OX40 Ligand and Programmed Cell Death 1 Ligand 2 Expression on Inflammatory Dendritic Cells Regulates CD4 T Cell Cytokine Production in the Lung during Viral Disease

Sarah E. Wythe, Jonathan S. Dodd, Peter J. Openshaw and Jürgen Schwarze

J Immunol 2012; 188:1647-1655; Prepublished online 20 January 2012;
doi: 10.4049/jimmunol.1103001
http://www.jimmunol.org/content/188/4/1647

Supplementary Material http://www.jimmunol.org/content/suppl/2012/01/20/jimmunol.1103001.DC1

References This article cites 48 articles, 34 of which you can access for free at: http://www.jimmunol.org/content/188/4/1647.full#ref-list-1

Why The JI? Submit online.
• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Fast Publication! 4 weeks from acceptance to publication

*average

Subscription Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription

Permissions Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2012 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
OX40 Ligand and Programmed Cell Death 1 Ligand 2
Expression on Inflammatory Dendritic Cells Regulates CD4
T Cell Cytokine Production in the Lung during Viral Disease

Sarah E. Wythe,*,† Jonathan S. Dodd,* Peter J. Openshaw,* and Jürgen Schwarze*,†

CD4 Th differentiation is influenced by costimulatory molecules expressed on conventional dendritic cells (DCs) in regional lymph nodes and results in specific patterns of cytokine production. However, the function of costimulatory molecules on inflammatory (CD11b+) DCs in the lung during recall responses is not fully understood, but it is important for development of novel interventions to limit immunopathological responses to infection. Using a mouse model in which vaccination with vaccinia virus vectors expressing the respiratory syncytial virus (RSV) fusion protein (rVVF) or attachment protein (rVVG) leads to type 1- or type 2-biased cytokine responses, respectively, upon RSV challenge, we found expression of CD40 and OX40 ligand (OX40L) on lung inflammatory DCs was higher in rVVF-primed mice than in rVVG-primed mice early after RSV challenge, whereas the reverse was observed later in the response. Conversely, programmed cell death 1 ligand 2 (PD-L2) was higher in rVVG-primed mice throughout. Inflammatory DCs isolated at the resolution of inflammation revealed that OX40L on type 1-biased DCs promoted IL-5, whereas OX40L on type 2-biased DCs enhanced IFN-γ production by Ag-reactive Th cells. In contrast, PD-L2 promoted IFN-γ production, irrespective of conditions, suppressing IL-5 only if expressed on type 1-biased DCs. Thus, OX40L and PD-L2 expressed on DCs differentially regulate cytokine production during recall responses in the lung. Manipulation of these costimulatory pathways may provide a novel approach to controlling pulmonary inflammatory responses. The Journal of Immunology, 2012, 188: 1647–1655.

Upon respiratory viral challenge, conventional lung dendritic cells (DCs) mature and migrate to the regional lymph nodes where they present Ag in the context of MHC to naive Ag-specific T cells (1–3). During this period of activation, naive CD4 T cells are educated to become Th1, Th2, Th17, or regulatory T cells (Tregs) or remain part of a large uncommitted population (Th0) that can become polarized at a later stage (4). It now seems that polarization is reversible and that both human and mouse Th1 and Th2 cells maintain flexibility and can respond to alternative polarizing conditions (5–7). These conditions consist of a combination of cytokines and costimulatory signals typically provided by APCs, in particular conventional DCs (5–7). In models of allergic airway inflammation, DCs were shown to be required to initiate, as well as maintain, recall T cell responses (8).

Many costimulatory molecules expressed by DCs have been shown to be important in regulating T cells during both primary and recall responses. There is evidence that cytokine production by effector CD4 T cells is solely dependent on DC-derived costimulatory signals, such as CD40 (9). However, naive and effector T cells differ in the type of costimulation required for activation/reactivation. For example, CD80 and CD86 are critical for induction, but not reactivation, of Th2 cells in an OVA-driven mouse model of asthma (10). In contrast, OX40 ligand (OX40L) is critical for reactivation of Th2 cells in this model (11).

Inflammatory monocytes are recruited to the lung during inflammation where they mature into CD11b+ (inflammatory) DCs (12). These cells were shown to increase in numbers after primary respiratory syncytial virus (RSV) infection, associated with increased CD86 expression, which outlasted viral illness and peak inflammation (13). Recently, inflammatory DCs were shown to be important in the formation of tertiary lymphoid structures in the lung and optimal humoral immune responses after viral infection (14). However, the functional consequences of costimulatory molecule expression on inflammatory DCs for effector CD4 T cell cytokine production in type 1- and type 2-polarizing environments in the lung have not been studied. In this study, we hypothesized that inflammatory DCs provide a dominant signal in modulating Ag-specific recall responses of CD4 T cells. To address this hypothesis, we used a well-characterized mouse model of type 1- and type 2-biased responses to RSV infection. In this model, the only variable is the Ag used to sensitize prior to RSV challenge: immunization with recombinant vaccinia virus vectors expressing the RSV fusion protein (rVVF) or the RSV attachment protein (rVVG) leads to type 1- or type 2-biased responses, respectively, during subsequent RSV infection (15, 16).

