The Innate Pulmonary Granuloma

Characterization and Demonstration of Dendritic Cell Recruitment and Function

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Granulomas are innate sequestration responses that can be modified by superimposed acquired immune mechanisms. The present study examined the innate stage of pulmonary granuloma responses to bead-immobilized Th1- and Th2-inducing pathogen antigens (Ags), *Mycobacteria bovis* purified protein derivative (PPD) and *Schistosoma mansoni* soluble egg Ags (SEA). Compared to a nonpathogen Ag, PPD and SEA bead elicited larger lesions with the former showing accelerated inflammation. Temporal analyses of cytokine and chemokine transcripts showed all Ag beads induced tumor necrosis factor- α mRNA but indicated biased interleukin (IL)-1, IL-6, and IL-12 expression with PPD challenge. All beads elicited comparable levels of CXCL9, CXCL10, CCL2, CCL17, and CCL22 mRNA, but PPD beads caused biased CXCL2, CXCL5, CCL3, and CCL4 expression whereas both pathogen Ags induced CCL7. Immunohistochemical, electron microscopic, and flow cytometric analyses showed that Ag beads mobilized CD11c⁺ dendritic cells (DCs) of comparable maturation. Transfer of DCs from PPD Ag-challenged lungs conferred a Th1 anamnestic cytokine response in recipients. Surprisingly, transfer of DCs from the helminth SEA-challenged lungs did not confer the expected Th2 response, but instead rendered recipients incapable of Ag-elicited IL-4 production. These results provide *in vivo* evidence that lung DCs recruited under inflammatory conditions favor Th1 responses and alternative mechanisms are required for Th2 commitment. (Am J Pathol 2004; 164:1021–1030)

Innate immunity has been increasingly recognized as a critical stage of the immune response. It provides initial host resistance to pathogens and determines the nature of subsequent immune events, thereby ultimately affect-

ing host morbidity and mortality.^{1,2} Granulomas are an overlooked type of innate immunity that can be modified by acquired immunity. These focal inflammatory lesions represent a primitive sequestration response observed in a wide variety of conditions. Those of the anamnestic, hypersensitivity type, involve superimposed antigen (Ag)-specific T-cell participation. Dendritic cells (DCs) are well-recognized components of granulomas and are thought to play a role in the generation and/or regulation of these lesions.^{3–7} Thus, granulomas are likely sites where innate and acquired immune responses merge.

DCs are critical participants in the innate response through their ability to link innate and acquired immunity.⁸ These Ag-presenting cells have been the subject of intense investigation and are known to recognize pathogen-associated molecular patterns by way of Toll-like receptors as well as provide co-stimulatory signals to Ag-specific T lymphocytes.⁹ Presently, there remains controversy regarding the mechanisms by which DCs are recruited to sites of infection and the means by which they shape the nature of acquired T-cell responses. In addition, because of their limited numbers, the study of DCs has depended heavily on DCs generated in culture, which likely do not fully reflect *in vivo* functions. A means to systematically study DC biology *in vivo* could significantly advance our understanding of innate responses and their relationship to acquired immunity. As potential sites of DC recruitment, granulomas might provide an ideal means to study DC biology.

We previously described models of polarized Th1 and Th2 cell-mediated anamnestic pulmonary granulomas in sensitized mice challenged with Ag-coated agarose beads.^{10–12} These highly synchronized models have allowed detailed analyses of cellular and molecular biological events involved in anamnestic T-cell-mediated gran-

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uloma formation. In the present study, we wished to determine whether a similar approach could be used to study critical events in the innate granulomatous response to mycobacterial and helminthic Ags represented by *Mycobacteria bovis* purified protein derivative (PPD) and *Schistosoma mansoni* soluble egg Ags (SEA). Respectively, these Ags induce Th1- and Th2-acquired immunity; therefore analysis of the innate phase of the response might help elucidate mechanisms directing polarized immune responses. In this report, we provide a comprehensive characterization of the innate Ag bead granuloma with special focus on DC recruitment and function. We demonstrate that Ag bead challenge invokes recruitment of MHCII+CD11c+ DCs. Furthermore different Ags invoked different patterns of cytokine and chemokine production with the potential to mobilize and activate DCs.

In draining lymph nodes, PPD bead challenge directly elicited a Th1 cytokine pattern, whereas SEA beads induced Th2 polarization in a two-stage manner with an initial Th0-like period as described for intact parasite eggs,¹³ therefore it was predicted that DCs from bead granulomas might similarly initiate polarized responses. Unexpectedly, DCs from PPD and SEA innate granulomas had similar phenotypes with regard to surface markers and cytokine expression. Transfer of PPD bead DCs conferred the predicted anamnestic Th1 response in naïve recipients, but surprisingly, SEA bead DCs failed to confer Th2 characteristics. Thus unlike that reported for resident lung DCs,^{14–16} granuloma DCs impaired Th2 commitment suggesting that SEA-induced Th2 commitment requires additional or alternative mechanisms. This study represents the first comprehensive analysis of innate pulmonary granuloma formation and the first *in vivo* analysis using directly transferred Ag-presenting DCs recruited during pathogen Ag-elicited inflammation in the lung.

