

# Innate Antifungal Immunity of Human Eosinophils Mediated by a $\beta_2$ Integrin, CD11b<sup>1</sup>

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Eosinophils produce and release various proinflammatory mediators and also show immunomodulatory and tissue remodeling functions; thus, eosinophils may be involved in the pathophysiology of asthma and other eosinophilic disorders as well as host defense. Several major questions still remain. For example, how do human eosinophils become activated in diseased tissues or at the site of an immune response? What types of host immunity might potentially involve eosinophils? Herein, we found that human eosinophils react vigorously to a common environmental fungus, *Alternaria alternata*, which is implicated in the development and/or exacerbation of human asthma. Eosinophils release their cytotoxic granule proteins, such as eosinophil-derived neurotoxin and major basic protein, into the extracellular milieu and onto the surface of fungal organisms and kill the fungus in a contact-dependent manner. Eosinophils use their versatile  $\beta_2$  integrin molecule, CD11b, to adhere to a major cell wall component,  $\beta$ -glucan, but eosinophils do not express other common fungal receptors, such as dectin-1 and lactosylceramide. The I-domain of CD11b is distinctively involved in the eosinophils' interaction with  $\beta$ -glucan. Eosinophils do not react with another fungal cell wall component, chitin. Because human eosinophils respond to and kill certain fungal organisms, our findings identify a previously unrecognized innate immune function for eosinophils. This immune response by eosinophils may benefit the host, but, in turn, it may also play a role in the development and/or exacerbation of eosinophil-related allergic human diseases, such as asthma. *The Journal of Immunology*, 2008, 181: 2907–2915.

Eosinophils were first described by Paul Ehrlich in 1879, and they are thought to have evolved as part of the innate/acquired immune response against parasitic helminths (1–3). Eosinophils are likely involved in the pathophysiology of various human diseases, such as bronchial asthma and atopic dermatitis (1–3). Eosinophils generate proinflammatory mediators, such as leukotrienes, reactive oxygen species, and various cytokines and chemokines, and release preformed inflammatory mediators, such as major basic protein (MBP),<sup>3</sup> eosinophil-derived neurotoxin (EDN), and eosinophil cationic protein (ECP) from granules, resulting in tissue damage and remodeling (4, 5). Deposition of released granule proteins has been demonstrated in diseased tissues, such as airway, skin, and heart, from patients with acute and chronic eosinophilic disorders (6–9). In contrast, how eosinophils become activated and release inflammatory mediators is largely unknown.

The association between eosinophilic inflammation and infection or colonization with fungi has been long recognized. For ex-

ample, in 1952, patients with allergic bronchopulmonary aspergillosis were reported, who demonstrated pulmonary eosinophilia and positive serological tests for *aspergillus* (10). The association between fungi and chronic eosinophilic airway inflammation has been described in patients with severe asthma and certain types of rhinosinusitis (11, 12). Furthermore, infiltration and degranulation of eosinophils were observed in lesions of patients with paracoccidioidomycosis (13). However, the immunological mechanisms underlying these relationships between fungi and eosinophilic inflammation have been poorly understood. Recently, major conceptual advances have been made in this regard. Chitin is a carbohydrate polymer, and it is found in the exoskeleton of insects and crustaceans (e.g., mites and cockroaches), the cell wall of fungi, as well as the pharynx, microfilarial sheath, and eggs of helminths. Importantly, in mice, the intranasal administration of chitin induced a Th2-like airway response and eosinophilia, and these responses were inhibited by a vertebrate chitinase (14). Thus, immune responses to chitin-encased insects and fungi (15, 16) may be part of the normal innate immune response, and inappropriate regulation of the system may contribute to asthma and allergic diseases.

These relationships among eosinophilia, asthma, and fungi prompted us to investigate the direct response of eosinophils to fungi. First, do human eosinophils recognize fungi and produce proinflammatory mediators? Second, if eosinophils do react to these organisms, which eosinophil receptor(s) is involved? To this end, we used a ubiquitous, nonpathogenic airborne fungus, *Alternaria alternata*, as a pathologically relevant model organism. Indeed, several epidemiological studies strongly implicate exposure or sensitization to *A. alternata* in the development and/or exacerbation of human asthma (17–19). We found that eosinophils exert a strong inflammatory response against germinating *A. alternata* and kill the fungus. A versatile  $\beta_2$  integrin adhesion molecule, CD11b, which is expressed by eosinophils, likely plays a key role in recognizing and/or interacting with  $\beta$ -glucan that is present on the surface of *A. alternata*.

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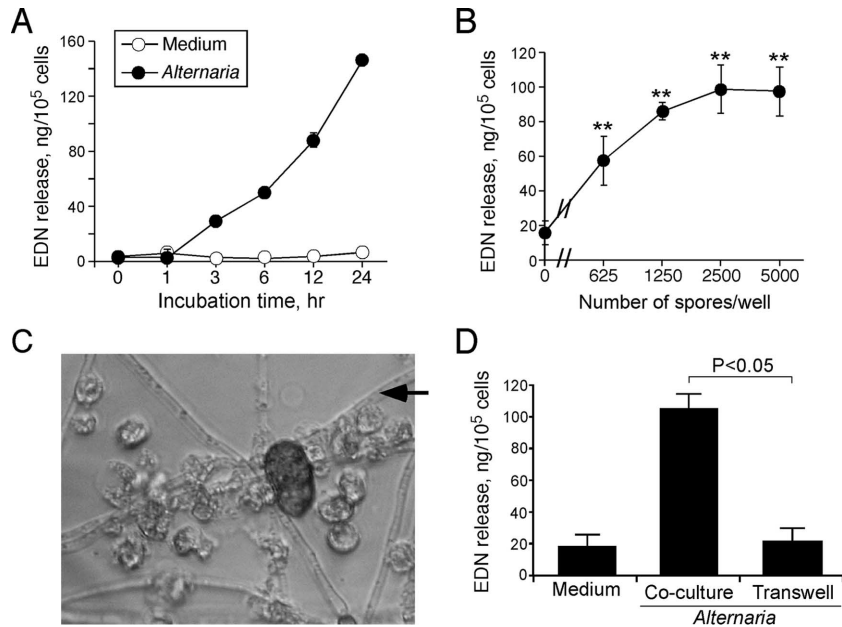
Received for publication February 22, 2008. Accepted for publication June 10, 2008.