We found that lung DCs matured in type 1- or type 2-biased environments differed significantly in their expression of CD40,
OX40L, and programmed cell death 1 ligand 2 (PD-L2). These differences were most pronounced 10 d after RSV challenge, at which time viral replication had passed, airway cytokine levels had returned to baseline, and the disease was resolving. However, DCs isolated from the lungs at this stage still biased Th1 cell responses according to the environment from which they came in the absence of detectable IL-12p70 in the immediate environment. The co-stimulatory molecule OX40L promoted IFN-γ production when expressed on DCs isolated from a type 2-biased environment and promoted IL-5 when expressed on DCs isolated from a type 1-biased environment. In contrast, PD-L2 was Th1 enhancing, irrespective of the environment from which the DCs came. Our data suggested that OX40L and PD-L2 expression on DCs at the time of isolation function to restore the cytokine balance in the lung. Modulation of these pathways in the airways has the potential to alleviate viral induced immunopathology.

Materials and Methods

Mice and virus stocks

Eight-week-old female BALB/c mice were purchased from Charles River Laboratories (Margate, Kent, U.K.). A DO11.10 mouse colony was established from breeding pairs donated by Prof. Claire Lloyd (Imperial College London) and bred in house. All mice were kept in specific pathogen-free conditions. RSV (A2 strain, grown on HEp2 cells following plaque purification) and recombinant vaccinia virus Western Reserve strain (rVV) expressing the RSV-fusion protein (F) or attachment protein (G) genes (or β-galactosidase [rVVβGal] as a control) in place of the thymidine kinase gene, were grown as previously described (17). All animal work was approved by the Home Office (London, U.K.).

Vaccination and RSV infection

Under anaesthesia with isoflurane, mice were scarified on the rump, and 3 × 10⁶ PFU (in 10 μl) rVV, rVVβ, or rVVβ-gal was administered. In brief, the rumps of mice were shaved, and the skin was deornified with an emery board. The viral inoculum was adsorbed to the deornified site, and the rumps of mice were shaved, and the skin was decornified with an emery board. The viral inoculum was adsorbed to the deornified site, and the mice were observed until fully recovered. Two weeks later, these mice and naive controls were infected intranasally with 5 × 10⁶ PFU (in 100 μl) RSV or were mock infected with HEp2 lysate, under anaesthesia with isoflurane, as described previously (15). Mice were weighed and monitored daily thereafter. At various time points postchallenge, mice were culled by isoflurane, as described previously (15). Mice were weighed and monitored daily thereafter. At various time points postchallenge, mice were culled by isoflurane, as described previously (15). All animal work was approved by the Home Office (London, U.K.).

Cell and tissue recovery

Bronchoalveolar lavage (BAL) was performed, as described (17), with 1 ml PBS. The BAL fluid (BALF) retrieved was centrifuged, and the supernatant was removed and stored at −80˚C prior to analysis. BALF cells were resuspended in PBS/1% BSA/0.1% sodium azide in preparation for flow cytometric analysis. Lungs were perfused with PBS via the heart prior to removal. They were then macerated and enzymatically digested by incubation with 0.1% collagenase solution for 1 h. Cells were washed with PBS, and single-cell suspensions were obtained by filtration. RBCs were lysed with ACK buffer. Live cells were enumerated by trypan blue exclusion.

Flow cytometric analysis of cell surface Ags

BALF, lung, and splenic cells were resuspended in PBS/1% BSA/0.1% sodium azide. After blocking FcRs with anti-CD16/32 Abs (BD Biosciences, Oxford, U.K.), surface stains were performed by incubating cells for 30 min at 4˚C with the appropriate combinations of the following Abs and isotype controls: anti-CD11c PE Cy5.5*(N418), hamster IgG PE Cy5.5 (550-60) (both from CaluBio, Laboratories, Burlingame, CA); anti-MHC class II (MHCI) allophycocyanin (M5/114.15.2), anti-ICOSL PE (BVD6-24G2); and for IL-5: purified (TRFK5) and biotinylated (TRFK4) rat IgG2b (eBioscience). Naive or effector CD4 T cells were then cocultured with OVA peptide-pulsed DCs for 96 h. Concentrations of IFN-γ, IL-4, IL-5, and IL-12p70 in culture supernatants were determined by ELISA.

Functional Ab blockade of OX40L and PD-L2 on DCs

DCs isolated from lungs of rVV- or rVVβ-primed mice 10 d after RSV challenge were incubated with 5 µg/ml (determined by prior titration) functional-grade blocking anti-OX40L (RM134L) and anti-PD-L2 (PJ25) Abs or the irrelevant isotype controls: rat IgG2a, and rat IgG2b (eBioscience). Naive or effector CD4 T cells were then cocultured with OVA peptide-pulsed DCs for 96 h. Concentrations of IFN-γ, IL-4, IL-5, and IL-12p70 in culture supernatants were determined by ELISA.

