *20*, 642–647.

Guilliams, M., Ginhoux, F., Jakubzick, C., Naik, S.H., Onai, N., Schraml, B.U., Segura, E., Tussiwand, R., and Yona, S. (2014). Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. Nat. Rev. Immunol. *14*, 571–578.

Holt, P.G. (1996). Primary allergic sensitization to environmental antigens: perinatal T cell priming as a determinant of responder phenotype in adulthood. J. Exp. Med. *183*, 1297–1301.

Honma, K., Udono, H., Kohno, T., Yamamoto, K., Ogawa, A., Takemori, T., Kumatori, A., Suzuki, S., Matsuyama, T., and Yui, K. (2005). Interferon regulatory factor 4 negatively regulates the production of proinflammatory cytokines by macrophages in response to LPS. Proc. Natl. Acad. Sci. USA *102*, 16001–16006.

Intlekofer, A.M., Banerjee, A., Takemoto, N., Gordon, S.M., Dejong, C.S., Shin, H., Hunter, C.A., Wherry, E.J., Lindsten, T., and Reiner, S.L. (2008). Anomalous type 17 response to viral infection by CD8+ T cells lacking T-bet and eomesodermin. Science *321*, 408–411.

Kumamoto, Y., Linehan, M., Weinstein, J.S., Laidlaw, B.J., Craft, J.E., and Iwasaki, A. (2013). CD301b$^+$ dermal dendritic cells drive T helper 2 cell-mediated immunity. Immunity *39*, 733–743.

León, B. (2017). T cells in allergic asthma: key players beyond the Th2 pathway. Curr. Allergy Asthma Rep. *17*, 43.

León, B., Ballesteros-Tato, A., Browning, J.L., Dunn, R., Randall, T.D., and Lund, F.E. (2012). Regulation of T(H)2 development by CXCR5+ dendritic cells and lymphotoxin-expressing B cells. Nat. Immunol. *13*, 681–690.

Lipscomb, M.W., Chen, L., Taylor, J.L., Goldbach, C., Watkins, S.C., Kalinski, P., Butterfield, L.H., Wesa, A.K., and Storkus, W.J. (2009). Ectopic T-bet expression licenses dendritic cells for IL-12-independent priming of type 1 T cells in vitro. J. Immunol. *183*, 7250–7258.

Lodge, C.J., Lowe, A.J., Gurrin, L.C., Hill, D.J., Hosking, C.S., Khalafzai, R.U., Hopper, J.L., Matheson, M.C., Abramson, M.J., Allen, K.J., and Dharmage, S.C. (2011). House dust mite sensitization in toddlers predicts current wheeze at age 12 years. J. Allergy Clin. Immunol. *128*, 782–788.e9.

Lugo-Villarino, G., Maldonado-Lopez, R., Possemato, R., Penaranda, C., and Glimcher, L.H. (2003). T-bet is required for optimal production of IFN-gamma and antigen-specific T cell activation by dendritic cells. Proc. Natl. Acad. Sci. USA *100*, 7749–7754.

Masoli, M., Fabian, D., Holt, S., and Beasley, R.; Global Initiative for Asthma (GINA) Program (2004). The global burden of asthma: executive summary of the GINA Dissemination Committee report. Allergy *59*, 469–478.

Negishi, H., Ohba, Y., Yanai, H., Takaoka, A., Honma, K., Yui, K., Matsuyama, T., Taniguchi, T., and Honda, K. (2005). Negative regulation of Toll-like-receptor signaling by IRF-4. Proc. Natl. Acad. Sci. USA *102*, 15989–15994.

Plantinga, M., Guilliams, M., Vanheerswynghels, M., Deswarte, K., Branco-Madeira, F., Toussaint, W., Vanhoutte, L., Neyt, K., Killeen, N., Malissen, B., et al. (2013). Conventional and monocyte-derived CD11b(+) dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. Immunity *38*, 322–335.

Reynolds, L.A., and Finlay, B.B. (2017). Early life factors that affect allergy development. Nat. Rev. Immunol. *17*, 518–528.

Schuijs, M.J., Willart, M.A., Vergote, K., Gras, D., Deswarte, K., Ege, M.J., Madeira, F.B., Beyaert, R., van Loo, G., Bracher, F., et al. (2015). Farm dust and endotoxin protect against allergy through A20 induction in lung epithelial cells. Science *349*, 1106–1110.

Siegrist, C.A. (2001). Neonatal and early life vaccinology. Vaccine *19*, 3331–3346.

Song, W., Wei, S., Liu, G., Yu, Z., Estell, K., Yadav, A.K., Schwiebert, L.M., and Matalon, S. (2011). Postexposure administration of a beta2-agonist decreases chlorine-induced airway hyperreactivity in mice. Am. J. Respir. Cell Mol. Biol. *45*, 88–94.

Steer, C.A., Martinez-Gonzalez, I., Ghaedi, M., Allinger, P., Mathä, L., and Takei, F. (2017). Group 2 innate lymphoid cell activation in the neonatal lung drives type 2 immunity and allergen sensitization. J. Allergy Clin. Immunol. *140*, 593–595.e3.

Stein, M.M., Hrusch, C.L., Gozdz, J., Igartua, C., Pivniouk, V., Murray, S.E., Ledford, J.G., Marques Dos Santos, M., Anderson, R.L., Metwali, N., et al. (2016). Innate immunity and asthma risk in amish and hutterite farm children. N. Engl. J. Med. *375*, 411–421.

Stranges, P.B., Watson, J., Cooper, C.J., Choisy-Rossi, C.M., Stonebraker, A.C., Beighton, R.A., Hartig, H., Sundberg, J.P., Servick, S., Kaufmann, G., et al. (2007). Elimination of antigen-presenting cells and autoreactive T cells by Fas contributes to prevention of autoimmunity. Immunity *26*, 629–641.

Sung, S.S., Fu, S.M., Rose, C.E., Jr., Gaskin, F., Ju, S.T., and Beaty, S.R. (2006). A major lung CD103 (alphaE)-beta7 integrin-positive epithelial dendritic cell population expressing Langerin and tight junction proteins. J. Immunol. *176*, 2161–2172.

Szabo, S.J., Kim, S.T., Costa, G.L., Zhang, X., Fathman, C.G., and Glimcher, L.H. (2000). A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell *100*, 655–669.

Takaoka, A., Yanai, H., Kondo, S., Duncan, G., Negishi, H., Mizutani, T., Kano, S., Honda, K., Ohba, Y., Mak, T.W., and Taniguchi, T. (2005). Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. Nature *434*, 243–249.

Taki, S., Sato, T., Ogasawara, K., Fukuda, T., Sato, M., Hida, S., Suzuki, G., Mitsuyama, M., Shin, E.H., Kojima, S., et al. (1997). Multistage regulation of Th1-type immune responses by the transcription factor IRF-1. Immunity *6*, 673–679.

Trinchieri, G. (2003). Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat. Rev. Immunol. *3*, 133–146.

Verstrepen, L., Bekaert, T., Chau, T.L., Tavernier, J., Chariot, A., and Beyaert, R. (2008). TLR-4, IL-1R and TNF-R signaling to NF-kappaB: variations on a common theme. Cell. Mol. Life Sci. *65*, 2964–2978.

Williams, J.W., Tjota, M.Y., Clay, B.S., Vander Lugt, B., Bandukwala, H.S., Hrusch, C.L., Decker, D.C., Blaine, K.M., Fixsen, B.R., Singh, H., et al. (2013). Transcription factor IRF4 drives dendritic cells to promote Th2 differentiation. Nat. Commun. *4*, 2990.

