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CD209+ Macrophages Mediate Host Defense against Propionibacterium acnes

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Propionibacterium acnes is a major etiological factor of acne, triggering an inflammatory response in part through the activation of TLR2. In this study, we demonstrate that activation of peripheral blood monocytes with P. acnes in vitro induced their differentiation into two distinct innate immune cell subsets, CD209+ macrophages and CD1b+ dendritic cells. Furthermore, P. acnes induced expression of mRNA for the cytokines IL-15 and GM-CSF, which differentiate CD209+ and CD1b+ cells, respectively. The CD209+ cells were more effective in uptake of P. acnes compared with the CD1b+ cells, and demonstrated a 2-fold greater antimicrobial activity against the phagocytosed bacteria. Although CD1b+ cells secreted inflammatory cytokines in response to both P. acnes and a TLR2 ligand control, the CD209+ cells responded only to P. acnes. The addition of all-trans retinoic acid, a commonly used agent for the treatment of acne, directly induced differentiation of monocytes into CD209+ macrophages and enhanced the P. acnes-mediated differentiation of the CD209+ subset. Therefore, the differentiation of monocytes into CD209+ macrophages and CD1b+ dendritic cells distinctly mediate the innate immune response to P. acnes. The Journal of Immunology, 2008, 180: 4919–4923.

To gain insight into the mechanism(s) by which P. acnes triggers both inflammation and antimicrobial responses, we studied the ability of the bacteria to induce these two functional subsets.

Materials and Methods  

Abs and ligands  
P. acnes strain ATCC 6919 was obtained from American Type Culture Collection and prepared by probe sonication. The level of endotoxin contaminating the P. acnes was quantified with a Limulus Amoebocyte lysate assay (BioWhittaker) and found to be <0.1 ng/ml. The following Abs were used: mAb specific to human TLR2 (clone 2392; Genentech), CD1b (Bd3.1; ATCC), CD209 (BD Pharmingen), and IgG controls (Sigma-Aldrich). All-trans retinoic acid (ATRA)† (Sigma-Aldrich) was prepared as previously described and used at 10−8 M (3). The TLR2/1 ligand (L) is a synthetic Mycobacterium tuberculosis-derived 19 kDa lipopeptide (EMC).

Monocyte isolation and stimulation  
Peripheral human blood was obtained from healthy donors with informed consent, as approved by the University of California-Los Angeles Institutional Review Board. PBMC were then isolated using Ficoll-Paque gradients (Amersham Biosciences) and adhered onto culture dishes for 3 h. RNA was isolated using the iSCRIPT cDNA synthesis kit (Bio-Rad). GM-CSF and IL-15 primers were previously published (2).

Flow cytometry  
Cells were labeled for surface proteins using specific Abs. A PE- or FITC-conjugated secondary Ab (CalTag Laboratories) was used for CD1b. Cells were fixed with 1% paraformaldehyde, acquired on a BD Biosciences FACS, and analyzed using WinMDI 2.8 (J. Trotter, Scripps Research Institute, San Diego, CA).

Quantitative PCR  
Monocytes were stimulated with media, TLR2/1L, or P. acnes sonicate for 3 h. RNA was isolated using Trizol reagent (Invitrogen Life Technologies), and cDNA was synthesized using the iSCRIPT cDNA synthesis kit (Bio-Rad). GM-CSF and IL-15 primers were previously published (2).

Abbreviations used in this paper: ATRA, all-trans retinoic acid; CTC, 5-cyano-2,3-dinitol tetrazolium chloride; MOI, multiplicity of infection; L, ligand.

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Green reactions were performed, and relative quantities of GM-CSF and IL-15 per sample were calculated as described (3).

Monocyte differentiation and enrichment

To enrich for CD209⁺ or CD1b⁺ cells, monocytes were cultured with rIL-15 (200 ng/ml) (R&D Systems) or rGM-CSF (10 U/ml) (Amgen) for 48 h and labeled with a CD209 or CD1b Ab. A MACS microbead-secondary Ab (IgG2a/b, IgG1; Miltenyi Biotec) was added for 30 min, on ice. Flow-through was designated as the negative population. Remaining cells were designated as the positive population.