Cytokine ELISAs

The concentrations of IFN-γ, IL-4, and IL-5 in BALF were determined using the following paired Abs: for IFN-γ: purified (AN-18) and biotinylated (BD Biosciences, Oxford, U.K.), surface stains were performed by incubating cells for 30 min at 4˚C with the appropriate combinations of the following Abs and isotype controls: anti-CD11c PE Cy5.5*(N418), hamster IgG PE Cy5.5 (550-60) (both from CaluBio, Laboratories, Burlingame, CA); anti-MHC class II (MHCI) allophycocyanin (M5/114.15.2), anti-ICOSL PE (BVD6-24G2); and for IL-5: purified (TRFK5) and biotinylated (TRFK4) (BD Biosciences). Thymic stromal lymphopoietin (TSLP) and IL-12p70 levels were assessed using a DuoSet (R&D Systems, Abingdon, U.K.). Ninety-six–well Maxisorp plates were coated with purified capture Ab (1:500 in sodium hydrogen carbonate buffer [pH 9.6]), BD; 1:200 in PBS [pH 7.2], R&D Systems) and incubated overnight at 4˚C (step 1). Following a blocking step with PBS/BSA (1% w/v) 2 h at room temperature (RT), samples and standards were added in triplicate and incubated overnight at 4˚C (step 2). Biotinylated Abs were diluted as for primary Abs, but in PBS/BSA (1% w/v) for 2 h at room temperature (RT), samples and standards were added in triplicate and incubated overnight at 4˚C (step 2). Biotinylated Abs were diluted as for primary Abs, but in PBS/BSA (1% w/v) for 2 h at room temperature (RT), samples and standards were added in triplicate and incubated overnight at 4˚C (step 2). Biotinylated Abs were diluted as for primary Abs, but in PBS/BSA (1% w/v) for 2 h at room temperature (RT), samples and standards were added in triplicate and incubated overnight at 4˚C (step 2).
for 1 h at RT (step 3). Extravidin-HRP was diluted 1:1000 in PBS/BSA (1% w/v)/Tween 20 (0.1% v/v) diluent, and plates were incubated for 30 min at RT (step 4). o-Phenylenediamine dihydrochloride substrate was added, and plates were observed for the colorimetric reaction (step 5). The reaction was stopped using 1 N H2SO4 (step 6). Wells were washed between steps 2 and 4 with PBS/Tween 20 (0.1% v/v). OD values were determined using a spectrophotometer ( Molecular Devices) at 490 nm reference wavelength. Cytokine concentrations were calculated from the standard curves. The range for each standard curve was as follows: 8–4000 pg/ml (IFN-γ), 8–2000 pg/ml (IL-4), 8–2000 pg/ml (IL-5), 10–2500 pg/ml (IL-12p70), and 8–1000 pg/ml (TSLP).

### Statistical analysis

All statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad; San Diego, CA). The Student t test or Mann–Whitney U test was used to determine whether two groups differed significantly from each other (for parametric or nonparametric data, respectively). One-way ANOVA was used when comparing more than two groups. If the groups differed significantly, a Tukey posttest was applied to determine where those differences lie. A p value < 0.05 was considered significant.

#### Results

**Memory responses elicit earlier weight loss than primary responses upon RSV challenge**

Weight loss is a reliable marker of illness and lung pathology in this model, and it is accelerated by prior sensitizing vaccination (15). After primary infection with RSV, mice began to lose weight on day 5 postinfection, with peak weight loss occurring at day 8 post-RSV challenge (p.c.) (Fig. 1A). In comparison, uninfected and mock-infected control mice did not lose weight over the same period of time. In contrast, mice previously vaccinated with rVVF or rVVG began to lose weight from day 1 p.c. (Fig. 1B), and weight peaked at day 6 p.c. (2 d earlier than in primary infection). Mice vaccinated with rVVβGal exhibited the same kinetic of weight loss upon RSV challenge as during primary infection.

**Type 1 and 2 cytokine responses to RSV challenge after rVVF or rVVG priming**

To confirm biased cytokine responses in primed mice, we measured the concentrations of IFN-γ, IL-4, IL-5, IL-12p70, and the Th2-inducing epithelial-derived cytokine TSLP (21) (Fig. 2D) in the BALF after RSV challenge. No IL-12p70 was detected in BALF in any of the groups over the duration of the response (data not shown). In mice vaccinated with rVVβGal, IFN-γ became detectable on day 4, peaked on day 7, and returned to baseline by day 10 p.c. IFN-γ responses measured in the BALF were accelerated and enhanced in mice primed with rVVF and rVVG; on days 4 and 7 p.c., the concentration of IFN-γ in the BALF was significantly greater in rVVF-vaccinated mice than in rVVG-vaccinated mice (p < 0.01) (Fig. 2A). By contrast, IL-4, which was detectable in both rVVF- and rVVG-vaccinated mice, but not in rVVβGal-vaccinated mice, was found at significantly higher levels in rVVF-vaccinated mice on day 4 p.c. (Fig. 2B). Together, these findings indicated type 1 bias after rVVF priming and type 2 bias after rVVG priming. IL-5 was detectable in all three groups. IL-5 levels peaked on day 7 p.c. at ~75 pg/ml in rVVF-vaccinated mice and at ~50 pg/ml in rVVβGal-vaccinated mice. IL-5 levels in rVVG-vaccinated mice peaked earlier (day 4 p.c.) and were significantly increased relative to other groups (~150 pg/ml), again indicating type 2 bias (Fig. 2C).

BALF TSLP levels were measured at 30–40 pg/ml prior to RSV challenge in all mice, probably representing basal production from the lung epithelia (22) (Fig. 2D). After RSV infection, TSLP decreased steadily in both the rVVβGal- and rVVF-vaccinated groups to ~10 pg/ml by day 10 p.c. However, after an initial decline, the concentration of TSLP in the BALF of rVVG-vaccinated mice increased to 50 pg/ml on day 4 p.c. (Fig. 2D).

To demonstrate a functional consequence of type 2 bias, eosinophil recruitment to the BALF was determined by flow cytometry at the peak of inflammation (Fig. 2E, 2F). On day 7 p.c., eosinophils constituted 13% of total BALF cells (23.7 ± 4.8 × 104 cells/ml) in the rVVG-vaccinated mice compared with <3% (4.7 ± 0.7 × 104 cells/ml) and 2% (0.7 ± 0.1 × 104 cells/ml) in mice primed with rVVF or rVVβGal, respectively (Fig. 2E, 2F). This confirmed a functional type 2 immune response in rVVG-primed mice that was not detected in the other groups.