Zhu, J., Jankovic, D., Oler, A.J., Wei, G., Sharma, S., Hu, G., Guo, L., Yagi, R., Yamane, H., Punkosdy, G., et al. (2012). The transcription factor T-bet is induced by multiple pathways and prevents an endogenous Th2 cell program during Th1 cell responses. Immunity *37*, 660–673.

Zhu, Z., Oh, S.Y., Zheng, T., and Kim, Y.K. (2010). Immunomodulating effects of endotoxin in mouse models of allergic asthma. Clin Exp Allergy. *40*, 536–546.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-B220 Clone RA3-6B2	BD Biosciences	Cat# 552094, RRID: AB_394335
Anti-CD4 Clone GK1.5	BD Biosciences	Cat# 563050, RRID: AB_2737973
Anti-CD11b Clone M1/70	BD Biosciences	Cat# 562127, RRID: AB_10893815
Anti-CD11c Clone HL3	BD Biosciences	Cat# 558079, RRID: AB_647251
Anti-CD44 Clone IM7	BD Biosciences	Cat# 560780, RRID: AB_1937316
Anti-CD45.1 Clone A20	BD Biosciences	Cat# 558701, RRID: AB_1645214
Anti-CD45.2 Clone 104	BD Biosciences	Cat# 560695, RRID: AB_1727493
Anti-CD69 Clone H1-2F3	BD Biosciences	Cat# 560689, RRID: AB_1727506
Anti-CD86 Clone GL1	BD Biosciences	Cat# 557426, RRID: AB_396698
Anti-CD103 Clone M290	BD Biosciences	Cat# 557495, RRID: AB_396732
Anti-CD138 Clone 281-2	BD Biosciences	Cat# 558626, RRID: AB_1645216
Anti-IgE Clone R35-72	BD Biosciences	Cat# 553414, RRID: AB_394847
Anti-Ly6c Clone AL-21	BD Biosciences	Cat# 560596, RRID: AB_1727555
Anti-Siglec-F Clone E50-2440	BD Biosciences	Cat# 565183, RRID: AB_2739097
Anti-Siglec-H Clone 440c	BD Biosciences	Cat# 747671, RRID: AB_2744232
Anti-CD64 Clone X54-5/7.1	Biolegend.	Cat# 139306, RRID: AB_11219391
Anti-MHC class II Clone M5/114.15.2	Biolegend.	Cat# 107620, RRID: AB_493527
Anti-IL-13 Clone 13A	eBiosciences	Cat# 12-7133-41, RRID: AB_10852712
Anti-IFNγ Clone XMG1.2	BD Biosciences	Cat# 557649, RRID: AB_396766
Anti-IL-5 Clone TRFK5	BioLegend	Cat# 504311, RRID: AB_2563161
Anti-IL-12 Clone C15.6	BD-Biosciences	Cat# 554479, RRID: AB_395420
Anti-TNF-α Clone MP6-XT22	BD-Biosciences	Cat# 554418, RRID: AB_395379
Anti-T-bet Clone 4B10	Biolegend	Cat# 644814, RRID: AB_10901173
Anti-GATA-3 Clone TWAJ	eBioscience	Cat# 25-9966-42, RRID: AB_2573568
Anti-Foxp3 Clone FJK-16 s	eBioscience	Cat# 25-5773-82, RRID: AB_891552
Anti-IRF1 Clone ab216114	Abcam	Cat# ab216114
Anti-IRF4 Clone IRF4.3E4	BioLegend	Cat# 646404, RRID: AB_2563005
Anti-IRF5 Clone ab212239	Abcam	Cat# ab212239
Anti-c-Rel Clone sc-6955	Santa Cruz	Cat# sc-6955 AF488, RRID: AB_670194
Anti-P-c-Jun Clone D47G9	Cell Signaling Technology	Cat# 8752S
Anti-IkBζ Clone LK2NAP	eBioscience	Cat# 46-6801-82, RRID: AB_11149496
Anti-CD16/32	BioXCell	Cat# CUS-HB-197, RRID: AB_2687830
Anti-TNF-α	BioXCell	Cat# BE0058, RRID: AB_1107764
Chemicals, Peptides, and Recombinant Proteins		
Recombinant mouse IL-12	PeproTech	Cat# 210-12
Recombinant mouse TNF-α	R&D Systems	Cat# 410-MT-010
EndoFit ovalbumin	InvivoGen	Cat# NC1005858
Diphtheria toxin	Sigma-Aldrich	Cat# D0564-1MG
PMA	Sigma-Aldrich	Cat# P8139-1MG
Calcimycin	Life Technologies	Cat# A1493
Brefeldin-A	Sigma-Aldrich	Cat# B7651-5MG
Methacholine	Sigma-Aldrich	Cat# 1396364

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical Commercial Assays		
Transcription factor staining buffer set	eBioscience	Cat# 00-5523-00
Anti-L3T4 (CD4) Microbeads, mouse	Miltenyi Biotec	Cat# 130-049-201, RRID: AB_2722753
CD11c MicroBeads UltraPure, mouse	Miltenyi Biotec	Cat# 130-108-338
CellTrace™ CFSE	ThermoFisher	Cat# C34554
AF647 labeling kit	Invitrogen	Cat# A20173
ProcartaPlex assay	Life Technologies	Cat# PPX-12
RNeasy Plus Micro Kit	QIAGEN	Cat# 74034
Deposited Data		
RNA-Seq. data. CD11b^{+} lung DC subset from adult (> 8-week-old) and infant (18-day-old) mice after HDM+LPS i.n. sensitization	This paper	GEO: GSE110046
Experimental Models: Organisms/Strains		
House-dust mite (Dermatophagoides pteronyssinus and Dermatophagoides farinae) extracts	Greer laboratories	N/A
Lipopolysaccharide from *Escherichia coli* 0111:B4	Sigma-Aldrich	Cat# L2630
Mouse: C57BL/6J (B6)	The Jackson Laboratory	Stock No: 000664
Mouse: B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ (CD45.1^{+} B6 congenic)	The Jackson Laboratory	Stock No: 002014
Mouse: C57BL/6-Tg(TcraTcrb)425Cbn/J (OT-II)	The Jackson Laboratory	Stock No: 004194
Mouse: B6.129-Il4^{tm1Lky}/J (B6.4get IL-4 reporter mice)	Laboratory of Dr. M. Mohrs (Trudeau Institute)	N/A
Mouse: B6.129S(C)-Batf3^{tm1Kmm}/J (*Baft3*$^{-/-}$)	The Jackson Laboratory	Stock No: 013755
Mouse: B6.129-Tbx21^{tm2Srnr}/J (*Tbx21*$^{fl/fl}$)	The Jackson Laboratory	Stock No: 022741
Mouse: C57BL/6J-Tg(Itgax-cre,-EGFP)4097Ach/J (*Itgax-cre,-EGFP*)	The Jackson Laboratory	Stock No: 007567
Mouse: B6.129S6-*Tbx21*^{tm1Glm}/J (*Tbx21*$^{-/-}$)	The Jackson Laboratory	Stock No: 004648
Mouse: B6(Cg)-*Tlr4*$^{tm1.2Karp}$/J (*Tlr4*$^{-/-}$)	The Jackson Laboratory	Stock No: 029015
Mouse: B6.129S7-*Il1r1*^{tm1Imx}/J (*Il1r1*$^{-/-}$)	The Jackson Laboratory	Stock No: 003245
Mouse: B6.129S-Tnfrsf1a^{tm1Imx} Tnfrsf1b^{tm1Imx}/J (*Tnfr1*$^{-/-}$*Tnfr2*$^{-/-}$)	The Jackson Laboratory	Stock No: 003243
Mouse: B6.FVB-Tg(Itgax-DTR/EGFP)^{57Lan}/J (*Itgax*-DTR,-EGFP)	The Jackson Laboratory	Stock No: 004512
Mouse: C57BL/6 T-bet-ZsGreen reporter (TBGR)	Laboratory of Dr. J. Zhu (NIAID, NIH)	N/A
Software and Algorithms		
Prism V7	GraphPad	https://www.graphpad.com/
FlowJo V9.9.6	TreeStar	https://www.flowjo.com
javaGSEA V3.0	Broad Institute	http://www.gsea-msigdb.org/gsea/index.jsp
R2017b V9.3	MATLAB	https://www.mathworks.com/products/matlab/whatsnew.html