Detection of cytokines

CD209⁺ or CD1b⁺ cells were activated with P. acnes or TLR2/1L for 18 h, which is the standard time point used in our laboratory for the study of TLR-induced cytokine expression (3). IL-12p40 levels in the supernatants were measured by ELISA (BD Pharmingen) as previously described (3). For additional chemokine and cytokine array testing including IL-8, supernatants were examined using the SearchLight cytokine array service (Pierce).

Phagocytosis assay

Monocytes were cultured with rIL-15 or rGM-CSF for 48 h. To remove traces of antibiotic in the cell cultures before the addition of bacteria, the cells were washed three times with 1× PBS and recultured in RPMI 1640 with 10% FCS and no antibiotics added. P. acnes strain ATCC 6919 was grown under anaerobic conditions in Reinforced Clostridial medium (Oxoid) for 3 days. The bacteria were enumerated by absorbance using a conversion factor of 7.5 × 10⁵ bacteria per ml = 1 OD unit at 600 nm. For the phagocytosis assay, bacteria were first incubated with 100 μl of 5-cyano-2,3-ditolyl tetrazolium chloride (CTC; Polysciences) per 1 ml of bacterial culture for 3 h at 37°C and then washed to remove excess CTC. Then, the P. acnes-CTC were incubated with the washed IL-15- and GM-CSF-derived cells at multiplicities of infection (MOIs) of 0.1, 0.5, or 1 for 45 min at 37°C. Following the 45-min incubation, the cells were harvested, washed, and labeled with FITC-conjugated Abs for CD1b or CD209 and analyzed using flow cytometry.

CFU assay

For CFU assay, IL-15 and GM-CSF cells were derived as detailed above and infected with nonlabeled P. acnes at a MOI of 1. Following 45 min of incubation with P. acnes, the cells were harvested and washed three times with 1× PBS. The harvested cells were split in half and either recultured for the 24-h time point or lysed in 30 μl of 3% saponin. The saponin lysates were diluted serially with 10-fold dilutions and plated onto Brucella Agar with 5% sheep blood, hemin, and vitamin K (Remel). After 24 h of incubation, the recultured cells were also lysed as described. The Brucella Agar plates were incubated for 4 days at 37°C under anaerobic conditions. Individual colonies were counted, and the number of CFUs was calculated. Cell viability was evaluated at both time points by trypan blue exclusion, and the viability was >90% in all conditions.

Statistical analysis

Statistical comparison between cell types in ELISA studies and antimicrobial studies were made using Student’s t test. Values of p < 0.05 were considered significant and are indicated in the figures.

Results

Generation of CD209⁺ and CD1b⁺ cells in vitro by P. acnes

To determine whether P. acnes is capable of inducing the expansion and differentiation of both CD209⁺ and CD1b⁺ cells, primary monocytes were treated with media, P. acnes, or a TLR2/1L, a triacylated lipopeptide, for 48 h. Since TLR2/1 activation has been shown to induce the differentiation of monocytes into two distinct cell subsets, CD209⁺ macrophages and CD1b⁺ dendritic cells, TLR2/1L was included as a control (2). Following treatment, the cell surface expression of both CD209 and CD1b on the monocytes was assayed using flow cytometry. Both P. acnes and the TLR2/1L were able to induce the rapid differentiation of monocytes into distinct CD209⁺ and CD1b⁺ cell populations to similar magnitudes (Fig. 1a). P. acnes- and TLR2/1L-induced CD209⁺ cells exhibit similar cell surface expression patterns of macrophage and dendritic cell markers, CD64, CD40, and CD11c, respectively, as did the induced CD1b⁺ population (Fig. 1b). Given that P. acnes contains a TLR2L (1), we investigated the role of TLR2 in the P. acnes-mediated monocytic differentiation by using a TLR2 blocking Ab. In the presence of the TLR2 blocking Ab, the induction of CD209⁺ cells was completely ablated (Fig. 1c); however, clear dependency on TLR2 for the differentiation of CD1b⁺ cells could not be found (data not shown).