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<sup>1</sup> This work was supported by National Institutes of Health Grants AI34486 and AI49235 and the Mayo Foundation.

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<sup>3</sup> Abbreviations used in this paper: MBP, major basic protein; CS, calf serum; DC, dendritic cell; ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; MPO, myeloperoxidase; PI, propidium iodide; RT, room temperature.



**FIGURE 1.** Human eosinophils are activated by whole *A. alternata* organisms and release granule protein. *A*, Isolated human eosinophils were cultured with germinating *A. alternata* spores (5000/well) for different time periods up to 24 h. After culture, the cell-free supernatants were harvested, and EDN released in the supernatants was measured by ELISA. Results show means  $\pm$  range of duplicate samples from an experiment, which is representative of two independent experiments. *B*, Isolated human eosinophils were cultured with different numbers of germinating *A. alternata* spores for 18 h, and EDN released in the cell-free supernatants was measured by ELISA. Results show means  $\pm$  SEM from three independent experiments. \*\*,  $p < 0.01$ , compared with medium alone (i.e., no *A. alternata* spores). *C*, Eosinophils were cultured with germinating *A. alternata* spores (5000/well) for 18 h. Photomicrograph was taken using an inverted microscope (original magnification,  $\times 400$ ); black arrow indicates the thread-like *A. alternata* hyphae; the eosinophils are translucent ovals; and an *A. alternata* spore appears as a dark oval. *D*, Physical contact between eosinophils and *A. alternata* was blocked with a Transwell system during 18 h of eosinophil-*A. alternata* culture; coculture wells are like those in *A*; and EDN released in the cell-free supernatants was measured by ELISA. Results show means  $\pm$  SEM from three independent experiments.

## Materials and Methods

### Reagents

Curdlan [linear  $\beta$ -(1,3)-glucan] was from WAKO. Anti-dectin-1 Ab and mouse IgG1 control Ab were from R&D Systems. Anti-CD11b mAb, clone 2LPM19c, was from Santa Cruz Biotechnology. Anti-CD11b mAbs, clones 5C6 and M1/70, were from Serotec and eBioscience, respectively. Antilactosylceramide mAb was from Ancell. Anti- $\beta$ -glucan Ab (mouse IgM) was a generous gift from Dr. Jonathan Reichner (Brown University, Medical School, Providence, RI). Control Mouse IgM and chitin from crab shells were from Sigma-Aldrich. Human cytokine Ab array V kit was from RayBiotech. Anti-CD32 mAb, clone IV3, was from Stem Cell Technologies and C3-depleted serum was from Calbiochem. Rhodamine (tetramethylrhodamine isothiocyanate)-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) (minimal cross-reaction to human serum proteins) was from Jackson ImmunoResearch Laboratories. *Alternaria alternata* (ATCC 11680) was cultured on potato dextrose agar (Sigma-Aldrich) at 25°C for 10 days before harvesting spores. After harvesting the spores by flooding the agar dishes with sterile water, they were counted by using a hemacytometer.

### Cell isolation

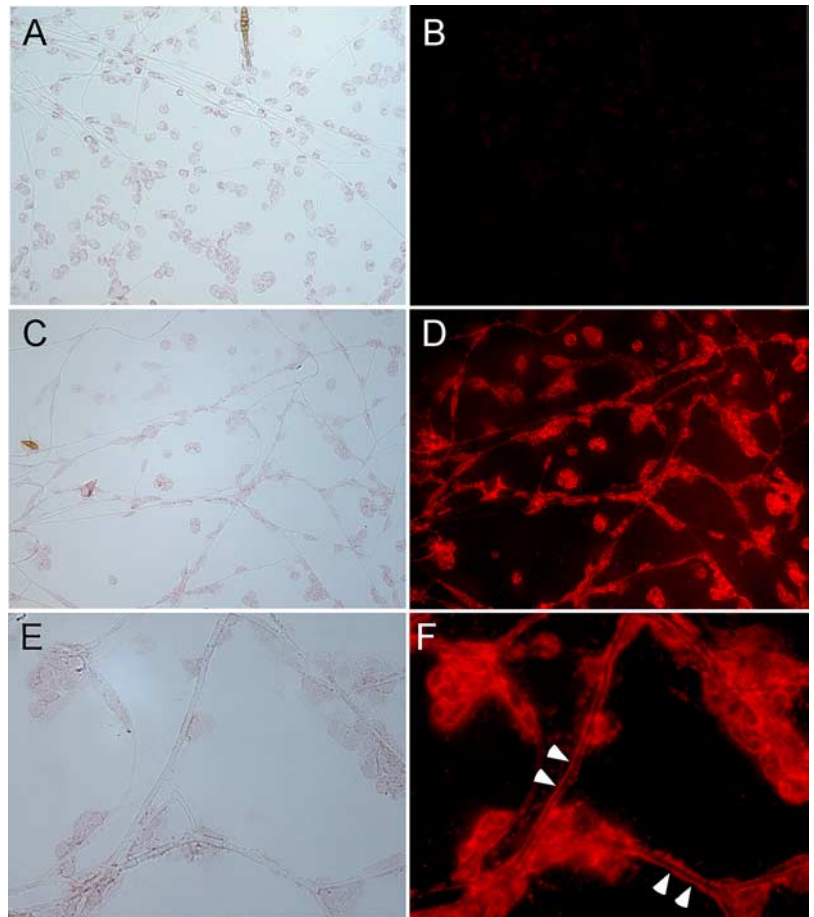
Eosinophils were isolated from the blood of 29 normal and mildly atopic volunteers using negative selection with anti-CD16 microbeads (Miltenyi Biotec) as previously described with only one slight modification (20). Granulocytes were incubated with an equal volume of anti-CD16-conjugated magnetic beads on ice for 30 min. This protocol consistently yielded  $>96\%$  eosinophil purity. Neutrophils were isolated from the blood of the same donors used for eosinophil isolation. The eosinophil isolation protocol referenced above was followed with 10  $\mu$ l of the granulocyte pellet being re-suspended in buffer and then counted using Randolph's stain. This consistently yielded  $\geq 95\%$  neutrophil purity and allowed us to do parallel experiments on neutrophils and eosinophils on the same day from the same donor. Dendritic cells (DCs) were generated by culturing CD14-positive monocytes, which were isolated from the blood of healthy volunteers, with GM-CSF (10 ng/ml) and IL-4 (10 ng/ml) (R&D systems) for 6 days in RPMI 1640 medium supplemented with 10% calf serum (CS). The study was approved by the Institutional

Review Board at the Mayo Clinic, Rochester, MN; all volunteers provided informed consent.