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Beatriz León (bleon@uab.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

The mouse strains used in these experiments include: C57BL/6J (B6), B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ (CD45.1^{+} B6 congenic), C57BL/6-Tg(TcraTcrb)425Cbn/J (OT-II), B6.129-Il4^{tm1Lky}/J (B6.4get IL-4 reporter mice), B6.129S(C)-Batf3^{tm1Kmm}/J (*Baft3*$^{-/-}$), B6.129-Tbx21^{tm2Srnr}/J (*Tbx21*$^{fl/fl}$), C57BL/6J-Tg(Itgax-cre,-EGFP)4097Ach/J (*Itgax-cre,-EGFP*), B6.129S6-*Tbx21*^{tm1Glm}/J (*Tbx21*$^{-/-}$),

B6(Cg)-*Tlr4*$^{tm1.2Karp}$/J (*Tlr4*$^{-/-}$), B6.129S7-*Il1r1*^{tm1Imx}/J (*Il1r1*$^{-/-}$), B6.129S-Tnfrsf1a^{tm1Imx} Tnfrsf1b^{tm1Imx}/J (*Tnfr1*$^{-/-}$*Tnfr2*$^{-/-}$) B6.FVB-Tg(Itgax-DTR/EGFP)^{57Lan}/J (*Itgax*-DTR,-EGFP) and C57BL/6 T-bet-ZsGreen reporter (TBGR). B6.4get mice were originally obtained from Dr. M. Mohrs (Trudeau Institute). TBGR mice were developed and kindly provided by Dr. J. Zhu (NIAID, NIH). All other mice were originally obtained from Jackson Laboratory and were bred and housed in the University of Alabama at Birmingham animal facility under specific pathogen-free conditions. Experiments were equally performed with male and female mice. The University of Alabama at Birmingham Institutional Animal Care and Use Committee approved all procedures involving animals.

METHOD DETAILS

Immunizations

HDM (Dermatophagoides pteronyssinus and D. farinae) extracts were obtained from Greer laboratories (< 12 EU/mg endotoxin). Mice were administered (i.n. or i.t.) with 50 μg of HDM extract ± 5 μg of LPS-free EndoFit OVA (InvivoGen) +/− LPS from *Escherichia coli* 0111:B4 (Sigma-Aldrich) +/− 150 ng of recombinant IL-12 (PeproTech) +/− 10-30 ng of recombinant TNFα (R&D Systems) daily for 1-3 days and challenged (i.n.) with 50 μg of HDM ± 5 μg of LPS-free EndoFit OVA. i.n. and i.t. administrations were given in 50 μL of PBS for adults and 18-20d-old infant mice and in 10 μL of PBS for 7d-old, 10d-old and 14d-old infant mice. In some experiments, mice were intraperitoneally administered (i.p.) with 250 μg of anti-TNFα mAb (BioXCell) at the time of initial sensitization. To deplete CD11c$^+$ cells, *Itgax*-DTR BM chimeras were i.p. treated with 60 ng DT (Sigma-Aldrich) on day 3 after sensitization. In some experiments HDM extract was labeled with AF647 labeling kit (Invitrogen) prior to administration to mice.

BM chimeras

Recipient mice were irradiated with 950 Rads from a high-energy X-rays source delivered in a split dose and reconstituted with 10^7 total BM cells. Mice were allowed to reconstitute for at least 8-12 weeks before HDM treatment.

Cell preparation and flow cytometry

Lungs were isolated, cut into small fragments and digested for 45 min at 37°C with 0.6 mg/mL collagenase A (Sigma) and 30 μg/mL DNase I (Sigma) in RPMI-1640 medium (GIBCO). Digested lungs, mLN or spleens were mechanically disrupted by passage through a wire mesh. Red blood cells were lysed with 150 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA. Fc receptors were blocked with anti-mouse CD16/32 (5 μg/mL; BioXCell5), followed by staining with fluorochrome-conjugated Ab. Fluorochrome-labeled anti-B220 (RA3-6B2), anti-CD4 (GK1.5), anti-CD11b (M1/70), anti-CD11c (HL3), anti-CD44 (IM7), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD69 (H1-2F3), anti-CD86 (GL1), anti-CD103 (M290), anti-CD138 (281-2), anti-IgE (R35-72y), anti-Ly6c (AL-21), anti-Siglec-F (E50-2440) and anti-Siglec-H (440c) were from BD Biosciences. Fluorochrome-labeled anti-CD64 (X54-5/7.1) and anti-MHC class II (M5/114.15.2) were from Biolegend.

For intracellular cytokine staining of T cells, cell suspensions were stimulated with PMA (20 ng/mL) plus Calcimycin (1 μg/mL) in the presence of Brefeldin-A (BFA, 10 μg/mL) for 4h. Restimulated cells were surface stained, fixed in 4% paraformaldehyde, permeabilized with 0.1% saponin, and stained with Abs against IL-13 (13A; eBiosciences), IFNγ (XMG1.2; BD-Biosciences) and IL-5 (TRFK5; BioLegend). For cytokine staining of DCs, cell suspensions were surface stained, fixed, permeabilized, and stained with anti-IL-12 (C15.6; BD-Biosciences). For TNFα staining, lung cell suspensions were incubated in the presence of BFA for 4h, fixed, permeabilized, and stained with anti-TNFα (MP6-XT22; BD-Biosciences). T-bet, GATA-3, Foxp3, IRFs, c-Rel, P-c-Jun and IkBζ intracellular staining was performed using the Mouse regulatory T cell staining kit (eBioscience) and Abs against T-bet (4B10; biolegend), GATA-3 (TWAJ; eBioscience), Foxp3 (.FJK-16 s; eBioscience), IRF1 (ab216114; Abcam), IRF4 (IRF4.3E4; BioLegend), IRF5 (ab212239; Abcam), c-Rel (sc-6955; Santa Cruz), P-c-Jun (D47G9; Cell Signaling Technology) and IkBζ (LK2NAP; eBioscience). Flow cytometry was performed on Attune NxT and FACSCanto II (BD-Biosciences) instruments.

Cell purifications, cell transfers and *in vitro* cultures

CD4$^+$ T cells were isolated by MACs (Miltenyi Biotec) from the spleens of naive OTII mice. CD11c$^+$ cells were isolated by MACs from pooled lungs or mLN of day 3 HDM+/−OVA+/−LPS-treated mice. All T and CD11c$^+$ cell preparations were more than 95% pure. HDM+/−LPS sensitization greatly reduced the proportion of CD11c$^+$ alveolar macrophages in the lungs (data not shown) and purification of CD11c$^+$ cells from the lungs of sensitized mice resulted in more than 80%–90% enrichment of CD11c$^+$ Siglec-F$^-$ MHC^{hi} lung DCs. Equivalent numbers (25×10^3) of naive OTII cells were transferred (i.v.) into naive congenic recipients. Equivalent numbers (1×10^6) of CD11c$^+$ lung cells were transferred (i.n.) into naive congenic infant recipients daily for 3 days. For *in vitro* cultures, OTII cells were labeled for 10 min at 37°C with CellTrace CFSE (ThermoFisher). $2x10^4$ DCs and $1x10^5$ CFSE-labeled OTII cells were cultured in 200 μL of complete medium in round-bottomed 96-well plates for 72 h at 37°C. Complete medium included RPMI 1640 supplemented with sodium pyruvate, HEPES, non-essential amino acids, penicillin, streptomycin, 2-mercaptoethanol and 10% heat-inactivated FBS from Fisher Scientific.