Since, the TLR2-triggered differentiation of CD209⁺ and CD1b⁺ cells is known to be mediated through the production of IL-15 and GM-CSF (2), we investigated whether these cytokines were similarly regulated by P. acnes. Monocytes were stimulated with either P. acnes or the TLR2/1L for 3 h, and gene expression levels of IL-15 and GM-CSF were assayed using real time quantitative PCR. The 3-h time point was chosen based on our previous results demonstrating optimal expression of IL-15 and GM-CSF mRNA at this time point following TLR stimulation (2). As shown, monocytes stimulated with P. acnes were capable of inducing the expression of IL-15 and GM-CSF mRNA to similar levels as induced by TLR2/1L (Fig. 1d). Cell surface expression of CD64, CD40, and CD11c on P. acnes- or TLR2/1L-generated CD209⁺ and CD1b⁺ subpopulations exhibited a similar trend to CD209⁺ or CD1b⁺ cells derived directly from culturing monocytes with rIL-15 or rGM-CSF, respectively (Ref. 2 and data not shown).
P. acnes induces proinflammatory cytokine production in both CD209+ and CD1b+ cells

To further study the functional role of differentiated monocytes in the immune response against P. acnes, CD209+ and CD1b+ subsets generated with rIL-15 or rGM-CSF, respectively, were activated with P. acnes, and the release of IL-12p40 and IL-8 levels were measured by ELISA and SearchLight cytokine array, respectively. We used rIL-15 or rGM-CSF stimulated monocytes as models for CD209+ macrophages and CD1b+ dendritic cells, respectively. Our previous study has demonstrated that rIL-15 or rGM-CSF induces differential expression of CD209 and CD1b, on monocytes similar to the cell populations induced by TLR2/1L directly (2). In addition, the rIL-15 and rGM-CSF derived cells have a similar expression pattern of activated macrophage and immature dendritic cell surface markers as compared with the CD209+ and CD1b+ cells differentiated with TLR2/1L.

Monocytes were cultured with either rIL-15 or rGM-CSF for 48 h, which is an optimal time point for the maximal differentiation of CD209+ and CD1b+ cells as previously described (2). The differentiated cells were then magnetically sorted based on positive selection of CD209 or CD1b expression, respectively. The sorted cells were then stimulated with media, P. acnes, or the TLR2/1L for 18 h, and the levels of IL-12p40 in the culture supernatants were measured using ELISA. The IL-12p40 subunit forms heterodimers as IL-12 or IL-23, a key mechanism in regulating the adaptive Th1 response (1). Similar to our previous study, the TLR2/1L induces higher levels of IL-12p40 in the CD1b+ as compared with the CD209+ subset (2). There was a minor level of IL-12p40 induction observed with TLR2/1L stimulation in the CD209+ population; however, this level was not statistically significant using the Student’s t test (Fig. 2a). In contrast, we found that P. acnes induced IL-12p40 production in both CD209+ and CD1b+ cells (Fig. 2a). The levels of P. acnes induced IL-12p40 in the CD209+ population as compared with the TLR2/1L levels were 7-fold higher with a value of $p > 0.05$. Both stimuli induced relatively similar levels of IL-8 from both CD209+ and CD1b+ cells, indicating that the lower levels of IL-12p40 induced by TLR2/1L in CD209+ cells were not due to insufficient levels of TLR2/1L (Fig. 2b). Since the CD209+ cells secrete higher levels of IL-12p40 in response to P. acnes but not the TLR2/1L, these data indicate that the CD209+ cells can contribute to inflammation in acne through a TLR2/1 independent mechanism.