### Eosinophil and neutrophil degranulation assay

To test whether eosinophils respond to live *A. alternata* and release inflammatory mediators, *A. alternata* spores ( $5 \times 10^3$ /well or numbers indicated in the figures) were suspended in HBSS supplemented with 25 mM HEPES, 0.01% gelatin, and heat inactivated 10%  $\alpha$ -CS, and allowed to germinate and to form hyphae in 24-well tissue culture plates overnight at 20°C. On the next day, freshly isolated eosinophils were suspended in HBSS with 25 mM HEPES and 10%  $\alpha$ -CS, and  $1 \times 10^5$  cells were added into each *A. alternata* well and incubated 18 h at 37°C. In some experiments, to examine the kinetics of the eosinophil response, the eosinophil and fungal mixture was cultured from 1 to 24 h. After incubation, cell-free supernatants from the plates were collected and stored at  $-20^\circ\text{C}$  to quantitate EDN released into supernatants. For the EDN ELISA, 96-well flat-bottom plates (Immulon 4HXB; Thermo Electron) were coated with 100  $\mu$ l of murine anti-human EDN mAb (5  $\mu$ g/ml in PBS, clone 167-6C5) and incubated at 4°C overnight; anti-human EDN mAbs (clones 167-6C5 and 167-2G4) were made at Mayo Clinic Rochester. The plates were then washed with wash buffer (0.01% Tween in PBS) using a microplate washer (BioTek Instruments). The wells were blocked with 200  $\mu$ l 1% BSA in assay buffer (0.05% Tween in PBS) for 30 min at room temperature (RT) and washed. Dilutions of the EDN standard or test sample (100  $\mu$ l) were added to the wells and incubated at RT for 2 h. The plates were then washed and incubated with 100  $\mu$ l of HRP-labeled anti-human EDN detection mAb (1/15,000 dilution in PBS, clone 167-2G4) for 30 min at RT. After washing, 100  $\mu$ l of freshly made 3,3',5,5'-tetramethylbenzidine substrate (Pierce Protein Research Products; Thermo Fischer Scientific) was added and incubated for 5–10 min. Immediately after stopping the reaction with 50  $\mu$ l of 1 M  $\text{H}_2\text{SO}_4$ , the absorbance at 450 nm was measured with a microplate reader (SpectraMax Plus; Molecular Devices). The lowest point of the standard curve was 0.09 ng/ml. All assays were conducted in duplicate. To detect cytokines and chemokines produced by activated eosinophils, cell-free supernatants were analyzed by the Human cytokine Ab array V kit, following the procedure recommended by the manufacturer.

**FIGURE 2.** Morphology of eosinophils and *A. alternata* organisms after culture. *A.* *alternata* hyphae and spores were incubated with eosinophils for 18 h. Specimens were fixed and stained with control rabbit IgG (*B*) or anti-MBP Ab (*D* and *F*) followed by Rhodamine-conjugated secondary Ab, as described in *Materials and Methods*. *A* and *C*, The brightfield photomicrographs show eosinophils clustered around the hyphae. *B*, Staining with control rabbit IgG shows no fluorescence signal. *D*, Staining with anti-MBP Ab shows intense MBP staining. *E* and *F*, Higher magnification view (from same field in *C* and *D*) shows clusters of eosinophils containing MBP and also extracellular MBP (arrowheads) coating the hyphae. Original magnification,  $\times 400$ . Figures are representative of two independent experiments.



To examine whether physical contact between *A. alternata* and eosinophils is necessary to activate eosinophils, 24-well Transwell inserts (pore size  $0.4 \mu\text{m}$ ; Costar) were used to prevent contact between *A. alternata* and eosinophils. In other experiments, eosinophils were incubated with supernatants from cultured *A. alternata*. To examine whether eosinophils respond to fungal cell wall components, serial dilutions of  $\beta$ -glucan particles (Curdlan) or chitin particles ( $10$ – $1000 \mu\text{g/ml}$ ) were used as stimuli. To examine the involvement of a  $\beta_2$  integrin, CD11b, in eosinophil activation in response to live *A. alternata* or  $\beta$ -glucan, eosinophils were preincubated with various anti-CD11b mAb or isotype matched control mouse Ig ( $5$ – $10 \mu\text{g/ml}$ ) for 30 min at RT and then exposed to stimulus.

To examine whether neutrophils respond to  $\beta$ -glucan and whether CD11b is involved in their interaction, neutrophils were preincubated with anti-CD11b mAbs or control mouse Ig and they were exposed to *A. alternata* by using the same conditions as described above for the eosinophil experiments. To quantitate neutrophil degranulation, the concentrations of myeloperoxidase (MPO) in cell-free supernatants were measured by using an MPO detection ELISA kit (Hycult Biotechnology) according to the manufacturer's directions.