BAL collection and measurement of cytokines

BAL was collected using 0.5-1 mL sterilized PBS per mouse. The BAL fluids were centrifuged at 5,000 × g for 10 min and the supernatants were frozen at −80°C. Cytokine measurements were performed using ProcartaPlex assay (Life Technologies) and Luminex xMAP technology as per manufacturer's instructions.

Lung histology

The lungs were fixed in buffered formalin (10%). Microtome sections of paraffin-embedded lungs were stained with periodic acid-Schiff (PAS) for visualization of goblet cells.

Pulmonary function assessment

To measure airway responsiveness, mice on day 5 post-challenge were mechanically ventilated and challenged with increasing concentrations of methacholine, as described previously (Song et al., 2011). Briefly, mice were anesthetized, intubated, connected to a ventilator (FlexiVent; SCIREQ, Montreal, PQ, Canada), and ventilated at a rate of 160 breaths per minute. Airway responsiveness was recorded at baseline and after a linear dose response with methacholine challenge (10–75 mg/mL; Sigma-Aldrich). Data were normalized to basal airway resistance and expressed as changes induced by methacholine. To measure arterial O_2 saturation (SpO2), the MouseOx Pulse-oximeter (Starr Life Sciences, Oakmont PA) was used on conscious mice 5 days-post-challenged, in accordance with manufacturer's instructions. Mice were shaved and the pulse-oximeter collar was placed behind the ears. To ensure accurate measurement, SpO2 data points were excluded from analysis if received an error code during measurement.

RNA-sequencing (RNA-seq). mCD11b$^+$DCs (B220$^-$CD64$^-$CD103$^-$CD11c$^+$MHCII^{hi}CD11b$^+$) from adult (7wks-old) and infant (2-3wks-old) mice were sorted from the mLN 24h after HDM+LPS sensitization using a FACSAria (BD Biosciences) after positive selection with anti-CD11c MACS beads (Miltenyi Biotec). RNA was isolated from the sorted cells using the RNeasy Plus Micro RNA purification kit (QIAGEN). Three or four replicates from three or four independent experiments for each condition were analyzed with RNA-seq. Library preparation and RNA sequencing was conducted through Genewiz. Libraries were sequenced using a 1Å~50-bp single end rapid run on the HiSeq2500 platform. Sequence reads were trimmed to remove possible adaptor sequences and nucleotides with poor quality (error rate < 0.05) at the end. After trimming, sequence reads shorter than 30 nucleotides were discarded. Remaining sequence reads were mapped to the *Mus musculus* mm10 reference genome using CLC genomics workbench v. 9.0.1. Differential gene expression was determined using DESeq2. Genes with an adjusted P value < 0.05 and an absolute log1.5-fold change (ratio ± 0.58) were considered significantly differentially expressed genes between adult and infant mCD11b$^+$DCs.

GSEA, hierarchical clustering and visualization of RNA sequencing results. GSEA was performed using the Molecular Signatures Database on the publically available MIT BROAD Institute server. We ranked the 173 genes obtained from RNA sequencing (Genewiz) according to a logarithmic transformation of each gene's P value multiplied by the sign of the corresponding logarithmic fold change, and subsequently used these rank lists to perform a gene set enrichment analysis (Broad Institute's GSEA Java app, version 3.0). Given gene sets can include both activated and repressed genes, we additionally performed an extended enrichment assessment of all identified gene sets. Separately, we performed hierarchical clustering analysis (Bar-Joseph et al., 2001; Eisen et al., 1998) of TNFα induced genes that are differentially expressed in adult versus infant mice, using MATLAB (version R2017b). Differential clusters are presented in form of an annotated heatmap based on standardized expression values, along with the resulting hierarchical clustering dendogram.

QUANTIFICATION AND STATISTICAL ANALYSIS

All plots and histograms were plotted in FlowJo v.9 software (Treestar). GraphPad Prism software (Version 7) was used for data and statistical analysis. Data were analyzed using the unpaired Student's t test. Values of $p < 0.05$ were considered significant ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

DATA AND SOFTWARE AVAILABILITY

The accession number for the raw data files for the RNA-Seq analyses reported in this paper is GEO: GSE110046.

Immunity, Volume *50*

Supplemental Information

Impaired Tumor-Necrosis-Factor-α-driven Dendritic Cell Activation Limits Lipopolysaccharide-Induced Protection from Allergic Inflammation in Infants

Holly Bachus, Kamaljeet Kaur, Amber M. Papillion, Tatiana T. Marquez-Lago, Zhihong Yu, André Ballesteros-Tato, Sadis Matalon, and Beatriz León