Materials and Methods

Mice

Female, 8- to 10-week-old, CBA mice were obtained from Jackson Laboratories, Bar Harbor, ME, and maintained under specific pathogen-free conditions. The University of Michigan Committee on Use and Care of Animals approved all animal studies.

Antigen Bead Challenge and DC Transfer Protocols

Primary innate lung inflammatory responses were elicited in naïve mice by intravenous injection of 6000 Sepharose 4B beads coupled to *M. bovis* purified protein derivative (PPD) (Department of Agriculture, Veterinary Division, Ames, IA) or to *S. mansoni* soluble schistosoma egg Ags (SEA) (the World Health Organization, Geneva, Switzerland), as described.¹⁰ Bovine serum albumin (BSA) (Sigma, St. Louis, MO) was coupled to beads as a control nonpathogenic Ag challenge. For secondary responses,

mice were rechallenged with respective Ag beads 16 days after primary injection. In DC-priming studies, CD11c+ DCs were isolated as below 3 days after primary bead injection and 2×10^5 DCs were adoptively transferred to each naïve recipient intravenously. Ten days later recipient mice were challenged by tail vein injection with respective Ag beads. Replicates consisted of independent experiments each using three to five mice per group.

Morphometry and Transmission Electron Microscopy

Granulomas were measured blindly from formalin-inflated lungs that were paraffin-embedded, sectioned, and then stained with hematoxylin and eosin. Granuloma area was measured by computerized morphometry. A minimum of 20 lesions was measured per lung. For transmission electron microscopy, lungs were inflated with 2.5% glutaraldehyde and then submitted for standard osmium fixation and plastic embedding. Thin sections were placed on grids and examined using an LEO electron microscope (Carl Zeiss, LEO Electron Microscopy, Thornwood, NY).

Immunohistochemistry

Frozen tissue sections (5 to 7 μ m thick) were mounted on poly-L-lysine-coated slides, fixed with acetone, and then rehydrated in phosphate-buffered saline (PBS). Sections were pretreated 10 minutes with 0.03% H₂O₂ and then avidin and biotin. The sections were covered with biotinylated 10 μ g/ml of hamster anti-mouse CD11c monoclonal antibody (BD Pharmingen, San Diego, CA). Biotinylated nonimmune hamster IgG served as a control. Slides were incubated for 1 hour at room temperature, then rinsed and overlaid with a 1- μ g/ml streptavidin peroxidase (Sigma), and incubated another 30 minutes at room temperature. The slides were rinsed again in PBS and overlaid with a 1:20 dilution of AEC staining solution (Sigma) for 20 minutes at room temperature. Finally, the sections were rinsed, counterstained with Gill's hematoxylin, and mounted with coverslips.

CD11c+ DC Isolation from Lungs and Granulomas

After perfusion with cold RPMI 1640, lungs were excised, placed in cold RPMI 1640 medium, and then homogenized in a Waring blender. Intact granulomas were collected over a no. 100 stainless steel mesh and rinsed with cold RPMI 1640. The filtrate, containing CD11c+ DCs not associated with beads, was also collected and spun down. Naïve, unchallenged lungs were similarly homogenized. Single cell suspensions were obtained by collagenase digestion. Briefly, granulomas or naïve lung homogenates were incubated with digestion medium containing RPMI (JRH Biosciences, Lenexa, KS), 10% fetal bovine serum (Intergen, Purchase, NY), 10 mmol/L glutamine, and 1000 U/ml type IV collagenase (Sigma

Chemical Co.). After 15 minutes of incubation with agitation at 37°C, the digest was passed through a no. 100 stainless steel mesh and washed four times by centrifugation in RPMI 1640. CD11c+ DCs were isolated from the dispersed cell preparations using an EasySep PE selection kit (StemCell Technologies, Vancouver, BC, Canada) combined with a phycoerythrin-labeled anti-CD11c antibody (BD PharMingen). The enrichment of the recovered CD11c+ cells was confirmed by flow cytometric analysis and was routinely greater than 95%.