CD209+ cells phagocytose P. acnes more efficiently than CD1b+ cells

Apart from the inflammatory cytokine response, the other key function of the innate immune response is to directly phagocytose and kill the microbial invader. Therefore, we investigated the ability of CD209+ and CD1b+ subsets to phagocytose and destroy P. acnes. Given that P. acnes is normally grown in bacterial medium under anaerobic conditions, we first determined whether P. acnes was viable in our standard cell culture conditions for 24 h. When grown in cell culture medium and conditions, P. acnes was found to be viable and capable of replication as determined by CFU assay (data not shown). We then labeled P. acnes with CTC, a monocrotaline redox dye, which produces a fluorescent formazan upon reduction by live bacterium detectable by flow cytometry. The CTC-labeled P. acnes (P. acnes-CTC) were added to IL-15 and GM-CSF cultured cells at a MOI of one for 45 min and then labeled with mAbs specific for CD209 and CD1b. Images of the cells were captured with the Imagestream flow cytometry system. Both CD209+ and CD1b+ cells phagocytosed the bacterium (Fig. 3a), and the average numbers of intracellular bacteria per cell were the same for both populations (data not shown). This data indicates that both CD209+ and CD1b+ cells are capable of phagocytosing P. acnes.

To compare the differential phagocytic capacity of two cell populations, we infected CD209+ and CD1b+ cells with P. acnes-CTC at a MOI of 0, 0.1, 0.5, or 1. Following 45 min of exposure to the bacteria, the cells were harvested and labeled with mAbs to CD209 and CD1b and analyzed with traditional flow cytometry. A 2-fold greater percentage of the CD209+ subset, compared with
the CD1b− subset, contained intracellular organisms (Fig. 3b). Therefore, the CD209+ subset is more phagocytic for P. acnes.

To ascertain the intracellular antimicrobial capacity of the CD209+ and CD1b− subsets, these subsets were infected with P. acnes at a MOI of 1 for 45 min or 24 h. Intracellular bacteria were harvested by lysing the cells with a mild detergent and the lysates were plated in serial dilutions. The number of CFU from each cell population was enumerated and normalized to the number of CD209+ or CD1b− cells in the culture at the start of the assay (CFU/cell). At 45 min, the number of CFU/cell recovered from the CD209+ cells was 4-fold higher in the CD1b− cells (Fig. 3c), confirming the results we obtained using flow cytometry that more CD209+ phagocytosed P. acnes (Fig. 3b). After 24 h, the CFU/cell recovered from the CD209+ cells were 64% lower than at day 0, whereas the CD1b− cells exhibited little to no change (Fig. 3c). These data demonstrate that the CD209+ cells mediate over 3-fold more (value of \( p < 0.05 \)) intracellular antimicrobial activity against phagocytosed P. acnes than CD1b− cells (Fig. 3d).

Since CD209+ cells contain 4-fold higher levels of CFUs at day 0 than CD1b− cells and 3-fold more antimicrobial activity of the phagocytosed bacterium, they, therefore, kill 12 times the absolute number of bacteria as compared with CD1b− cells.

To test the possibility of extracellular antimicrobial mechanisms, IL-15 and GM-CSF derived cells were stimulated with either media or irradiated P. acnes at a MOI of 1 for 24 h. P. acnes were then grown in the conditioned supernatants collected from the cells for 24 h and enumerated by CFU assay. There was no evidence of decreased P. acnes viability in any condition tested (data not shown), which suggested that there are no extracellular P. acnes killing mechanisms by these two cell types. Our findings suggest that CD209+ macrophages can efficiently phagocytose and inhibit the growth of P. acnes, and therefore play a role in host defense against P. acnes.

ATRA modulates monocyte differentiation toward CD209+

Previously, we reported that ATRA, a commonly used acne treatment, could decrease P. acnes-induced inflammatory cytokines by down-regulation of TLR2/1 expression and function (3). Given that ATRA modulates the differentiation of promyeloleukemic monocytes (4), the ability of ATRA to induce the differentiation of primary human monocytes into CD209+ and CD1b− cell subsets was investigated. Monocytes were treated with a titration of ATRA ranging from 10−9 M to 10−7 M, or carrier control, for 48 h and assayed for cell surface expression of CD209 using flow cytometry. ATRA directly triggered the differentiation of monocytes into CD209+ cells but did not induce CD1b− cells (Fig. 4a).