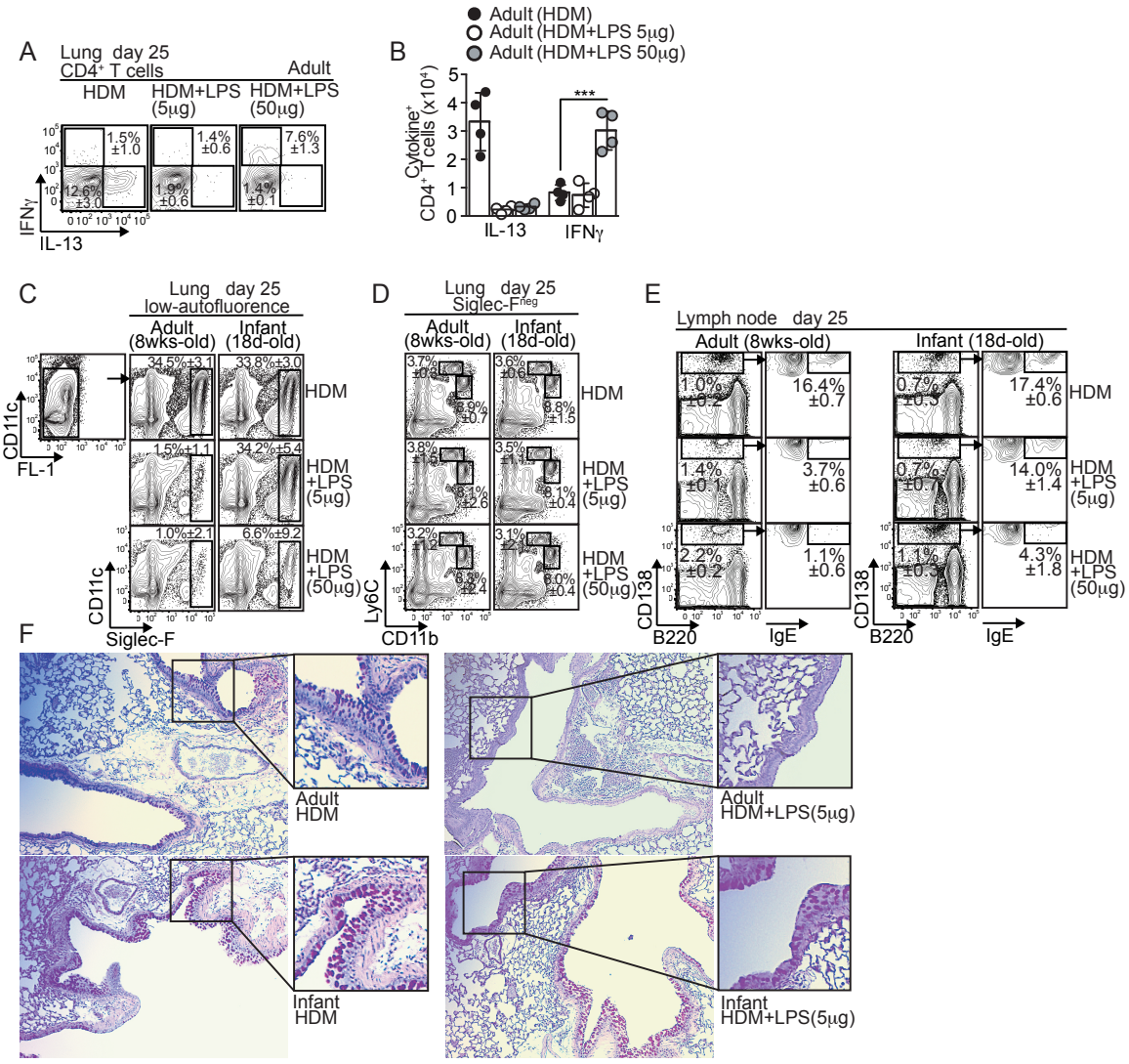


Figure S1. Related to Figure 1. Low-dose LPS sensitization in 18d-old infants does not prevent HDM-induced asthma features in adult life. B6 adult and 18d-old infant mice were i.n treated with 50μg of HDM plus different concentrations of LPS for 3 consecutive days starting on day 1. On day 20, mice were i.n challenged with 50μg of HDM daily for 3 days and analyzed on day 25. (**A-B**) Frequencies (**A**) and numbers (**B**) of IL-13- and IFNγ-expressing $CD4^+$ T cells in the lungs determined by ICS after restimulation for 4 h. (**C-D**) Frequencies of eosinophils (autofluorescence^{lo} $CD11c^{lo}$ Siglec-F^+) (**C**), neutrophils (Siglec-F^-CD11b^{hi}-$Ly6C^{int}$) and monocytes (Siglec-$F^-CD11b^{int}Ly6C^{hi}$) (**D**) in the lungs. (**E**) Frequencies of IgE^+ ACS in the mLN. (**F**) Lung sections stained with Periodic Acid-Schiff (PAS) to identify globlet cells. *P < 0.05 **P < 0.01 ***P < 0.001 (unpaired Student's t test). Data are representative of at least two independent experiments (mean and S.D. of 4-5 mice per group).

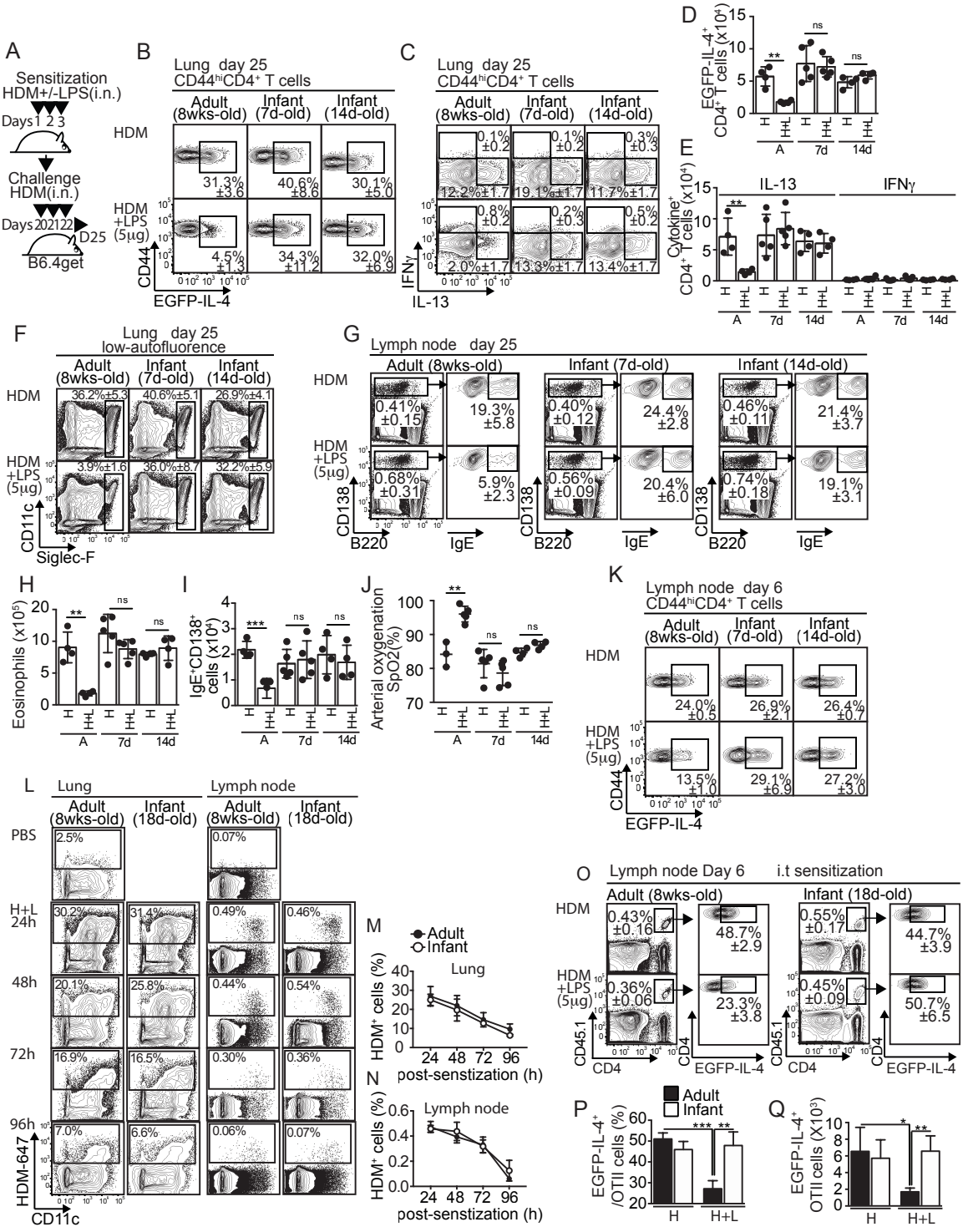


Figure S2. Related to Figure 1. Low-dose LPS sensitization in 7 to 18d-old infants does not prevent HDM-induced Th2 priming and development of Th2 cell-mediated asthmatic features later in life. (**A-J**) B6.4get adults, 7d-old neonates and 14d-old infants were i.n treated with 50µg of HDM+/-5µg of LPS for 3 consecutive days starting on day 1. On day 20, mice were i.n challenged with 50µg of HDM daily for 3 days and analyzed on day 25. Frequencies (**B-C**) and numbers (**D-E**) of IL-4-expressing (EGFP$^+$) CD4$^+$ T cells (**B, D**) and IL-13- and IFNγ-expressing CD4$^+$ T cells (**C, E**) in the lungs. Frequencies (**F**) and numbers (**H**) of eosinophils in the lungs. Frequencies (**G**) and numbers (**I**) of IgE$^+$ ACS in the mLN. SpO2 as determined by pulse-oximetry (**J**). (**K**) B6.4get adults, 7d-old neonates and 14d-old infants were i.n treated with 50µg of HDM+/-5µg of LPS for 3 consecutive days starting on day 1 and analyzed on day 6. Frequencies of IL-4-expressing (EGFP$^+$) CD4$^+$T cells in the mLN. (**L-N**) B6 adult and 18d-old infant mice were i.n treated with Alexa Fluor 647-labeled HDM+5µg of LPS on day 1 and analyzed at different time points. Frequencies of HDM-bearing cells in the lungs (**L-M**) and mLNs (**L, N**). (**O-Q**) Adult and 18d-old infant naïve B6 mice were adoptively transferred with 25x10^3 CD45.1$^+$ OTII.4get cells at day 0, i.t treated with 50µg of HDM+5µg of OVA+/-5µg of LPS for 3 consecutive days starting on day 1 and analyzed by flow cytometry on day 6. Frequencies (**O-P**) and numbers (**Q**) of donor IL-4-expressing (EGFP$^+$) OTII T cells in the mLN of recipient mice. *P < 0.05 **P < 0.01 ***P < 0.001 (unpaired Student's t test). Data are representative of two independent experiments (mean and S.D. of 3-5 mice per group).