Flow Cytometry

Monoclonal antibodies used to identify mouse DC populations included fluorescein isothiocyanate-, phycoerythrin-, and Cy-chrome-conjugated anti-CD11c (HL3), phycoerythrin-conjugated anti-I-Ak (11-5.2), anti-CD8a (53-6.5), anti-CD11b/Mac-1a (M1/70), anti-CD40 (3/23), anti-CD80 (16-10-A1), anti-CD86 (GL-1) (all from BD PharMingen), anti-DEC-205 (NLDC-145) and anti-F4/80 (A3-1) (both from Serotec). All isotype controls and anti-CD16/CD32 (2.4G2) were from BD Pharmingen. After blocking with anti-CD16/CD32 for 5 minutes, cells were stained with fluorescent-labeled antibodies or isotype control antibodies in 2% fetal bovine serum-PBS buffer. A FACScan flow cytometer with CellQuest software (B-D, San Jose, CA) was used for data acquisition and analysis. Depending on the number of cells available, 10,000 to 100,000 events per sample were analyzed.

Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Transcript Analysis

Real-time RT-PCR analyses were performed as described.¹⁷ Poly(A) pure mRNA was isolated from cells using mRNA isolation kits (Ambion, Austin, TX) and reverse-transcribed using Reverse Transcription System kits (Promega, Madison, WI). Real-time PCR was performed on samples before and after reverse transcription to assess genomic DNA contamination levels in a sample preparation. DNase treatment was applied whenever contamination was detected. mRNA was considered detectable only when the signal was 10-fold more abundant than any contamination level. The ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) was used for real-time PCR analysis with comparative CT method. TaqMan (Applied Biosystems) pre-developed reaction kits were used for most target genes. *De novo* designed primer probe sequences have been previously reported.¹⁸ In all cases, the thermal cycling condition was programmed according to the manufacturer's instructions. Data are expressed as arbitrary units as described previously.¹⁷

Lymph Node Cell Culture and Cytokine Assays

Mediastinal lymph nodes were collected aseptically at the time of lung harvest and teased into a single cell suspension. After washing, the cells were cultured in

RPMI 1640 medium (JRH Biosciences) containing 10% fetal bovine serum (Intergen), 10 mmol/L glutamine, and 100 µg/ml streptomycin and 100 U/ml penicillin (RPMI-fetal bovine serum) at 5×10^6 cells/ml in 100-mm dishes with 5 µg/ml of PPD or SEA for 48 hours at 37°C in a humidified 5% CO₂ atmosphere. Supernates were collected by centrifugation and stored at -45°C before performing cytokine assays. All cytokines used in this study were obtained as purified carrier-free recombinant proteins from PeproTech Inc., Rocky Hill, NJ, and R&D Systems, Minneapolis, MN. Interleukin (IL)-2, IL-4, and interferon (IFN)-γ were measured by standard enzyme-linked immunosorbent assay using commercially available reagents (R&D Systems and BD Pharmingen); sensitivities ranged from 15 to 50 pg/ml.

Statistics

Analysis of variance was used for intergroup comparisons with $P < 0.05$ considered to indicate significance. Pairwise tests were done with Dunnett error protection at 95% confidence interval.

Results

Time Course and Histomorphology of Innate Antigen Bead Granuloma Formation

We previously demonstrated that pulmonary Ag bead challenge of Ag-sensitized mice offers an effective means to study sequential events in the development of anamnestic, Th1- and Th2-mediated hypersensitivity-type granulomas. In the present study, we wished to determine whether this approach could be used to study key events of the innate response by challenging naïve mice with beads bearing pathogen-associated Ags known to ultimately evoke Th1 and Th2 responses, specifically mycobacterial purified protein derivative (PPD) and soluble schistosomal egg Ags (SEA). In the first phase of the study we characterized the histology of Ag bead-challenged lungs throughout a 20-day period (Figure 1). Pathogen Ag-coated beads elicited focal reactions that were consistent with granulomas and approximately half of the area reported for the anamnestic Ag bead response.¹⁹ Interestingly, the reaction to mycobacterial PPD occurred more rapidly than the SEA response and more neutrophils were observed in these lesions on day 2. The SEA-elicited lesions tended to decline more slowly and were noted to leave more matrix scarring during the involutional period. Both pathogen Ags elicited more vigorous responses than a control nonpathogen Ag BSA.

Further analyses revealed that CD11c+ cells were a notable component of granulomas. As shown in Figure 2, immunohistochemical staining showed a rim of CD11c+ cells around all bead types; this was most prominent on day 3 after Ag bead injection. Scattered resident interstitial CD11c+ were also present in naïve and noninflamed lungs. Electron microscopy identified mononuclear cells with complex dendritic processes in close proximity to

Figure 1. Histology and time course of innate Ag bead granuloma formation. **A:** Histological appearance of the innate response to PPD-, SEA-, and BSA control (CON)-coated agarose beads during a 20-day study period. **B:** Time course of innate granulomatous inflammation in response to PPD, SEA, or BSA bead. Points are mean granuloma cross-sectional area $\pm$ SEM with three to five mice per point with 20 granulomas measured per lung. Dotted line indicates average size of bead only. Original magnifications, $\times 400$.

beads either adherent to endothelial cell basement membrane or in direct contact with bead surfaces (Figure 2).