#### Morphological assessment of eosinophil and *A. alternata* interaction

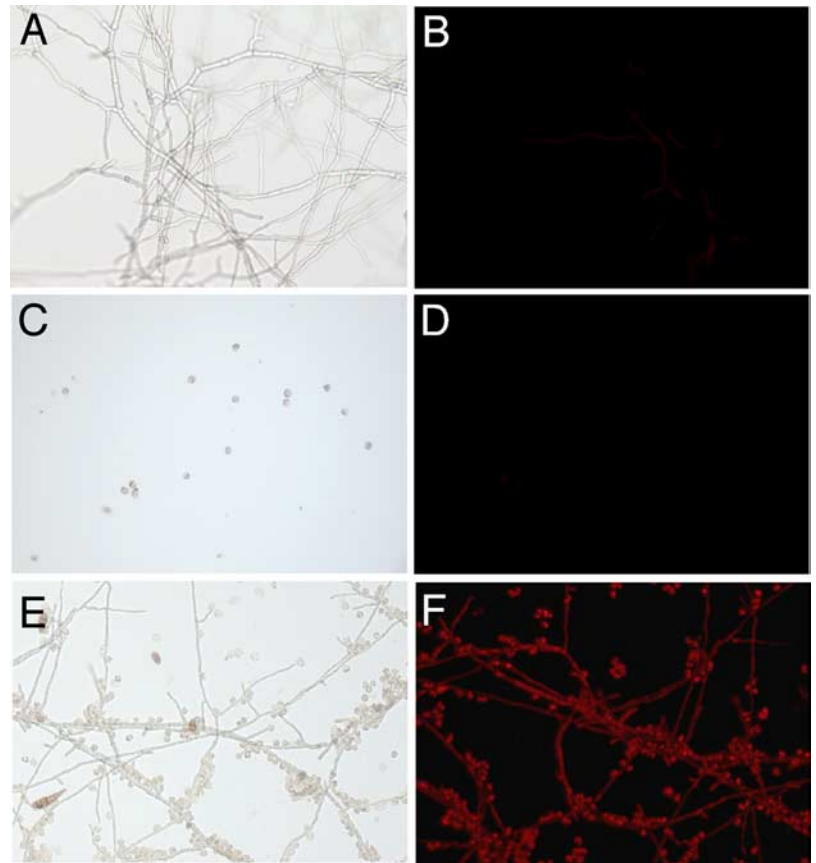
To monitor the interaction between eosinophils and *A. alternata* in detail, we used immunohistochemistry. After eosinophils were cultured 18 h with *A. alternata*, they were mounted on positively charged slides by cytopsin and stained with rabbit anti-human MBP or control rabbit IgG (21). All specimens were incubated in 10% normal goat serum and 1% chromotrope 2R to block nonspecific binding by the second-stage Ab. Rhodamine-conjugated goat anti-rabbit IgG was used as the secondary Ab. The slides were observed using an AxioPhot fluorescence microscope (Carl Zeiss) and recorded by AxioCam HR digital camera and AxioVision 4.0 software (Carl Zeiss). The same camera settings (e.g., exposure time) were used to record images for both control IgG and anti-MBP, and the images presented have not been manipulated.

#### Eosinophil fungicidal activity

*A. alternata* spores ( $5 \times 10^3/\text{well}$ ) were allowed to germinate and to form hyphae in HBSS with 25 mM HEPES, 0.01% gelatin, and heat inactivated 10%  $\alpha$ -CS overnight at  $20^\circ\text{C}$  in 24-well tissue culture plates. The next day,  $1 \times 10^5$  freshly isolated eosinophils were suspended in HBSS buffer with 25 mM HEPES and 10%  $\alpha$ -CS and added into the wells with or without *A. alternata* and incubated 18 h at  $37^\circ\text{C}$ . Cytopsin preparations of eosinophils and *A. alternata* were stained with propidium iodide (PI) for 1 min at RT, following a previously published method with minor modifications (22). Images were recorded immediately by fluorescence microscopy. The viability of *A. alternata* hyphae was determined using fluorescence microscopy to observe  $\geq 50$  hyphae with low magnification ( $\times 100$ ). Hyphae with a length  $\geq$  half of the field were evaluated. Because fungal hyphae are elongated, they were judged PI-positive if roughly  $>75\%$  of the entire visible length of the hyphae was stained with PI. They were judged PI-negative if  $<5\%$  was stained with PI. No equivocal staining with PI (i.e., less than 75% but more than 5% of the piece is stained with PI) was observed.

#### FACS analysis

To examine the expression of dectin-1, lactosylceramide, and CD11b by eosinophils, neutrophils and monocyte-derived DCs, freshly isolated eosinophils ( $1 \times 10^6$  cells), eosinophils primed with IL-5 (1 ng/ml) for 1 h at  $37^\circ\text{C}$ , or freshly isolated neutrophils or cultured DCs were incubated with Fc $\gamma$ R blocking reagent (Miltenyi Biotech) and incubated with anti-dectin-1, anti-lactosylceramide, anti-CD11b mAbs ( $10 \mu\text{g/ml}$ ), or appropriate control mouse Ig for 30 min, followed by FITC-conjugated goat F(ab')<sub>2</sub> anti-mouse IgG Ab (Sigma-Aldrich) for 1 h on ice. To detect intracellular proteins, cells were first fixed and permeabilized using a fixation/permeabilization kit (Caltag Laboratories) followed by primary and secondary Abs, as described above. Cells were fixed with 1% paraformaldehyde and were analyzed using FACSscan (BD Biosciences) and Becton Dickinson lysis II software.



**FIGURE 3.** Eosinophils damage *A. alternata* organisms. Photomicrographs show germinating *A. alternata* organisms after culture with medium alone (A and B), eosinophils cultured with medium alone (C and D), or *A. alternata* organisms after culture with isolated eosinophils (E and F) for 18 h. The preparations of the *A. alternata* in medium (B), of the eosinophils in medium (D), and of the eosinophil-*A. alternata* mixture (F) were stained with PI and observed using fluorescence microscopy. Original magnification,  $\times 100$ . Figures are representative of six independent experiments.

#### Immunofluorescence microscopy of *A. alternata* hyphae

To detect the expression of  $\beta$ -glucan on the surface of *Alternaria*, the spores were allowed to germinate in HBSS with 25 mM HEPES, 0.01% gelatin, and heat inactivated 10%  $\alpha$ -CS overnight at 20°C, as described above. After cytospin, the *A. alternata* was fixed with 3.7% formaldehyde for 20 min at RT. *A. alternata* was then incubated with anti- $\beta$ -glucan mAb or control Ab for 1 h at RT, followed by Texas red-conjugated goat F(ab')<sub>2</sub> anti-mouse IgM Ab (Jackson ImmunoResearch Laboratories). The images were recorded by using a fluorescence microscope, as described above.

#### Statistical analysis

All the error bars represent SEM. Two-sided differences between two samples were analyzed with the Student *t* test. A value of  $p < 0.05$  was considered significant.