Figure S3. Related to Figure 2. Kinetic analysis of specific CD4+ T cells and mDCs in the mLN after low-dose LPS sensitization. (**A-D**) Adult and 18d-old infant naïve B6 mice were adoptively transferred with 25x10³ CD45.1+ OTII.4get cells at day 0, i.n treated with 50μg of HDM+5μg of OVA+/-5μg of LPS for 3 consecutive days starting on day 1 and analyzed by flow cytometry on days 4,5 and 6. Frequencies of donor OTII T cells (**A**) and frequencies of GATA-3 and T-bet-expressing cells (**B**), MFI of GATA-3 expression (**C**) and MFI of T-bet expression (**D**) in donor OTII T cells from mLN of recipient mice. (**E-K**) B6 adult and 18d-old infant mice were i.n treated with HDM+/-5μg of LPS (**E-G**) or with Alexa Fluor 647-labeled HDM+/-5μg of LPS (**H-K**) for 3 consecutive days and analyzed at different time points. Frequencies and numbers of CD103+ mDCs (**E-F**), CD11b+ mDCs (**E,G**), HDM-bearing mDCs (**H-I**), HDM-bearing CD103+ mDCs (**H,J**) and HDM-bearing CD11b+ mDCs (**H,K**). Data are representative of three independent experiments (mean ± SD of 4 mice per time point).

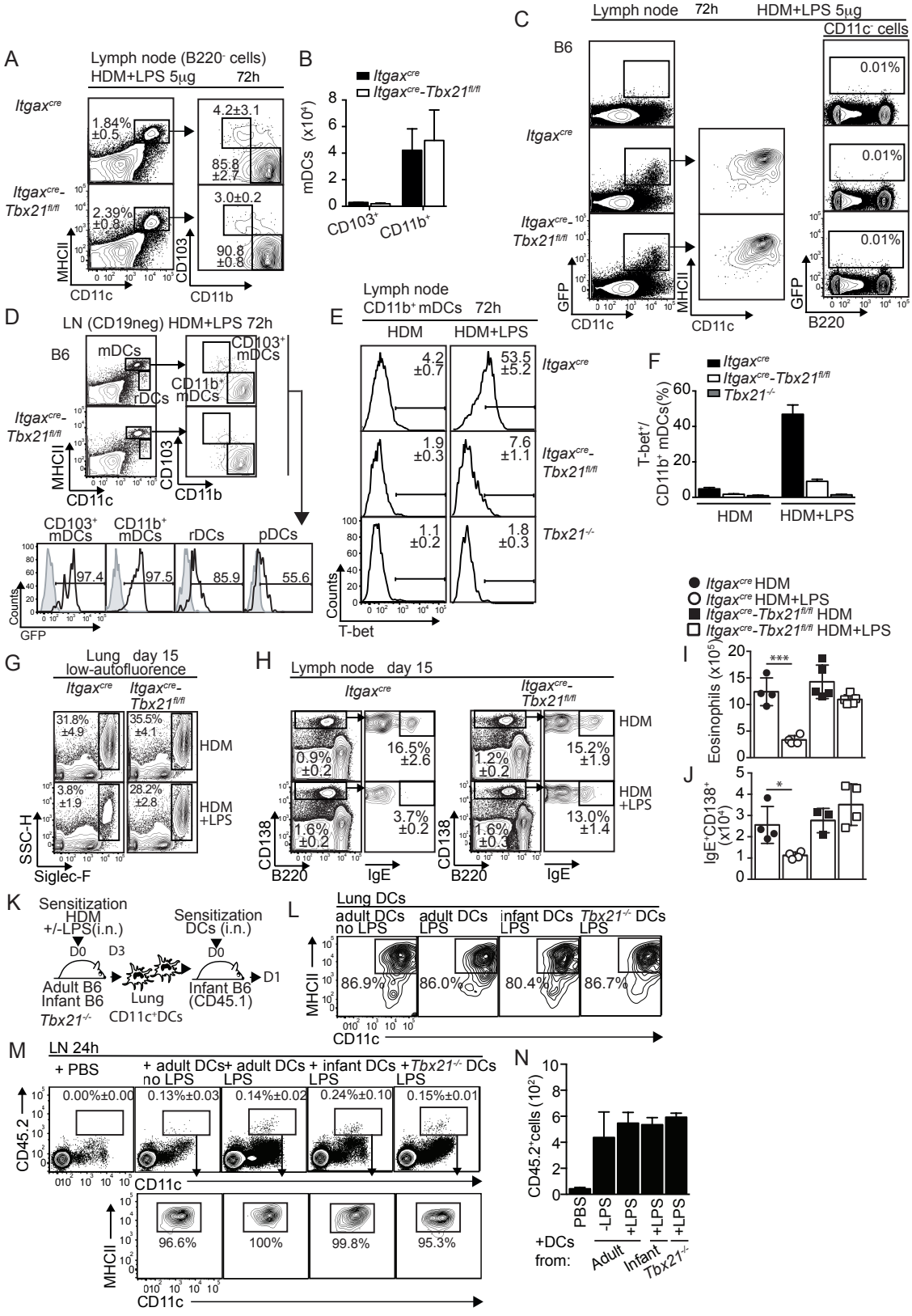


Figure S4. Related to Figure 4. Analysis of DCs in *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ mice and analysis of lung mDCs in recipient mice after i.n. transference. (**A-F**) *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ and control mice were i.n. HDM or HDM+5µg of LPS sensitized on day 1 and analyzed on day 4. Frequencies (**A**) and numbers (**B**) of CD103$^+$ mDCs and CD11b$^+$ mDCs in the mLN. Expression of GFP in total mLN cells and in CD11c$^-$ mLN cells and expression of CD11c and MHCII in GFP$^+$ cells from mLN (**C**). Expression of GFP in gated CD103$^+$ mDCs, CD11b$^+$ mDCs, rDCs and pDCs (Siglec-H+, CD11cl^o, B220$^+$ CD19$^-$). Gray filled overlays indicate control staining from B6 mice (**D**). Frequencies of T-bet expression in CD11b$^+$ mDCs from mLN (**E-F**). (**G-J**) *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ and control mice were i.n treated with 50µg of HDM+/-5µg of LPS for 3 consecutive days starting on day 1. On day 10, mice were i.n challenged with 50µg of HDM daily for 3 days and analyzed on day 15. Frequencies (**G**) and numbers (**I**) of eosinophils in the lungs. Frequencies (**H**) and numbers (**J**) of IgE$^+$ ACS in the mLN. (**K-N**) Day 3 CD11c$^+$ lung mDCs from HDM/OVA+/-LPS-treated adult and infant B6 mice or *Tbx21*$^{-/-}$ mice were purified and i.n. transferred into B6 (CD45.1$^+$) infant mice (K). Frequencies of CD11c$^+$MHCII^{hi} lung mDCs in purified lung preparations before transference (**L**). Frequencies (**M**) and numbers (**N**) of donor mDCs in the mLN 24h after transference. *P < 0.05 ***P < 0.001 (unpaired Student's t test). Data are representative of at least two independent experiments (mean and S.D. of 3-5 mice per group).