**Type 2 biased cytokine responses to RSV challenge after rVVF or rVVG priming**

To confirm biased cytokine responses in primed mice, we measured the concentrations of IFN-γ, IL-4, IL-5, IL-12p70, and the Th2-inducing epithelial-derived cytokine TSLP (21) (Fig. 2D) in the BALF after RSV challenge. No IL-12p70 was detected in BALF in any of the groups over the duration of the response (data not shown). In mice vaccinated with rVVβGal, IFN-γ became detectable on day 4, peaked on day 7, and returned to baseline by day 10 p.c. IFN-γ responses measured in the BALF were accelerated and enhanced in mice primed with rVVF and rVVG; on days 4 and 7 p.c., the concentration of IFN-γ in the BALF was significantly greater in rVVF-vaccinated mice than in rVVG-vaccinated mice (p < 0.01) (Fig. 2A). By contrast, IL-4, which was detectable in both rVVF- and rVVG-vaccinated mice, but not in rVVβGal-vaccinated mice, was found at significantly higher levels in rVVF-vaccinated mice on day 4 p.c. (Fig. 2B). Together, these findings indicated type 1 bias after rVVF priming and type 2 bias after rVVG priming. IL-5 was detectable in all three groups. IL-5 levels peaked on day 7 p.c. at ~75 pg/ml in rVVF-vaccinated mice and at ~50 pg/ml in rVVβGal-vaccinated mice. IL-5 levels in rVVG-vaccinated mice peaked earlier (day 4 p.c.) and were significantly increased relative to other groups (~150 pg/ml), again indicating type 2 bias (Fig. 2C).

BALF TSLP levels were measured at 30–40 pg/ml prior to RSV challenge in all mice, probably representing basal production from the lung epithelia (22) (Fig. 2D). After RSV infection, TSLP decreased steadily in both the rVVβGal- and rVVF-vaccinated groups to ~10 pg/ml by day 10 p.c. However, after an initial decline, the concentration of TSLP in the BALF of rVVG-vaccinated mice increased to 50 pg/ml on day 4 p.c. (Fig. 2D).

To demonstrate a functional consequence of type 2 bias, eosinophil recruitment to the BALF was determined by flow cytometry at the peak of inflammation (Fig. 2E, 2F). On day 7 p.c., eosinophils constituted 13% of total BALF cells (23.7 ± 4.8 × 104 cells/ml) in the rVVG-vaccinated mice compared with <3% (4.7 ± 0.7 × 104 cells/ml) and 2% (0.7 ± 0.1 × 104 cells/ml) in mice primed with rVVF or rVVβGal, respectively (Fig. 2E, 2F). This confirmed a functional type 2 immune response in rVVG-primed mice that was not detected in the other groups.

**Increases in inflammatory DC numbers following RSV challenge are not affected by type 1- or type 2-biased environments**

To examine whether priming with different Ags caused a change in the magnitude of the inflammatory DC (MHCII+CD11c+CD11b+) response, we enumerated lung inflammatory DCs during the course of RSV infection. The number of inflammatory DCs (23) increased ≥8-fold postinfection, peaking at 1.2–1.6 × 106 cells/lung on day 7 p.c. (Fig. 3). Vaccination seemed to slightly delay the peak of DC numbers to day 10 p.c., but it did not significantly affect the absolute numbers of DCs at any time point.

**DC costimulatory molecule expression differs between type 1- and type 2-biased environments**

We next tested whether inflammatory DCs expressed a different repertoire of costimulatory molecules in different environments. The percentage of DCs expressing costimulatory molecules was generally greater in vaccinated compared with mock-vaccinated (rVVβGal) mice. Prior to RSV challenge, <30% of DCs expressed the costimulatory molecules CD80/86, commonly associated with maturation (data not shown); <10% expressed CD40 (Fig. 4A), <5% expressed OX40L (Fig. 4B), and <20% expressed PD-L2 (Fig. 4C). In contrast, >40% expressed ICOSL, and >60%
expressed PD-L1 (data not shown). There were no significant differences in costimulatory molecule expression between the groups prior to RSV infection. Postinfection, the percentage of DCs expressing CD80/86 increased to 60%, and those expressing ICOSL and PD-L1 increased to 70 and 80%, respectively, by day 10 p.c. There were no significant differences between rVVF- and rVVG-vaccinated mice (data not shown).

In contrast, CD40, OX40L, and PD-L2 were differentially expressed following RSV challenge (Fig. 4, Supplemental Fig. 1). By day 2 p.c., CD40 expression increased to 36 ± 2.7% of lung DCs in mice vaccinated with rVVF, significantly more than in the other two groups (Fig. 4A, Supplemental Fig. 1). Expression then decreased to 15.6 ± 1.6% of lung DCs by day 7 p.c. and remained at similar levels thereafter. This contrasted with rVVG-vaccinated mice, in which CD40 expression increased more slowly and reached 25.0 ± 3.2% on day 7 p.c. At this time point, there were significantly more CD40+ DCs in the lungs of rVVG-vaccinated mice (p < 0.01) than in the other groups, and this difference was maintained at day 30 p.c.