## Results

### Human eosinophils recognize the fungus, *A. alternata*, and produce inflammatory responses

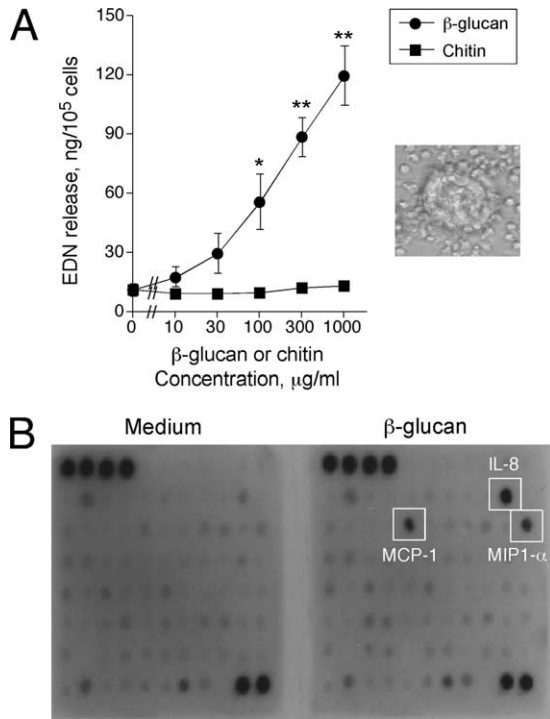
We first examined whether human eosinophils recognize *A. alternata* organisms and release inflammatory mediators. *A. alternata* spores (5000/well) were allowed to germinate and to form hyphae overnight. Freshly isolated eosinophils ( $1 \times 10^5$ /well) were added to the culture and incubated for up to 24 h. Eosinophil degranulation was observed after 3 h of incubation and increased in a time-dependent manner (Fig. 1A); at 24 h,  $\sim 16\%$  of total cellular EDN was released into supernatants. No EDN was released when eosinophils were incubated with medium alone. Dose-response experiments showed that even 625 spores/well were sufficient to induce EDN release at 18 h (Fig. 1B). Morphologically, eosinophils clustered around and bound to *A. alternata* fungal hyphae (Fig. 1C). Because eosinophils appeared bound to *A. alternata* hyphae, we examined whether adhesion between eosinophils and *A. alternata* is necessary for eosinophil activation. Indeed, separation of

eosinophils and *A. alternata* by a Transwell system significantly inhibited eosinophil degranulation induced by *A. alternata* ( $p < 0.05$ ,  $n = 3$ ) (Fig. 1D). Furthermore, eosinophils incubated with culture supernatants of *A. alternata* released minimal EDN ( $8.4 \pm 2.9$  and  $9.9 \pm 1.7$  ng/ $10^5$  cells with medium alone and with *A. alternata* supernatants, respectively, mean  $\pm$  SEM,  $n = 3$ ).

To examine the interaction between eosinophils and *A. alternata* more closely, we used immunohistochemistry to look for eosinophil degranulation in response to the fungal spores and hyphae. As shown in brightfield images (Fig. 2A, C, and E), eosinophils appear to be aggregated on the hyphae and spores. When viewed with a Rhodamine filter, the field in Fig. 2C shows the intense anti-MBP staining (Fig. 2D) that appears localized to the eosinophils and to the hyphae and spores; staining with control rabbit IgG showed no fluorescence (Fig. 2B). Interestingly, using higher magnification, deposition of extracellular MBP is clearly apparent on the fungal hyphae (Fig. 2F). Thus, human eosinophils recognize *A. alternata* in vitro and release proinflammatory and cytotoxic granule proteins, such as EDN and MBP, in a contact-dependent manner.

### Eosinophils damage *A. alternata*

To examine the outcome of the eosinophils' interaction with *A. alternata*, the hyphae, which had been cultured with medium alone or with eosinophils for 18 h, were stained with PI and examined by bright-field and fluorescence microscopy. *A. alternata* hyphae incubated with medium alone were refractile and appeared healthy (Fig. 3A); these hyphae did not stain with PI, indicating minimal damage to the cell wall (Fig. 3B). In addition, eosinophils incubated without *A. alternata* hyphae did not stain with PI (Fig. 3, C and D). In contrast, *A. alternata* hyphae incubated with eosinophils stained strongly with PI (Fig. 3, E and F), suggesting that the

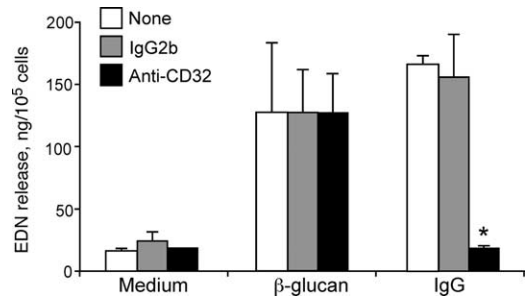


**FIGURE 4.** Eosinophils respond to  $\beta$ -glucan but not to chitin. *A*, Isolated human eosinophils were incubated with medium alone or with serial dilutions of purified chitin particles or purified  $\beta$ -glucan particles (10 ~ 1000  $\mu$ g/ml) for 18 h. EDN released into cell-free supernatants was measured by ELISA. Results show means  $\pm$  SEM from four independent experiments. \*,  $p < 0.05$ ; and \*\*,  $p < 0.01$ , compared with medium alone. The photomicrograph *inset* shows the morphology of eosinophils incubated with the larger  $\beta$ -glucan particles (original magnification,  $\times 400$ ). *B*, Eosinophils were incubated with medium alone or purified  $\beta$ -glucan particles (500  $\mu$ g/ml) for 18 h. Cell-free supernatants were collected and analyzed by a cytokine protein microarray, as described in the *Materials and Methods*. Four dots in the *top row (left)* and two dots in the *bottom row (right)* indicate positive controls provided by the manufacturer. Results are representative from two independent experiments.

integrity of the fungal cell walls has been compromised. The eosinophils, which were incubated with *A. alternata* hyphae, also stained positive with PI (Fig. 3*F*), consistent with previous observations in degranulated eosinophils (23). Four separate experiments showed that damage to fungal cell wall (e.g., PI-positive hyphae) was observed in  $95 \pm 3\%$  of *A. alternata* incubated with eosinophils, compared with only  $5 \pm 2\%$  in *A. alternata* incubated with medium alone (mean  $\pm$  SEM,  $n = 4$ ). Thus, eosinophils likely possess fungicidal activity against *A. alternata*.