Recruited cells were characterized by flow cytometry. Figure 3, A and B, shows the number of F4/80+, CD11c+, CD11c+MHCII+, Gr-1^{hi} cells measured as a percentage of CD45+ leukocytes in dispersed PPD and SEA bead-challenged lungs. Antigen bead challenge increased proportions of F4/80+, CD11c+, and Gr-1^{hi} cells corresponding to the accumulation of inflammatory cells observed histologically. The number of CD11c+ cells reached maximum on day 3 after injection, respectively, comprising 43% and 25% of CD45+ cells in the PPD and SEA models. (Figure 2, A and B). Unlike naïve lung CD11c+ cells, the majority of Ag-elicited CD11c+ cells expressed high levels of MHCII. As reported by others,^{16,20} a majority of CD11c+ cells in the lung displayed CD11b and weak F4/80 co-expression consistent with myeloid origin (data not shown). Although both CD11c+ and F4/80+ populations had weak Gr-1 expression, Gr-1^{bright} cells were a distinct population that did not overlap with CD11c+ populations and were more dominant in PPD bead-challenged lungs, which corroborated histological findings. The CD11c+ population in

the lung was heterogeneous with regard to DEC-205, CD8a, and CD11b expression, but was negative for B220 and CD4. Two distinct populations were identified, CD11c^{bright} CD11b^{dim}, and CD11c^{dim} CD11b^{bright}.^{16,20} Interestingly, the former did not increase with Ag challenge and retained an immature phenotype. In summary, Ag bead challenge mobilized CD11c+CD11b+MHCII+ cells consistent with DCs of myeloid origin.

Cytokine and Chemokine Expression During Innate Ag Bead Granuloma Formation

To begin a mechanistic analysis of events associated with the innate granuloma, we measured cytokine mRNA expression in lungs using quantitative real-time RT-PCR assay of pathogen Ag beads and compared this to non-pathogen BSA bead challenge. Preliminary time course studies indicated that most transcript induction occurred during the first 72 hours after bead challenge. Figure 4 shows the induction of several known innate response-related cytokines, IL-1 β , tumor necrosis factor (TNF)- α , GM-CSF, IL-6, IL-12p35, and IL12p40. The patterns revealed Ag-related differences in transcript induction. Specifically, PPD bead challenge was associated with the highest transcript levels of IL-1 β , GM-CSF, IL-6, and IL-12p35. In addition, although IL-12 p40 and TNF- α transcripts were detected in all groups, PPD challenge tended to evoke higher levels. SEA bead challenge evoked minimal IL-12p35 mRNA that was comparable to BSA bead challenge. However, SEA beads did induce more IL-6 than BSA beads. The finding of greater IL-12 induction in the PPD response suggests innate Ag recognition and cytokine induction could potentially promote the Th1-biased immune response²¹ observed in the adaptive phase of the PPD response. Transcripts for cytokines that promote Th2 responses, IL-4 and IL-10, were not significantly increased in the lungs of any bead-challenged group during the 3-day study period (not shown).

A similar expression analysis of chemokine mRNA was performed. It likewise revealed Ag-related differences. Figure 5 shows the induction patterns of 12 chemokines, most of which displayed significant up-regulation over virtually nil baseline levels in naïve lungs (time 0). Of these, CXCL2, CXCL5, CCL3, and CCL4 were more dominant in PPD bead-challenged lungs indicating a PPD-dependent effect. Interestingly, CXCL9 and CXCL10, both IFN- γ -amplified chemokines, did not display biased expression with primary PPD bead challenge as observed in the anamnestic response,¹² pointing to a requirement for adaptive immunity to fully establish chemokine polarization patterns. In addition, although most of chemokine transcripts achieved maximal levels by 24 hours, CXCL9 did not reach maximum until 48 to 72 hours.

CCL2 and CCL17 were induced with either pathogen Ags or BSA within 24 hours indicating an Ag nonspecific response. In contrast, CCL7 was augmented by the presence of PPD or SEA suggesting underlying innate Ag

Figure 2. Immunohistochemical and morphological detection of dendritic-like mononuclear cells in innate Ag bead granulomas. Cryostat sections were obtained from day 3 bead-challenged lungs and stained for CD11c. **A:** PPD bead; **B:** SEA bead; **C:** BSA bead. Note red-brown staining cells clustered around beads. Similar patterns were obtained for all Ag beads. Electron microscopy of innate Ag bead granulomas. **D:** PPD bead; **E:** SEA bead; **F:** BSA bead. Note the presence of large mononuclear cells with complex cytoplasmic processes. These were present within granulomas and in close proximity to all Ag bead types. Original magnifications: $\times 400$ (**A–C**); $\times 9300$ (**D–F**).

recognition. Unlike that reported for the anamnestic response,¹⁸ CXCL11, CCL8, and CCL22 were weakly expressed in the innate response, again pointing to a role for adaptive phase mediators in the amplification of chemokine responses during secondary granuloma formation.