There were similar kinetics of expression for OX40L (Fig. 4B, Supplemental Fig. 1), peaking on day 2 p.c. in rVVF-vaccinated mice, followed by decreased expression, and a delayed peak expression on day 10 p.c. in rVVG-vaccinated mice. The percentage of OX40L+ DCs also remained significantly higher in rVVG-vaccinated mice up to day 30 p.c. In contrast, the percentage of PD-L2+ DCs was significantly higher in rVVG-vaccinated mice (p < 0.01) at all time points assessed, peaking on day 7 p.c. at 70.0 ± 3.4% of lung DCs in rVVG-vaccinated mice compared with 40.0 ± 0.4% in rVVF-vaccinated mice (Fig. 4C, Supplemental Fig. 1).

We also measured the known ligands for the costimulatory molecules CD40L, OX40, and PD1 on CD4 T cells in the lung. CD40L (Supplemental Fig. 4A) and PD1 (Supplemental Fig. 4B) increased after RSV challenge, peaking on day 7, with significantly more CD4 T cells expressing CD40L and PD1 in rVVG-primed mice compared with rVVF-primed mice (p < 0.001). However, by day 10 p.c., both CD40L and PD1 were undetectable. In contrast, OX40 expression peaked on day 10 p.c. in both rVVG- and rVVF-vaccinated mice, and there were no significant differences between the two groups at this time point (Supplemental Fig. 4A). Interestingly, on day 30 p.c., CD40L and PD1, but not OX40, were again expressed on CD4 T cells, with significantly more CD4 T cells expressing CD40L and PD1 in rVVG-primed mice compared with rVVF-primed mice (p < 0.001).

Isolated lung DCs from type 1- and type 2-biased environments are viable and induce biased cytokine responses in naive and effector CD4 T cells

Having shown that the immune environment in which inflammatory DCs mature programs them to express a specific repertoire of costimulatory molecules, we investigated whether these DCs can regulate cytokine production by CD4 T cells. To specifically investigate the role of DC-expressed costimulatory molecules on CD4 T cells, we isolated viable, functional inflammatory DCs by sorting MHCIIhi, CD11c+, F4/80lo, CD11b+ cells (Fig. 5A); in these cells, viability was consistently >95%, and sorted cells developed dendrites in response to LPS stimulation (not seen on alveolar macrophages under the same conditions) (Fig. 5B). Next, we assessed the ability of these DCs pulsed with OVA peptide to express PD-L1 (data not shown). There were no significant differences in costimulatory molecule expression between the groups prior to RSV infection. Postinfection, the percentage of DCs expressing CD80/86 increased to 60%, and those expressing ICOSL and PD-L1 increased to 70 and 80%, respectively, by day 10 p.c. There were no significant differences between rVVF- and rVVG-vaccinated mice (data not shown).

In contrast, CD40, OX40L, and PD-L2 were differentially expressed following RSV challenge (Fig. 4, Supplemental Fig. 1). By day 2 p.c., CD40 expression increased to 36 ± 2.7% of lung DCs in mice vaccinated with rVVF, significantly more than in the other two groups (Fig. 4A, Supplemental Fig. 1). Expression then decreased to 15.6 ± 1.6% of lung DCs by day 7 p.c. and remained at similar levels thereafter. This contrasted with rVVG-vaccinated mice, in which CD40 expression increased more slowly and reached 25.0 ± 3.2% on day 7 p.c. At this time point, there were significantly more CD40+ DCs in the lungs of rVVG-vaccinated mice (p < 0.01) than in the other groups, and this difference was maintained at day 30 p.c.

The mice were vaccinated with rVVF, rVVG, or rVVβGal. Fourteen days later, mice were challenged with RSV intranasally. Mice were sacrificed, and BALF was harvested at various time points postchallenge. BALF concentrations of IFN-γ (A), IL-4 (B), IL-5 (C), and TSLP (D) were determined by ELISA. E, BALB eosiophilia was determined by flow cytometry. Top panel, BALF granulocytes: forward scatter 360-760/ B220 /CD3 . Bottom panel, Eosinophils: CD3+ B220+ MHCII+ CCR3+; F, Eosinophils as percentage of total BALF cells. Data in A–E are representative of three independent experiments (n ≥ 5/group) and show means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, rVVF versus rVVG, ANOVA (Tukey posttest).
bias naive and effector CD4 T cells. Naive OVA-specific CD4 T cells were isolated from the spleens of DO11.10 mice (>91% pure) (Supplemental Fig. 2A, 2B). DCs pulsed with an irrelevant peptide did not induce any cytokine production by CD4 T cells from DO11.10 mice (Fig. 5C–E), but OVA peptide-pulsed DCs from both rVVF- and rVVG-vaccinated mice induced IFN-γ production by DO11.10 T cells. This was significantly increased if DCs were obtained from rVVF-primed mice (Fig. 5C). Conversely, DCs isolated from rVVG-vaccinated mice induced significantly greater levels of IL-5 (Fig. 5D), OVA peptide-pulsed DCs from both rVVF- and rVVG-vaccinated mice induced IL-4 production from DO11.10 T cells (Fig. 5E). Subsequent coculture experiments were carried out using an OVA peptide concentration of 10 μM.

Because inflammatory (CD11b+) DCs have been shown to form close contacts with effector CD4 T cells in organized lymphoid tissue in the lung after viral infection, we asked whether these DCs can influence cytokine production by nonnaive Ag-reactive cells. Ag-reactive CD4 T cells with an intermediate Th1/Th2 profile were generated by culturing whole splenocyte populations from OVA-sensitized mice in the presence of OVA peptide (24). CD4 T cells were then isolated by immunomagnetic separation (>97% pure) (Supplemental Fig. 2C, 2D) and cocultured with DCs. No cytokine production was detected in the presence of an irrelevant peptide (data not shown). With OVA peptide, IL-12p70 was below the level of detection (data not shown), and only low concentrations of IL-4 (<10 pg/ml) (Fig. 5H) could be measured. In contrast, IFN-γ (Fig. 5F) and IL-5 (Fig. 5G) were detected, with a trend toward increased IFN-γ production when CD4 T cells were cocultured with rVVF-DCs and increased IL-5 levels when CD4 T cells were cocultured with rVVG-DCs.