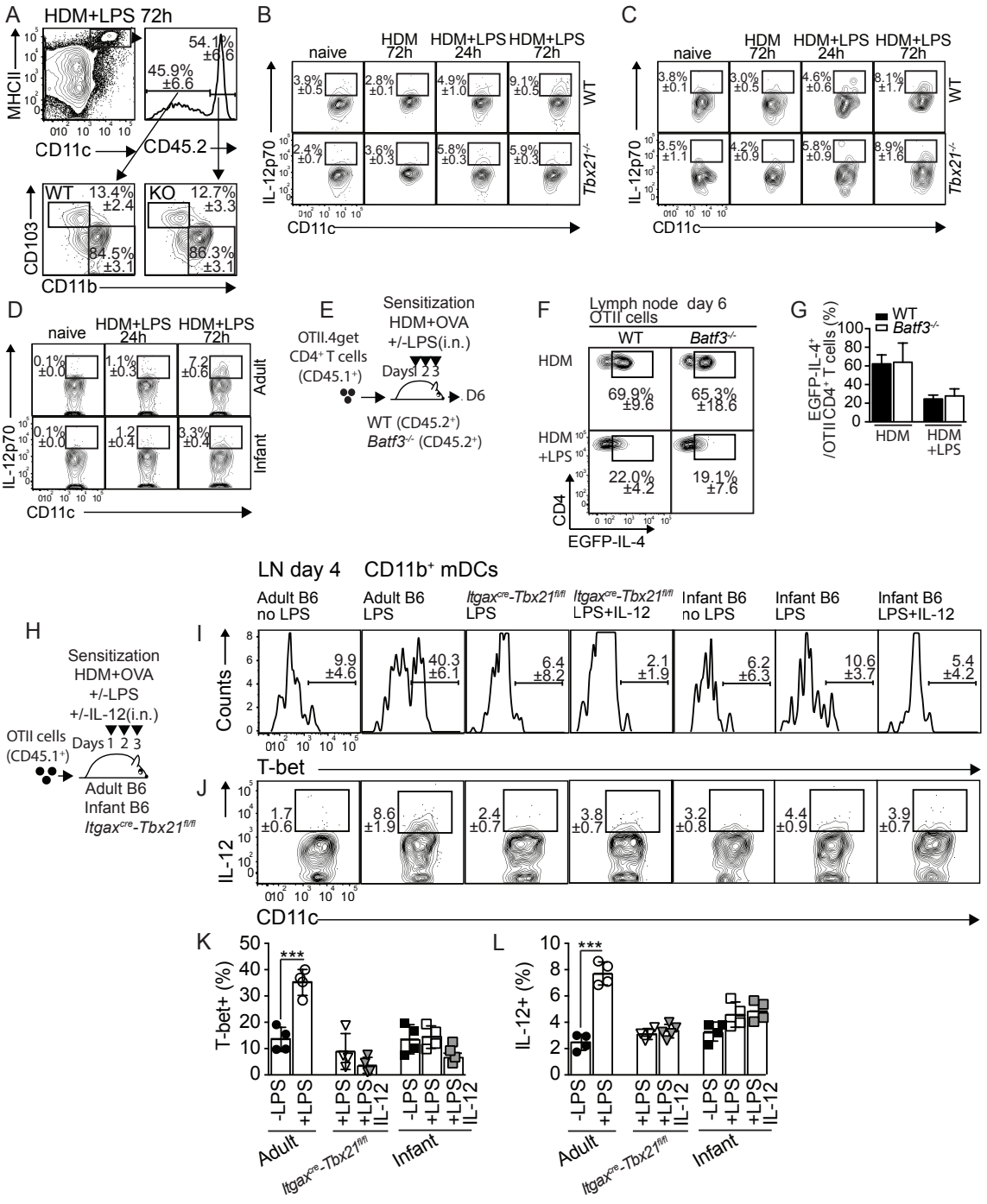


Figure S5. Related to Figure 5. *Tbx21*$^{-/-}$ and infant CD11b^{+} mDCs are intrinsically impaired in their ability to produce IL-12 after low-dose LPS exposure. (**A-C**) B6 (CD45.1^{+}) mice were irradiated and reconstituted with 1:1 mixture of BM from B6 (CD45.1^{+}) and *Tbx21*$^{-/-}$ (CD45.2^{+}) donors. Frequency of WT and *Tbx21*$^{-/-}$ CD11b^{+} and CD103^{+} mDCs in the mLN of day 3-HDM+LPS-treated reconstituted chimeric mice (**A**). Frequencies of IL-12p70-expressing WT and *Tbx21*$^{-/-}$ CD11b^{+} mDCs (**B**) and CD103^{+} mDCs (**C**) in the mLN of HDM+/-LPS-treated reconstituted chimeric mice. (**D**) Frequency of IL-12p70-expressing CD11b^{+} mDCs in the mLN of HDM+/-LPS-treated adult and infant B6 mice. (**E-G**) *Batf3*$^{-/-}$ and control mice were adoptively transferred with 25x10^{3} CD45.1^{+} OTII.4get cells at day 0 and i.n treated with 50µg of HDM+5µg of OVA+/-5µg of LPS for 3 consecutive days starting on day 1 (**E**). Frequencies of IL-4-expressing (EGFP^{+}) cells in donor OTII T cells from the mLN on day 6 (**F-G**). (**H-L**) Adult and 18d-old infant B6 mice or *Itgax*^{cre}-*Tbx21*$^{fl/fl}$ mice were i.n treated with 50µg of HDM+5µg of OVA+/-5µg of LPS+/-150ng of rIL-12p70 for 3 consecutive days starting on day 1 (**H**). Frequencies of T-bet (**I,K**) and IL-12p70 (**J,L**) expression in CD11b^{+} mDCs from mLN on day 4. ***P < 0.001 (unpaired Student's t test). Data are representative of two independent experiments (mean and S.D. of 3-4 mice per group).