The above described cytokine and chemokine transcript analyses indicated that innate granuloma formation is associated with rapid cytokine and chemokine induction. Moreover, there was evidence of innate pathogen Ag recognition resulting in different induction patterns for some of these mediators.

Induction of Differential Adaptive Immunity During Innate Ag Bead Granuloma Formation

Having defined key immunopathological events occurring during the innate granulomatous response we next analyzed draining lymph nodes (DLNs) for evidence of evolving adaptive immunity. Figure 6 shows the cytokine profiles of DLN cultures prepared 4 days after primary or secondary rechallenge after primary Ag bead exposure. The primary DLN response in PPD bead-challenged mice consisted of IL-2 and IFN- γ ; this Th1 pattern was accentuated with rechallenge on day 16. The primary response to SEA beads was a Th0 profile with concomitant IL-2, IFN- γ , IL-4, IL-5, and substantial IL-10. By day 16 this had polarized toward a Th2 profile with decreased IL-2 and IFN- γ and increased IL-5 and IL-13. Thus, the PPD response showed early and direct commitment to a Th1

profile, whereas the SEA response evolved from a Th0 to a Th2 dominant profile similar to sensitization with intact parasite eggs.¹³

Characterization of Innate Ag Bead Granuloma DC Populations

Because DCs were potentially responsible for initiating the observed DLN responses, we examined granuloma DC populations for phenotypic differences. We first compared the activation/maturation status of Ag bead-elicited lung DCs, by measuring surface expression of MHCII, CD40, CD80, and CD86 by flow cytometry. The CD11c+ cells shifted from low to high MHCII, CD40, CD80, and CD86 expression after Ag bead injection. Table 1 compares the mean fluorescent intensity (MFI) of the CD11c+ populations in naïve unchallenged and 3-day Ag bead-challenged lungs. MHCII and CD40 expression increased 5- and 10-fold, respectively, among 3-day Ag bead-challenged lung DCs. CD80 and CD86 expression increased approximately twofold. It should be noted that DCs from BSA bead granulomas showed similar activation profiles (not shown).

We next isolated CD11c+ cells from lungs using a standard immunomagnetic bead system and then performed real-time PCR transcript analysis for chemokines and chemokine receptor genes reportedly associated with activation.²² Compared to naïve lungs, CD11c+

Figure 3. Flow cytometric characterization of CD45⁺ leukocytes appearing in lungs after mycobacterial (PPD) and schistosomal (SEA) Ag bead challenge. Naïve mice received an intravenous injection of Ag beads. The lungs were harvested at the indicated time points, enzymatically dispersed, then CD45⁺ leukocytes were stained and subjected to flow cytometric analysis. **A:** PPD bead challenge; **B:** SEA bead challenge. Bars are means $\pm$ SD derived from three to six individual mice.

cells from Ag bead-challenged lungs expressed CCL4 (MIP1 β), CCL17 (MDC), CCL22 (TARC) transcripts that were 3-, 4-, and 20-fold higher (Table 1). Not all chemokines were affected because CCL3 (MIP1 α) did not change after challenge. Interestingly, IL-12p35, IL-12p40, and IL-10 transcripts were negligible and did not show up-regulation among CD11c⁺ cells from challenged lungs. This was also the case for IL-4, IFN- γ , IL-1 β , IL-6, and TNF- α transcripts (data not shown). All CD11c populations expressed CCR1, CCR5, and CXCR4 transcripts with evidence of augmented CCR5 mRNA in challenged mice. Unlike DCs in lymphoid tissues, lung DCs did not express CCR7. In addition, CCR3, CCR6, CCR8, CCR9, CXCR1/2, CXCR3, and CXCR5 transcripts were negligible (not shown). Notably, CCR2 transcripts increased nearly 100-fold among CD11c⁺ cells from challenged lungs compared to those of naïve lungs.

Unexpectedly, the phenotypes of SEA and PPD bead lung DCs were strikingly similar despite the disparate effects of the eliciting Ags. Taken together, the data indicated that inflammatory CD11c⁺ DCs from challenged lungs expressed genes consistent with activated DCs present in peripheral nonlymphoid tissues.²²

Figure 4. Cytokine transcript expression during innate Ag bead granuloma formation. PolyA pure mRNA was isolated from unchallenged (time 0) and Ag bead-challenged lungs then subjected to real-time PCR analysis for the indicated cytokine genes throughout a 3-day study period. **Bars** are mean arbitrary units $\pm$ SD for three to four individual lungs per point. Note different patterns of cytokine induction.