**OX40 and PD1 are absent on naive DO11.10 CD4 T cells but are up-regulated on Ag-activated CD4 T cells**

Having shown differential upregulation of OX40L and PD-L2 on inflammatory lung DCs, which cause biased cytokine responses in coculture with naive and Ag-activated CD4 T cells, we then assessed whether their known ligands were expressed on these T cells. Less than 1% of naive CD4 T cells expressed OX40 (the true ligand for OX40L, and <2% expressed PD1 (the only identified ligand for PD-L2) (Supplemental Fig. 3Aii). In contrast, ~10% of splenic CD4 T cells from OVA-sensitized mice expressed OX40, and 2% expressed PD1. Following 5 d of Ag stimulation in vitro, the percentage of CD4 T cells expressing OX40 and PD1, individually or in combination, increased to >40% of the total CD4 T cell population (Supplemental Fig 3Biv). In vivo, 10 d post-RSV challenge, ~14 × 10⁴ CD4 T cells in the lung expressed OX40 compared with ~3 × 10⁴ cells at

**FIGURE 3.** Inflammatory DC number in the lungs is not affected by type 1- or type 2-biased environments. BALB/c mice were vaccinated by scarification of the rump with rVVF, rVVG, or rVVbGal and challenged with RSV. A, Mice were sacrificed on days 0, 2, 7, 10, and 30 p.c., and the number of inflammatory DCs in the lung was determined. B, The percentage of inflammatory DCs (CD11c⁺ MHCII⁺ CD11b⁺) in the lung expressing CD40 (A), OX40L (B), and PD-L2 (C) was determined by flow cytometry. Representative dot plots are shown in Supplemental Fig. 1. Data are representative of two independent experiments (n ≥ 5/group) and show means ± SD.

**FIGURE 4.** DC costimulatory molecule expression differs between type 1- and type 2-biased environments. BALB/c mice were vaccinated by scarification of the rump with rVVF, rVVG, or rVVbGal and challenged with RSV. Mice were sacrificed, and the lungs were harvested on days 0, 2, 7, 10 and 30 p.c. The percentage of inflammatory DCs (CD11c⁺ MHCII⁺ CD11b⁺) in the lung expressing CD40 (A), OX40L (B), and PD-L2 (C) was determined by flow cytometry. Representative dot plots are shown in Supplemental Fig. 1. Data are representative of two independent experiments (n ≥ 5/group) and show means ± SD. **p < 0.01, ***p < 0.001, rVVF versus rVVG, ANOVA (Tukey posttest).
baseline, thus confirming the possibility for OX40–OX40L interaction in both rVVF- and rVVG-primed mice in the lung at this time point. In contrast, PD1 and CD40L peaked at day 7 p.c. but were absent at day 10 p.c. (Supplemental Fig. 4).

**PD-L2 promotes IFN-γ production, but OX40L counterregulates cytokine production by effector CD4 T cells**

Using functional Ab blockade of OX40L and PD-L2 in these cocultures, we then investigated how these differentially expressed costimulatory molecules regulate cytokine production by CD4 T cells. In cocultures with DO11.10 CD4 T cells, which were stimulated with a full peptide dose-response curve, no significant effect of OX40L or PD-L2 blockade on cytokine production was observed (data not shown). In cocultures with effector CD4 T cells, blocking OX40L on rVVG-DCs significantly reduced IFN-γ production ($p < 0.05$) (Fig. 6Ai), but it had no effect on IL-5 production (Fig. 6Bii). Conversely, blocking OX40L on rVVF-DCs did not affect IFN-γ (Fig. 6Ai), but it significantly reduced IL-5 production (Fig. 6Bi). In contrast, blocking PD-L2 significantly reduced IFN-γ concentrations, irrespective of the DCs used to activate effector CD4 T cells (Fig. 6C). It also significantly enhanced IL-5 levels but only in cocultures with rVVF-DCs and not rVVG-DCs (Fig. 6Di). IL-4 levels were not changed by blocking of either costimulatory molecule (data not shown).

**Discussion**

In this study, we showed that CD40, OX40L, and PD-L2 expression on lung DCs differ both in timing and magnitude during type 1- and type 2-biased immune responses to RSV infection and that these molecules modulate cytokine production by effector, but not naive, CD4 T cells. We confirmed the previously reported role for PD-L2 in promoting IFN-γ production, but showed that OX40L counterregulates ongoing cytokine production by effector CD4 T cells. This study highlighted these two molecules as strong candidates for novel therapeutic targets to modulate CD4 T cell responses to respiratory pathogens and potentially other infectious agents.