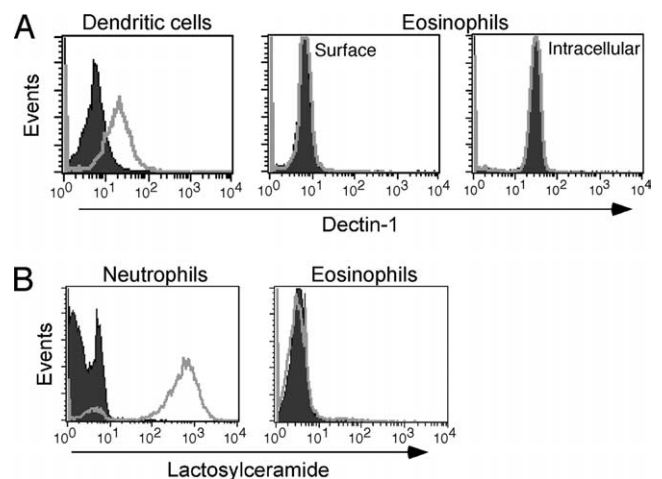
*$\beta$ -glucan, not chitin, is recognized by eosinophils*

The fungal cell wall is composed of various carbohydrates, including manno-glycoprotein,  $\beta$ -glucan, and chitin (24). In particular, the immune recognition of chitin has been recently implicated in the pathogenesis of allergic airway inflammation in mouse models and in patients with asthma (25, 26). Therefore, to identify the fungal cell surface molecule(s) recognized by human eosinophils without the confounding presence of other cell wall molecules, we examined whether isolated chitin or  $\beta$ -glucan can activate eosinophils. After cells were incubated with serial dilutions of chitin or  $\beta$ -glucan (Curdlan) particles ( $\sim 100 \mu$ m diameter), the cell-free supernatants were analyzed for EDN and chemokines. Eosinophils did not degranulate in response to chitin particles up to 1000  $\mu$ g/ml (Fig. 4*A*). In contrast, eosinophils responded vigorously to  $\beta$ -glu-

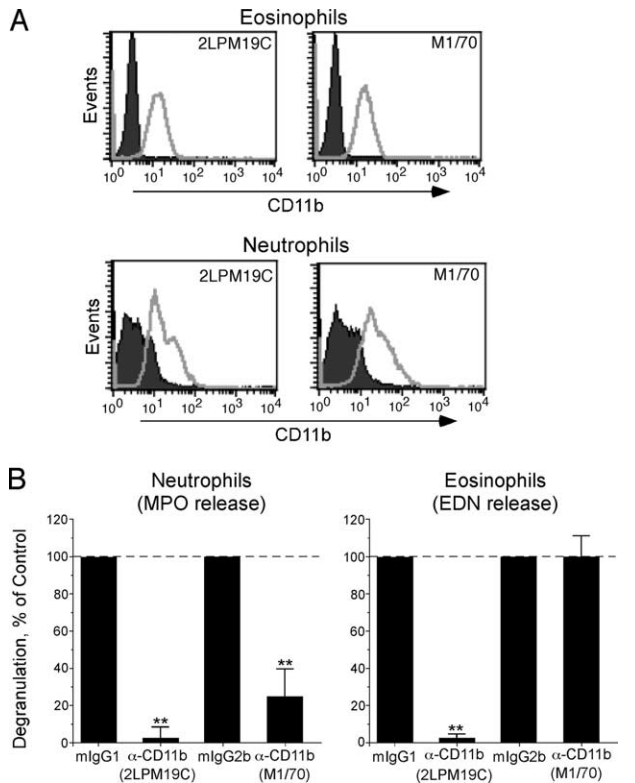


**FIGURE 5.** Fc $\gamma$ RII (CD32) is not involved in the eosinophils' response to purified  $\beta$ -glucan. Isolated human eosinophils were preincubated with medium alone (None), control mouse IgG2b, or anti-CD32 mAb (10  $\mu$ g/ml) for 30 min at RT. Cells were then stimulated with medium alone, purified  $\beta$ -glucan particles (100  $\mu$ g/ml), or human IgG (50  $\mu$ g/ml) immobilized onto the tissue culture plates (IgG) for 18 h. Cell-free supernatants were collected and measured for EDN by ELISA. Results show means  $\pm$  SEM from three independent experiments. \*,  $p < 0.05$  compared with mouse IgG2b treatment stimulated with immobilized human IgG.

can particles at 100  $\mu$ g/ml and released EDN into the extracellular milieu in a concentration-dependent manner ( $p < 0.05$  and  $< 0.01$ ,  $n = 4$ ). Morphologically, eosinophils adhered to the glucan particles (Fig. 4*A, inset*), similarly to the observations with fungal hyphae (vide supra Fig. 1*C*). Furthermore, several chemokines, including MCP-1, IL-8, and MIP-1 $\alpha$ , were clearly detectable by protein microarray in cell-free supernatants of eosinophils after their incubation with  $\beta$ -glucan particles (Fig. 4*B*). Thus, eosinophils appear to release granule proteins and chemokines, when exposed to  $\beta$ -glucan alone in the absence of other fungal cell wall molecules; in other words,  $\beta$ -glucan is sufficient to activate eosinophils. Furthermore, there is likely a selectivity in the eosinophils' responses to different fungal carbohydrate structures, such as  $\beta$ -(1,3)-linked glucose polymers (i.e.,  $\beta$ -glucan) compared with  $\beta$ -(1,4)-linked *N*-acetylglucosamine polymers (i.e., chitin).