Ag Bead DCs Promote Th1 but Not Th2 Characteristics on Transfer

Lung derived DCs are believed to be critical for CD4⁺ T-cell priming; yet only lymphoid tissue but not peripheral tissue DCs are considered to be capable of initiating

Figure 5. Chemokine transcript expression during innate Ag bead granuloma formation. PolyA pure mRNA was isolated from unchallenged (time 0) and Ag bead-challenged lungs then subjected to real-time PCR analysis for the indicated chemokine genes throughout a 3-day study period. **Bars** are mean arbitrary units $\pm$ SD for three to four individual lungs per point.

Table 1. Antigen Bead-Elicited CD11c⁺ Cells Express Characteristics of Activated Peripheral Dendritic Cells

Marker or gene	Expression level (mean $\pm$ SD)		
	Unchallenged lung DCs	3-day PPD, bead DCs	3-day SEA, bead DCs
Fluorescence (MFI)			
MHCII	126 $\pm$ 8	616 $\pm$ 69*	625 $\pm$ 81*
CD40	6 $\pm$ 2	67 $\pm$ 3*	72 $\pm$ 15*
CD80	35 $\pm$ 6	62 $\pm$ 8*	72 $\pm$ 6*
CD86	26 $\pm$ 9	46 $\pm$ 7*	47 $\pm$ 5*
mRNA (AU)			
CCL3	1825 $\pm$ 589	1902 $\pm$ 396	1249 $\pm$ 80
CCL4	339 $\pm$ 71	1212 $\pm$ 181*	1289 $\pm$ 513*
CCL17	53 $\pm$ 17	336 $\pm$ 64*	197 $\pm$ 67*
CCL22	68 $\pm$ 24	1367 $\pm$ 115*	1867 $\pm$ 835*
IL-12p35	12 $\pm$ 16	33 $\pm$ 22	11 $\pm$ 1
IL-12p40	8 $\pm$ 4	6 $\pm$ 1	6 $\pm$ 3
IL-10	<5	<5	<5
CCR1	2941 $\pm$ 405	2978 $\pm$ 848	1510 $\pm$ 893
CCR2	9 $\pm$ 5	900 $\pm$ 288*	817 $\pm$ 26*
CCR5	212 $\pm$ 43	716 $\pm$ 124*	409 $\pm$ 149*
CXCR4	940 $\pm$ 161	1308 $\pm$ 232*	1812 $\pm$ 737*

* $P < 0.05$ compared to unchallenged resident dendritic cells.

T-cell activation.²³ Ag bead-elicited lung DCs had some features of mature activated DCs (Table 1). Because SEA and PPD Ags induced divergent CD4⁺ T-cell responses, we wished to determine whether the Ag bead-elicited lung DCs could similarly induce polarized Th responses. Ag bead DCs were isolated and directly transferred to syngeneic recipients (2×10^5 /recipient). Ten days later, recipients were challenged with respective Ag beads then DLNs were harvested and cultured on day 4 after challenge (day 14). Parallel challenged naïve mice were used for controls. Figure 7 shows cytokine profiles of DLN cultures after challenge. As noted above primary PPD bead challenge of naïve mice resulted in a detectable early Th1 profile, whereas SEA beads induced a Th0 pattern with concomitant IL-2, IFN- γ , and IL-4 production (Figure 7, right column). Compared to naïve mice, priming with PPD bead DCs conferred an enhanced Th1 pattern with twofold and fourfold increases of IL-2 and IFN- γ (Figure 7, left column). This effect was associated with an anamnestic inflammatory response to PPD beads and could not be transferred with DCs from non-Ag beads (data not shown). Surprisingly, priming with SEA bead lung DCs did not confer the expected Th2 cytokine profile, but rather elicited IL-2 and IFN- γ but not IL-4, resembling a more Th1-like profile. The experiment was repeated four times and in one, DC-primed mice were further boosted with soluble SEA before SEA bead challenge and still the IL-4 response was blocked by DC transfer. It was noted that the IFN- γ promoting ability of SEA bead DCs was weaker than their PPD counterparts, suggesting that SEA lung DCs could not invoke a vigorous Th1 response. Taken together, the data indicated that bead-elicited, activated lung DCs were capable of conferring a Th1 adaptive response, but were unable to establish a Th2 response.