**FIGURE 5.** Isolated lung DCs from type 1- and type 2-biased environments are viable and induce polarized cytokine responses in naive and effector CD4 T cells. BALB/c mice were vaccinated by scarification of the rump with rVVF or rVVG and challenged with RSV. On day 10 p.c., mice were sacrificed, and lungs were harvested. A, Cells were pooled from the mice within each group, and DCs were isolated from a CD11c-enriched population by FACS sorting of CD11c+MHCIIhi cells. Top panel, CD11c-enriched cells pre-FACS sorting (left) and post-FACS sorting (right). Bottom panel, Expression of F4/80 on FACS-sorted cells (left) and expression of CD11b on FACS-sorted cells (right). B, Isolated lung DCs and macrophages obtained from the BALF of naive mice were cultured in the presence of LPS for 24 h, stained with H&E, and imaged under phase microscopy (original magnification ×40). The arrow highlights dendrite formation. Naive CD4 T cells were isolated from the spleens of DO11.10 mice (Supplemental Fig. 2). A total of $1 \times 10^4$ DCs/well were pulsed for 1 h with increasing concentrations of OVA peptide (323–339) or an irrelevant peptide control (amyloid β residues 25–35) and then cocultured with $1 \times 10^5$ cells/well for 96 h. The supernatants were assayed for IFN-γ (C), IL-5 (D), and IL-4 (E). A total of $1 \times 10^5$ effector CD4 T cells was cocultured with $1 \times 10^5$ DCs pulsed with OVA peptide or an irrelevant peptide control (10 μM) for 96 h. The supernatants were assayed for IFN-γ (F), IL-5 (G), and IL-4 (H). Data in C–E are representative of five independent experiments and show means ± SD. *$p < 0.05$, **$p < 0.001$, OVA peptide-pulsed rVVF-DCs versus rVVG-DCs, ANOVA (Tukey posttest).
were determined by ELISA. Data are representative of two independent
the relevant isotype controls. IFN-

m from rVVF-primed (i) or rVVG-primed (ii) mice was pulsed for 1 h with 10 µM OVA peptide (323–339) and cocultured with 10^5 effector CD4 T cells for 96 h in the presence of anti-OX40L (A, B), anti–PD-L2 (C, D), or the relevant isotype controls. IFN-γ (A, C) and IL-5 (B, D) concentrations were determined by ELISA. Data are representative of two independent experiments and show means ± SD. *p < 0.05, **p < 0.01, Student t test.

It is known that DCs play a critical role in both activation and
reactivation of T cells and can deliver polarizing signals to T cells
mediated by cytokines or costimulatory molecules. Of the costimulatory molecules that we investigated, only CD40, OX40L, and PD-L2 differed in their expression between the two environments. Ligation of CD40 was shown to be critical for full activation and function of DCs, and it has also been closely associated with the induction of Th1 responses through increasing IL-12p70 production (25, 26). Although, in this study, CD40 expression was significantly increased in the type 1 environment on day 2 postchallenge, no IL-12p70 could be detected in the airways, which may suggest a dominant role for costimulatory molecules in T cell polarization. In support of this, inflammatory DCs isolated from type 1-biased environments induced Ag-specific IFN-γ responses in both naive and effector CD4 T cells without detectable IL-12p70 in the immediate culture environment. Interestingly, effector T cells produced much less IFN-γ than did naïve T cells. Because IL-5 levels were comparable, this was not due to the cells being Th2 biased. One possible explanation is that the reduction in IFN-γ occurred as the result of repeated stimulation of the cells with peptide; alternatively, it could be due to the reduced percentage of Ag-specific CD4 T cells in the effector T cell cocultures (27).

To delineate functional roles for OX40L and PD-L2 on lung DCs in viral inflammation, these molecules were blocked with Abs during coculture with CD4 T cells. Because CD40 is critical for DC activation, it would not constitute a good therapeutic target; therefore, we focused on delineating the function of OX40L and PD-L2. Blocking these molecules had no effect on cytokine production by naïve CD4 T cells. We sought to explain this by assessing whether they expressed ligands for OX40L and PD-L2. OX40L binds exclusively to OX40 to exert its functional effects (28). In contrast, although the only identified ligand for PD-L2 is PD1, PD-L2 was shown to exert T cell-stimulatory functions through binding an as-yet-unidentified ligand (29, 30). We could detect OX40 and PD1 on effector, but not naïve, T cells, suggesting that the lack of an effect of blocking OX40L and PD-L2 on DCs in cocultures with naïve CD4 T cells could be attributed to an absence of the ligands for these molecules. These results suggested that the factors other than those measured in this study are responsible for the observed bias in cytokine production from naïve CD4 T cells.

In contrast to naïve CD4 T cells, PD-L2 blockade on inflammatory DCs in coculture with effector CD4 T cells reduced IFN-γ production, irrespective of the environment from which the DC came, and it increased IL-5 production if blocked on DCs from a type 2 environment, indicating Th1-promoting effects of PD-L2. Our findings supported those of other studies that showed PD-L2 is upregulated on DCs in response to Th2 cytokines (31) but serves to downregulate them. For example, in a model of allergic airway inflammation, adoptive transfer of DCs activated through PD-L2 into sensitized mice abrogated IL-4 production by T cells and completely protected the mice from allergic airway inflammation upon allergen challenge (32). Further, in a mouse model of leishmaniasis, in which host resistance is associated with Th1 responses and downregulation of Th2 responses, PD-L2 deficiency resulted in exacerbated disease and increased parasite burden, indicative of an increased Th2 response (33).

In contrast to PD-L2, blockade of OX40L on DCs from a type 2 environment in coculture with effector CD4 T cells reduced the production of IFN-γ, whereas if blocked on DCs from a type 1 environment, OX40L reduced IL-5 production. This may be explained by the finding that OX40–OX40L interactions can influence the immediate cytokine environment that can then influence Th differentiation. For example, although OX40 engagement preferentially leads to generation of Th2 cells, which can be driven by autocrine IL-4 and related to enhanced calcium/NFATc signaling (21, 34–36), in the presence of IL-12 and IFN-α, OX40–OX40L interactions lead to the induction of Th1 responses (21, 37, 38). It also should be noted that reverse signals through OX40L result in the production of proinflammatory cytokines, including IL-12, IL-6, IL-1, TNF, and IFN-α (39–41), from DCs, potentially through alterations in downstream signaling involving PKCζ, c-Jun, and c-Fos (42).