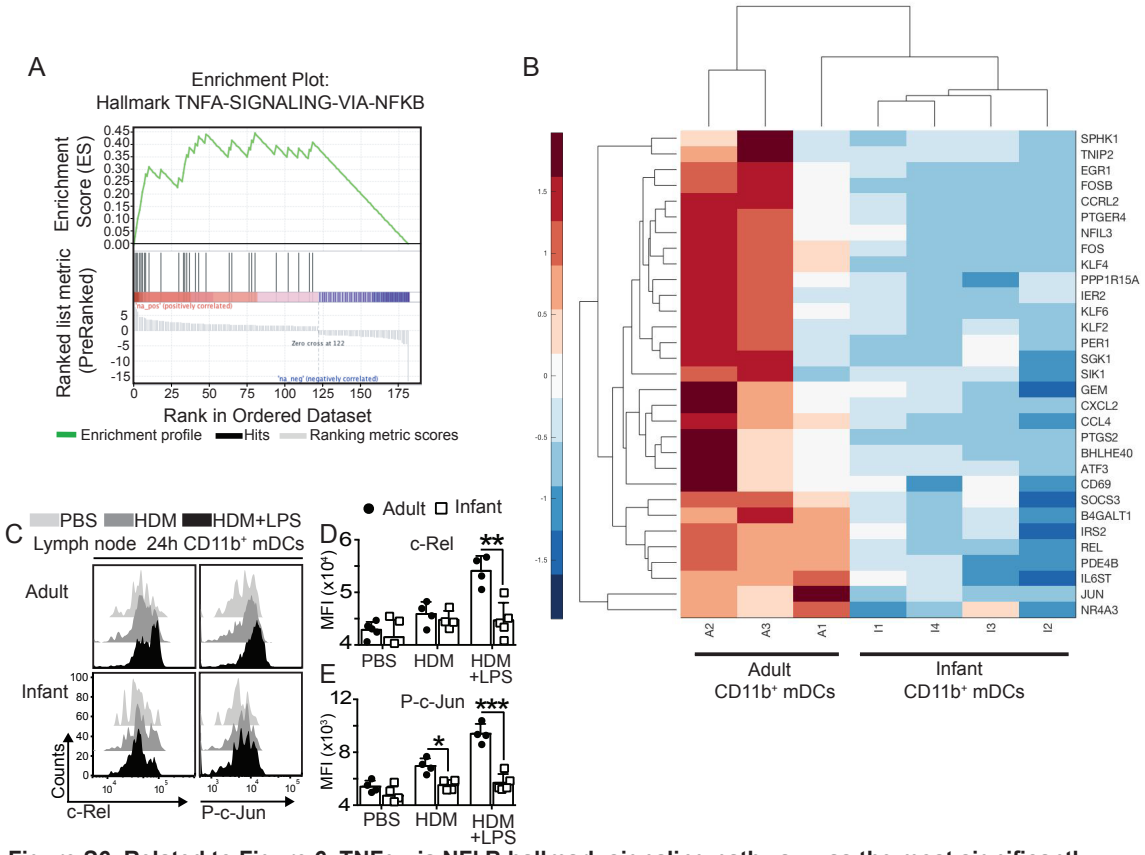


Figure S6. Related to Figure 6. TNFα-via NFkB hallmark-signaling pathway was the most significantly down-regulated pathway in infant CD11b⁺ mDCs after low-dose LPS sensitization. (**A-B**) CD11b⁺ mDCs were sorted from the mLN of adult and 18d-old infant mice 24 after HDM+LPS^{lo} sensitization and RNA-seq was performed. (**A**) GSEA showing enrichment in the TNF-α via NFkB hallmark-signaling pathway in adults vs. infant CD11b⁺ DCs (adjusted p-value <0.05). (**B**) Heat map displaying the expression of the TNFα-via NFkB hallmark signaling pathway genes that are differentially expressed in adult CD11b⁺ mDCs relative to infant CD11b⁺ mDCs. Replicates for each cell type were obtained from three or four independent experiments. (**C-E**) B6 adult and 18d-old infant mice were i.n treated with HDM+/-5µg of LPS. Expression c-Rel (**C,D**) and P-c-Jun (**C,E**) in CD11b⁺ mDCs from the mLN on day 1. *P < 0.05 **P < 0.01 ***P < 0.001 (unpaired Student's t test). Data are representative of two independent experiments (mean and S.D. of 4-5 mice per group).

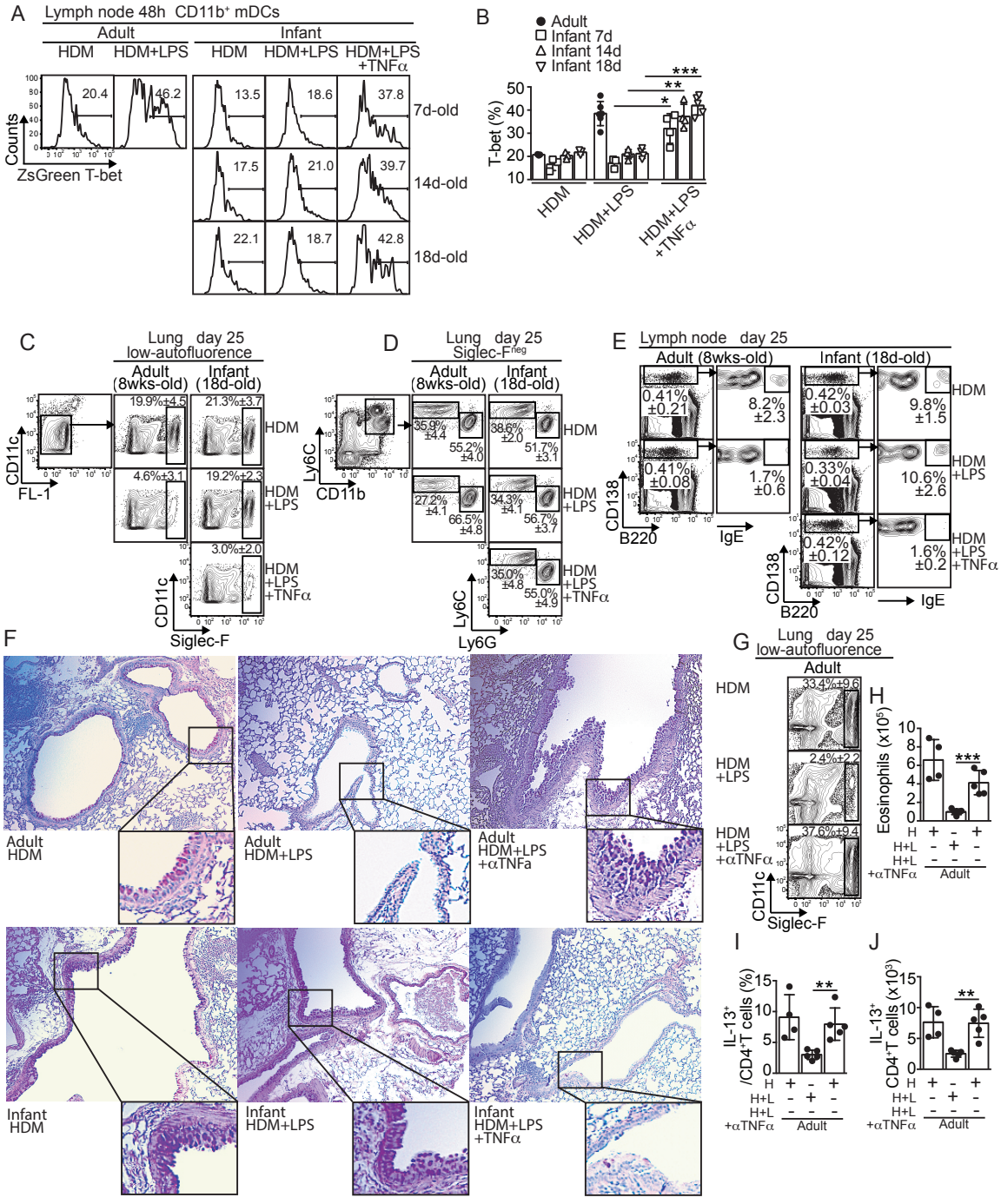


Figure S7. Related to Figure 7. Asthma features in the presence and absence of TNF-α. (**A-B**) T-bet-ZsGreen reporter adults, 7d-old neonates, 14d-old infants and 18d-old infants were i.n treated with HDM+/-5μg of LPS+/-30ng of TNF-α. Frequencies of T-bet-expressing (ZsG+) mLN CD11b$^+$ mDCs on day2. (**C-J**) B6 adult and 18d-old infant mice were i.n treated with HDM+/-5μg of LPS+/-30μg of TNF-α. Some adult mice also receive 250μg of anti-TNFα (i.p.). On day 20, mice were i.n challenged with HDM and analyzed on day 25. Frequencies of eosinophils (autofluorescencelo CD11c^{lo} Siglec-F$^+$) (**C, G**), neutrophils (Siglec-F$^-$CD11b^{hi}Ly6C^{int}) and monocytes (Siglec-F$^-$CD11b^{int}Ly6C^{hi}) (**D**) and numbers of eosinophils (**H**) in the lungs. Frequencies of IgE$^+$ ACS (**E**) in the mLN. Lung sections stained with PAS to identify globlet cells (**F**). Frequencies (**I**) and numbers (**J**) of IL-13-expressing CD4$^+$ T cells in the lungs determined by ICS after restimulation for 4 h. *P < 0.05 **P < 0.01 ***P < 0.001 (unpaired Student's t test). Data are representative of two independent experiments (mean and S.D. of 3-5 mice per group).