Discussion

Granulomas are macrophage-rich inflammatory responses that can sequester a wide variety of pathogens

as well as envelop insoluble nonantigenic foreign bodies.²⁴ Although macrophages and their derivatives are a defining cellular component of granulomas, these lesions can vary histomorphologically depending on the nature of the eliciting agent. Granulomas represent an overlooked form of innate immunity that can be modified by superimposed acquired immune mechanisms if the eliciting agent carries appropriate stimulatory Ags. The present study was undertaken to begin a detailed sys-

Figure 6. Draining mediastinal lymph node cytokine profiles after primary and secondary mycobacterial (PPD) or schistosomal (SEA) Ag bead challenge. PRIM, lymph node cultures prepared on day 4 after primary Ag bead challenge of naïve mice; SEC, lymph node cultures prepared 4 days after (day 20) rechallenge of mice on day 16 of previous Ag bead challenge. Lymph node cultures were stimulated with respective Ags for 48 hours; supernates were then harvested and assayed for cytokines by enzyme-linked immunosorbent assay. Lymph nodes were obtained from four to five mice. Note the primary and secondary PPD response is primarily IL-2 and IFN- γ . The primary SEA response is a mixed Th0 pattern whereas the secondary response is strongly polarized to a Th2 pattern.

Figure 7. Adoptively transferred Ag bead-elicited lung DCs induce Th1 but not Th2 commitment. Cytokine profiles were determined in draining lymph nodes from primed and naïve mice on day 4 after Ag bead challenge. Standard enzyme-linked immunosorbent assay was used to measure Ag-elicited cytokine production in 48-hour culture supernatants. **Left column,** mice primed intravenously with 2×10^5 purified DCs derived from day 3 PPD bead- or SEA bead-challenged lungs, and then challenged with respective Ag beads by intravenous injection 10 days later. **Right column,** naïve mice challenged with Ag beads without presensitization. **Bars** are means $\pm$ SD of IL-2, IL-4, and IFN- γ production of triplicate determinations. Cytokines were not detected without Ag stimulation. Experiments were repeated four times with three to four mice per group in each experiment.

tematic analysis of events associated with the innate phase of granuloma formation with a special focus on the participation of DCs. We used models of pulmonary challenge of naïve mice with agarose bead immobilized Ags. The Ags, *M. bovis* purified protein derivative (PPD) and *S. mansoni* soluble egg Ags, (SEA), were derived from pathogens that are well-recognized granulomagenic agents as well as respective inducers of Th1- and Th2-polarized immune responses. Because the lesions are synchronously induced, immune events could be characterized and compared in parallel.

Our histomorphological analysis revealed a transient innate inflammatory response to Ag-coated beads with subtle kinetic differences that suggested Ag-dependent effects. Mycobacterial PPD elicited rapid focal inflammation that reached a peak on day 3 that quickly resolved thereafter. The helminth Ag, SEA, reached maximum on day 4 and showed a slower resolution rate. Both pathogen Ag bead responses were greater than that for BSA beads suggesting an element of innate recognition of pathogen-associated molecular patterns. The response to BSA beads likely results from a nonspecific release of mediators from cells because of local tissue injury/irritation combined with activation of complement and coagulation pathway factors. DCs may process BSA Ags but no Toll-like receptor recognition has been described for bovine albumin.

Kinetic analyses of cytokine and chemokine transcript expression likewise provided evidence of innate Ag-rec-

ognition. TNF- α , IL-1, and IL-6 transcripts were all induced by 24 hours and PPD beads elicited the greatest levels. BSA beads primarily induced only TNF- α whereas SEA beads induced TNF- α and low levels of IL-1 and IL-6. By 48 hours there was enhanced expression of IL-12 p35 and p40 transcripts in the PPD response but not in the other responses. The delayed IL-12 expression would be consistent with derivation from a recruited population or might reflect a maturation/activation interval. As discussed below, the selective induction of IL-12 mRNA by PPD beads is possibly relevant in linking the innate PPD response to subsequent acquired Th1 cell-mediated immunity.²¹ Sources of IL-12 could include mononuclear phagocytes, which bear Toll-like receptors (TLR). PPD contains lipoprotein-lipopolysaccharide complexes²⁵ that could be recognized by such receptors.²⁶

Chemokine transcripts were primarily induced by 24 hours with the exception of CXCL9, CCL3, and CCL8, which appeared by 48 to 72 hours. As reported for the secondary response, CXCL2, CXCL5, CCL3, and CCL4 were biased to the innate PPD granuloma,²⁷ suggesting that innate recognition signals may help establish this bias. It is known that the neutrophil chemotactins, CXCL2 and CXCL5, are induced by endotoxin therefore it is conceivable that PPD lipopolysaccharide complexes could potentially mediate direct cellular activation and mediator production by way of the Toll-like LPS receptor, TLR-4.^{28,29} The enhanced expression of neutrophil chemotactins in the innate PPD granuloma corresponds to the increased neutrophil influx that we observed in these lesions. Transcripts for CCL3 showed delayed expression kinetics that paralleled IL-12. This would be fully consistent with our demonstration that CCL3 is dependent on endogenous IL-12 expression during PPD granuloma formation.¹⁸ CCL3 and CCL4 are potentially chemotactic for monocytes, DCs, and CCR5+ Th1 cells, providing coordinating recruitment signals. In addition, several other chemokines displayed rapid Ag nonspecific induction, these included CXCL9/10 and CCL17, ligands for known Th1 and Th2 cell-associated chemokine receptors. Thus, cells of the acquired immune system are adapted to respond to chemokines generated by the innate response, providing a means for rapid local mobilization of immune cells. CCL2 was also among the rapidly elicited Ag-nonspecific chemokines and transcripts or its receptor, CCR2, was notably enhanced among granuloma DCs. This would be fully consistent with reports implicating CCL2 in the trafficking of DCs.^{30,31}