Therefore, we speculated that, in our system, modulation of inflammatory DC phenotype in vivo biases the DCs to secrete different cytokines upon OX40L engagement, which then influences the cytokine profile of the T cells following OX40–OX40L interaction. However, an alternative hypothesis is that reverse signals from OX40L engagement result in cytokine secretion into the immunological synapse, leading to differences in T cell cytokine production. Our data showed that OX40–OX40L interaction on rVVF-primed DCs promoted IL-5 production, whereas on rVVG-primed DCs it promoted IFN-γ. These data supported our hypothesis that, at the time of DC isolation, the DCs are functioning to restore the cytokine balance in the lung. To our knowledge, our study is the first to describe such a regulatory role for OX40L. Expression of OX40 was also measured on lung DC T cells following RSV challenge. Expression of OX40 peaked on day 10 p.c. in both rVVF- and rVVG-primed mice, confirming the possibility of OX40–OX40L engagement between CD4 T cells and inflammatory DCs in the lung. However, PD1 was absent at
this time. The absence of PD1 in vivo does not contradict our hypothesis because, as previously mentioned, PD-L2 is most likely binding another ligand by which it exerts its T cell-stimulatory functions.

Other studies investigated the role of OX40–OX40L interactions in the induction of immune responses, be they primary or memory (43–45). For example, induction of a primary Th2 response by TSLP-activated human monocyte-derived DCs was shown to occur in an OX40L-dependent manner (21). Moreover, in a mouse model of chronic Leishmania major infection, blocking OX40L on DCs reduced Th2 cytokines and anti-L. major IgG1 and IgE (46).

In contrast, in the current study, we investigated the role of OX40L expression on DCs after viral clearance and at the beginning of resolution of pulmonary inflammation when inflammatory DC numbers were at their greatest. We showed that, in this phase of the response to RSV, OX40L expression on inflammatory DCs provides a negative-feedback signal, irrespective of the DCs’ cytokine bias. The early expression of OX40L with CD40 on DCs in type 1-biased responses to RSV, taken together with their delayed expression in type 2-biased responses, may indicate that, during active viral infection, the function of inflammatory DCs is to stimulate antiviral effector T cell functions, which can be suppressed or delayed by the presence of type 2 cytokines. However, after viral clearance, the function of these DCs changes from one of activation to one that aids resolution of inflammation and formation of optimal protective immunological memory. A recent study showed that, 10 d after viral challenge to the lung, inflammatory DCs are found in tertiary lymphoid organs (TLOs) in the lung in close contact with effector T and B cells (14). The interactions between inflammatory DCs and B cells within these TLOs were shown to be essential for optimal antiviral humoral responses. Although the precise function of inflammatory DCs within TLOs has not been studied, it has been suggested for a long time that DCs play an important role in terminating T cell responses both directly, through interactions with negative regulators of T cell activation, such as PD1 (47) and CTLA-4, as well as indirectly, through induction of Treg responses (48). Our data support the hypothesis that the function of DCs and their costimulatory molecules is time and location dependent.

Therefore, we speculate that, after prior antigenic exposure and then airway pathogen challenge, Ag-specific memory CD4 T cells are recruited to the lung and interact with DCs, which serve to reactivate their effector function, enabling them to secrete a previously determined cytokine profile. This initial wave of T cell responses biases the cytokine environment, which, in turn, feeds back onto both resident and recruited inflammatory DCs, resulting in upregulation of a specific repertoire of costimulatory molecules that further shapes antiviral T cell responses. Once the virus has been cleared, inflammatory DCs become essential in the formation of TLOs, where they are in close contact with effector T and B cells in the lung. At this stage, we speculate that DC expression of OX40L (independently of the cytokine environment) and PD-L2 (in a dominant type 2 environment) works with effector CD4 T cells to restore the cytokine balance in the lung. This neutralization of the dominant cytokine profile may inhibit ongoing inflammatory responses and prevent further cell recruitment, thereby promoting disease resolution.

It had not been demonstrated previously that costimulatory molecules expressed on DCs isolated from type 1- or type 2-biased environments can influence the cytokine production of activated naive and effector CD4 T cells without the addition of polarizing cytokines into their immediate environment. Therefore, these novel findings demonstrated the central role of DC-expressed costimulatory ligands in governing CD4 T cell effector function. Because effector CD4 T cells exhibit functional plasticity in their ability to produce cytokines (5–7), our observations support the hypothesis that costimulatory molecules on DCs determine and modify the quality of T cell cytokine production in ongoing recall responses. This study highlights a new mechanism by which DCs control effector T cell responses during established inflammation in the lung. Thus, our study suggests that manipulation of OX40L and PD-L2 costimulatory pathways has the potential to reduce T cell-dependent immunopathology. Such manipulation could provide the basis for novel immunomodulatory treatments for respiratory disease, as well as for other inflammatory conditions in which an imbalance of CD4 T cell-derived cytokines is responsible.

Acknowledgments

We thank Dr. Cecilia Johansson for critical review of this manuscript and Prof. Brigitte Askonas for advice and guidance.

Disclosures

The authors have no financial conflicts of interest.

References