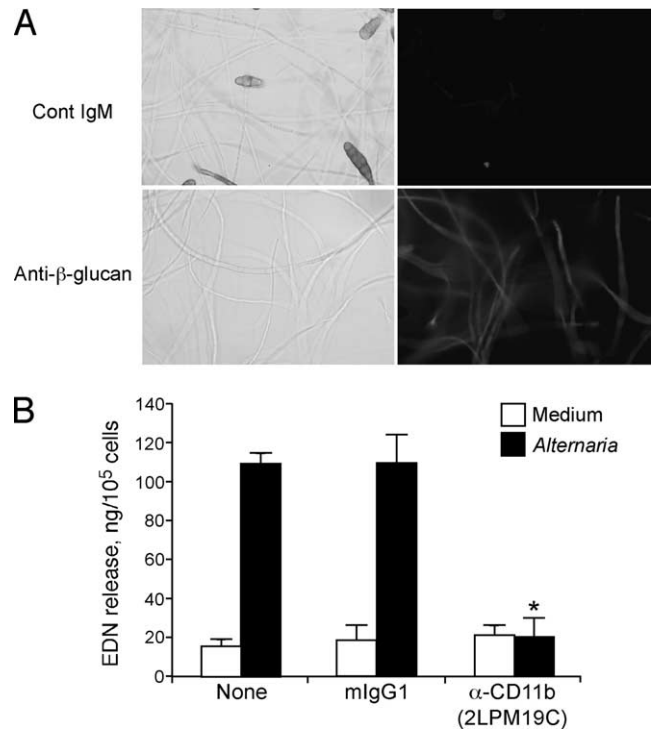
**FIGURE 6.** Eosinophils do not express dectin-1 or lactosylceramide. *A*, Human monocyte-derived DCs or freshly isolated eosinophils were incubated with anti-dectin-1 mAb, followed by PE-conjugated secondary Ab, and were analyzed by flow cytometry. For intracellular staining, eosinophils were fixed and permeabilized, as described in *Materials and Methods*, before staining. *B*, Freshly isolated neutrophils or eosinophils were stained with anti-lactosylceramide mAb, followed by PE-conjugated secondary Ab, and were analyzed by flow cytometry. Figures are representative histograms from three independent experiments.



**FIGURE 7.** A  $\beta_2$  integrin, CD11b, expressed by eosinophils is involved in the cells' response to  $\beta$ -glucan. *A*, Freshly isolated human eosinophils or neutrophils were incubated with anti-CD11b mAbs (clone 2LPM19c and clone M1/70). Cells were stained with PE-conjugated secondary Ab and analyzed by flow cytometry. Representative histograms from two independent experiments are shown. *B*, Neutrophils or eosinophils were preincubated with medium alone, control mouse IgGs (mIgG1 or mIgG2b), or anti-CD11b mAbs (clone 2LPM19c or clone M1/70) (10  $\mu$ g/ml) for 30 min at RT and then incubated with medium alone or purified  $\beta$ -glucan particles (100  $\mu$ g/ml) for 18 h. After incubation, cell-free supernatants were collected and analyzed for MPO (neutrophils) and EDN (eosinophils). Data were normalized to the values with appropriate control mouse IgGs plus  $\beta$ -glucan particles as 100%. Spontaneous levels of MPO and EDN release without  $\beta$ -glucan particles were an average of 38 and 31%, respectively, of the values with control mouse IgGs plus  $\beta$ -glucan particles, and these values were subtracted before normalization. Results show means  $\pm$  SEM from three independent experiments. \*\*,  $p < 0.01$  compared with values with control IgGs.

*In human eosinophils, a  $\beta_2$  integrin, CD11b, is involved in interactions with  $\beta$ -glucan and *A. alternata**

Having established that eosinophils respond to  $\beta$ -glucan, we investigated the molecular mechanisms involved in the eosinophils' interaction with  $\beta$ -glucan and ultimately with *A. alternata* organisms. Initially, because eosinophils express a low affinity IgG receptor, Fc $\gamma$ RII (CD32) (23), we suspected potential roles for the products of acquired immunity, such as Abs. Perhaps the CS used in the eosinophil-*A. alternata* culture contains Abs to  $\beta$ -glucan, and eosinophils may recognize  $\beta$ -glucan through these bound IgG Abs. To test this hypothesis, eosinophils were preincubated with blocking anti-CD32 mAb and then incubated with  $\beta$ -glucan particles or with IgG immobilized onto the tissue culture plates, as a positive control. Eosinophils incubated with  $\beta$ -glucan particles showed marked degranulation; however, the degranulation induced by  $\beta$ -glucan was not affected by anti-CD32 mAb (Fig. 5). Immobilized IgG induced a comparable level of eosinophil degranulation as that observed with  $\beta$ -glucan particles, and degran-



**FIGURE 8.** *A. alternata* organisms express  $\beta$ -glucan on their hyphal surface, and CD11b is involved in the eosinophils' responses to whole *A. alternata* organisms. *A*, *A. alternata* fungal spores were allowed to germinate for 18 h, and then hyphae were stained with control mouse IgM or anti- $\beta$ -glucan followed by Texas red-conjugated secondary Ab. *Left panels*, Brightfield photomicrographs; *right panels*, fluorescence photomicrographs. Original magnification,  $\times 400$ . Figures are representative from three independent experiments. *B*, *A. alternata* spores germinated for 18 h; isolated eosinophils were preincubated with medium alone, control mouse IgG1, or anti-CD11b Ab (clone 2LPM19c) (10  $\mu$ g/ml) for 30 min at RT, and then cultured with medium alone or *A. alternata* organisms for 18 h. After incubation, cell-free supernatants were analyzed for EDN. Results show means  $\pm$  SEM from three independent experiments. \*,  $p < 0.05$  compared with control mouse IgG1 plus *A. alternata*.

ulation induced by IgG was abolished by anti-CD32 mAb. Furthermore, inactivation of complement by heat-inactivating the CS for 56°C for 30 min or by using C3-depleted human sera did not affect eosinophil degranulation induced by  $\beta$ -glucan particles (data not shown). Thus, neither Abs nor complement are likely to be involved in the eosinophils' response to  $\beta$ -glucan particles.

Based on these observations, we suspected potential roles for innate receptors, which recognize the molecular patterns of microbes. Three molecules reportedly recognize  $\beta$ -glucan, including dectin-1, lactosylceramide, and a  $\beta_2$  integrin, CD11b (27). Dectin-1 was clearly detected by FACS on the surface of human DCs, but it was undetectable on the cell surface or the intracellular compartments of human eosinophils (Fig. 6A). Another candidate receptor, lactosylceramide, was abundant on the surface of human neutrophils, but not on the surface of human eosinophils (Fig. 6B). Furthermore, neither dectin-1 nor lactosylceramide were detectable on IL-5-primed eosinophils (data not shown).