Some chemokines including CXCL11, CCL8, and CCL22, which show strong expression during secondary Ag bead granuloma formation,¹⁸ were weakly represented in the innate granuloma indicating that adaptive immune signals profoundly amplify their expression. Interestingly, the innate response to SEA beads did not display a distinctly biased chemokine pattern. Toll receptors are reportedly not required for the generation of Th2 responses³² and the relatively modest cytokine response elicited by SEA beads might support this notion. However, CCL7 transcripts were expressed at levels comparable to that elicited by PPD, which were threefold higher

than for nonpathogen Ag bead challenge suggesting some form of innate recognition of SEA. Recently, TLR-2 has been implicated in recognition of lysophosphatidylserine residues derived from schistosome eggs,³³ providing precedent for innate recognition of helminth Ags. Taken together, our findings suggest the existence of a reflexive innate chemokine response designed to recruit all potentially relevant immune-related cells; however that response can be tailored to a pathogen likely through recognition of pathogen-associated molecular patterns.

Our study extends the findings of Lyonaga and colleagues⁴ who reported PPD bead granulomas as a site of DC recruitment. We demonstrate that DCs undergo maturation at the site of recruitment displaying expression of class II major histocompatibility proteins and other co-stimulatory molecules when challenged with either pathogen or nonpathogen Ag. Furthermore we show that such DCs when directly transferred to naïve recipients have the capacity to transfer a Th1 anamnestic response. Under noninflamed conditions, the lung is thought to favor a Th2 default response.^{15,16} However, pathogens often breach the mucosa evoking inflammation and DC mobilization. Studies of rat lungs indicate that resident DCs internalize Ag efficiently but weakly express MHCII and co-stimulatory molecules and confer Th2 responses on adoptive transfer. However, after overnight culture with Ag and GM-CSF in combination with TNF- α or CD40L those DCs became potent antigen-presenting cells (APC) that induced Th1 responses on adoptive transfer.¹⁵ Our models of Ag bead challenge elicit an environment rich in mediators that apparently similarly activated CD11c+ DCs.

In unprimed mice, SEA beads behaved like parasite eggs inducing an initial Th0 that progressed to a Th2 cytokine pattern;¹³ therefore it was surprising that SEA bead DCs were unable to transfer a Th2 response. The SEA bead DCs were clearly biologically active as they prevented Th2 differentiation even on Ag boosting. Others have reported successful transfer of Th2 responses using culture generated DCs loaded with SEA.³⁴ However, these cells were not exposed to inflammatory mediators that occur under conditions of parasite egg deposition. Because SEA DCs could not initiate Th2 differentiation then SEA must invoke other pathways to establish a Th2 response. A number of potential mechanisms will need to be investigated such as Ag-presenting B cells, alternative DC subpopulations, and regulatory T cells.^{35,36}

The less vigorous Th1 response induced by SEA DCs as compared to PPD DCs may reflect our observed differences in IL-12. Although isolated lung DCs expressed negligible IL-12 transcripts, PPD induces greater overall IL-12 production in lungs and DLNs compared to SEA (not shown). Although APC-derived IL-12^{37–39} is considered critical for Th1 differentiation,²³ recent evidence suggests that the induction and maintenance of high levels of T-bet are necessary for initial Th1 commitment and IL-12/STAT4 signaling amplifies the amount of IFN- γ produced by individual cells.⁴⁰ Thus, transferred SEA DCs may provide signals for T-bet induction but in a milieu deficient in IL-12, resulting in less IFN- γ production.

In conclusion, we describe models of pulmonary Ag bead-elicited innate granuloma formation that offer a potential *in vivo* approach to systematically analyze critical mechanisms of the DC response to pathogen Ags as well as determine their relationship to acquired immunity. We further provide the first demonstration of the *in vivo* Ag-presenting function of lung DCs recruited during pathogen Ag-elicited innate granuloma formation. We show that Ag bead-recruited DCs can confer Th1 responses on adoptive transfer and do not favor Th2 responses even when exposed to a potent Th2 Ag. These findings have important implications with regard to manipulating adaptive immunity in the lung.

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