Since ATRA is able to modulate the differentiation of monocytes, we investigated whether or not it could affect P. acnes-, TLR2/1L-, and IL-15-mediated cellular differentiation. Monocytes were stimulated with P. acnes, TLR2/1L, and IL-15 while coinoculated with or without ATRA at 10−8 M for 48 h, following which the expression of CD209 was assayed using flow cytometry. Coincubation with ATRA enhanced P. acnes−, TLR2/1L−, and IL-15-induced CD209 expression (Fig. 4b). The percent of CD209+ cells induced by P. acnes, TLR2/1L, and IL-15 were increased by 11, 25, and 17% (value of \( p < 0.05 \)), respectively (Fig. 4c). In contrast, ATRA down-regulated GM-CSF-induced CD1b expression (value of \( p < 0.05 \)) but had little to no effect on either P. acnes− or TLR2/1L-induced CD1b− cells (Fig. 4b). Although ATRA is already known to induce the differentiation of transformed neoplastic cells (5–7), its ability to differentiate primary monocytes into CD209+ macrophages directly as well as modulate TLR-induced differentiation is novel. These data imply that ATRA can enhance innate immunity by expanding the cell population that can phagocytose and inhibit the growth of P. acnes.

Discussion

The innate immune system serves two main functions, the first is to rapidly respond to and destroy microbial invaders and the second is to instruct the adaptive immune response. In this study, we provide new evidence demonstrating: 1) the ability of the CD209+ macrophages to phagocytose P. acnes bacterium; 2) the ability of CD209+ cells to kill P. acnes; and 3) that ATRA, a commonly used treatment for acne, modulates differentiation of the CD209+ cell subpopulation. Although both cells produced IL-12p40 in response to P. acnes, strikingly, CD209+ cells were more capable than CD1b− cells in their ability to phagocytose and mediate an antimicrobial activity against P. acnes. Therefore, the ability of P. acnes to induce the differentiation of monocytes into CD209+ macrophages and CD1b− dendritic cells contributes to innate immunity in human disease.

The ability of CD209+ macrophages to phagocytose and exhibit antimicrobial activity against P. acnes indicates that macrophages mediate the direct host defense function of the innate immune response in acne. When incubated with P. acne, a 2-fold greater percentage of the CD209+ subset, compared with the CD1b− subset, was able to phagocytose P. acnes. A previous study suggested...
that both neutrophils and monocytes readily phagocyte extracellular *P. acnes*, but the ability of these cells to directly kill *P. acnes* following phagocytosis was not clear (8). We demonstrate that following uptake of the bacteria, the CD209*<sup>+</sup>* cells exhibited marked antimicrobial activity, approximately a 64% reduction in viable bacilli that was not evident in the CD1b*<sup>+</sup>* cells. The ability to kill *P. acnes* has important clinical relevance, since acne patients have significantly higher numbers of *P. acnes* on their skin when compared with age-matched control subjects (9). Furthermore, treatments that reduce the absolute numbers of cutaneous *P. acnes* result in clinical improvement (10–12), suggesting that nat-

more, treatments that reduce the absolute numbers of cutaneous *P. acnes* that both neutrophils and monocytes readily phagocytose extracellular *P. acnes*, activates monocytes via TLR2 to re-

ect different in these cells populations in the pathogenesis and host defense against *P. acnes*. Finally, the ability of ATRA to both enhance the differentiation of monocytes into macrophages that can ingest and kill organism while at the same time down-regulating proinflam-

matory cytokine responses represents a potential adjuvant to the treatment of infectious disease in which inflammatory-mediated tissue injury represents a clinical problem.

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**Disclosures**

The authors have no financial conflict of interest.

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