Because both the I-domain and lectin-domain of CD11b are involved in glucan recognition by phagocytic cells (28–30), we examined the expression of CD11b and its specific domains by human eosinophils and neutrophils. Two anti-CD11b Ab, namely anti-I-domain mAb (clone 2LPM19c) and lectin-function blocking mAb (clone M1/70), clearly detected CD11b expressed on the eosinophils' surface (Fig. 7A). Similar levels of CD11b expression

were also observed on neutrophils. To examine the functional relevance of these CD11b molecules, neutrophils or eosinophils were preincubated with anti-CD11b Abs and exposed to  $\beta$ -glucan particles. Neutrophil MPO release induced by  $\beta$ -glucan was inhibited by both the anti-I-domain mAb (clone 2LPM19c) and, as expected, by the lectin-function blocking mAb (clone M1/70) (Fig. 7B), suggesting that both the I-domain and lectin-domain are involved in the neutrophils' response to  $\beta$ -glucan. The anti-I-domain mAb (clone 2LPM19c) also abolished eosinophil degranulation induced by  $\beta$ -glucan particles (Fig. 7B). In contrast, the lectin-function blocking mAb (clone M1/70) showed no effects on  $\beta$ -glucan-induced eosinophil degranulation, suggesting that the I-domain but not the lectin-domain of CD11b is involved in the eosinophils' response to  $\beta$ -glucan.

Finally, we examined whether these same CD11b molecules are involved in the eosinophils' interaction with *A. alternata* organisms. Immunohistochemical analysis using anti- $\beta$ -glucan Ab clearly showed that  $\beta$ -glucan is expressed on the surface of *A. alternata* hyphae (Fig. 8A). Furthermore, eosinophil degranulation induced by *A. alternata* was completely inhibited by preincubation of eosinophils with anti-I-domain mAb (clone 2LPM19c) (Fig. 8B). Thus, CD11b, in particular its I-domain, likely plays a pivotal role in how eosinophils recognize and/or interact with  $\beta$ -glucan as well as *A. alternata* organisms.

## Discussion

The key observation in this study is the immunological response of human eosinophils against the common environmental nonpathogenic fungus *Alternaria alternata*. Eosinophils may possess anti-helminth immune effector functions (2, 31–33). Indeed, the cytotoxic granule proteins of eosinophils (e.g., MBP) bind strongly to the parasite targets and directly damage schistosomules of *Schistosoma mansoni* (34) and other parasites (35). Interestingly, MBP and ECP also exhibit robust antimicrobial activities against certain bacteria, such as *Staphylococcus aureus* and *Escherichia coli* (36). Furthermore, EDN and ECP possess anti-viral activities against respiratory syncytial virus (37). Although the immunological activities of eosinophil granule proteins have been relatively well characterized, little has been described regarding the immunological functions of eosinophils. We found that eosinophils respond vigorously to the common environmental fungus *A. alternata*. Eosinophils bound to whole *A. alternata* organisms, in particular their hyphae (Fig. 1), released cytotoxic granule proteins (Figs. 1 and 2) and damaged the fungus (Fig. 3). Although the biological significance of these observations needs to be verified in animal models and in humans, these findings strongly suggest that eosinophils have the capacity to recognize and exert immunological responses to certain fungi. Thus, the innate immune functions of eosinophils may be more diverse than previously recognized.

$\beta$ -glucan is one of the major cell wall components of fungus and is detected by several pattern recognition receptors, such as dectin-1 and lactosylceramide, as well as by CD11b (38).  $\beta$ -glucan is involved in antifungal immune responses in vivo (39, 40). Previously, we found that the soluble protease-like molecules secreted by *A. alternata* activate eosinophils likely through the protease-activated receptor-2 (41). We now report that eosinophils directly recognize purified  $\beta$ -glucan and release granule proteins (Fig. 4A), and these eosinophils actively produce and release several chemokines (Fig. 4B). Thus, there seems to be at least two fundamental, but nonexclusive, mechanisms involved in the eosinophils' responses to fungal organisms, including the recognition of secreted and soluble proteases and of  $\beta$ -glucan expressed on the cell wall. Chitin is also a major cell wall component of fungi. A recent report showed that chitin activates macrophages; this activation is essen-

tial to recruit IL-4-producing eosinophils and basophils into the lungs in vivo in mice (14). In contrast, purified chitin did not activate eosinophils, even at high concentrations in our study (Fig. 4). Furthermore, an authentic receptor for chitin, namely the mannose receptor, was undetectable on eosinophils (data not shown). Therefore, different innate immune cells, such as macrophages/DCs, neutrophils, and eosinophils, may recognize different fungal cell wall components (and maybe different genera of fungi) and may produce distinct immunological functions, including the recruitment of inflammatory cells, production of inflammatory mediators, and elimination of the organisms.

Eosinophils likely use a versatile  $\beta_2$  integrin, CD11b, to recognize and/or to interact with  $\beta$ -glucan and *A. alternata* hyphae, while they lack expression of dectin-1 and lactosylceramide (Fig. 6). Furthermore, unlike neutrophils, the eosinophil shows limited expression of TLR-2 (42), which can recognize yeast zymosan (43). Indeed, anti-CD11b blocking mAb (clone 2LPM19c) completely inhibited eosinophil activation induced by purified  $\beta$ -glucan and whole *A. alternata* organisms (Figs. 7B and 8B), suggesting a pivotal role for CD11b in the eosinophils' response to filamentous fungi. We initially suspected that serum components, which directly bind to  $\beta$ -glucan, might be involved in how eosinophils recognize fungi. For example C3bi, one of the major serum proteins in the complement system, opsonizes target pathogens, which then can be recognized by the I-domain of eosinophil CD11b (44, 45). Serum could also contain anti- $\beta$ -glucan Ab, which binds to  $\beta$ -glucan on *A. alternata*, and the bound Ab is subsequently recognized by the Fc receptor of eosinophils, leading to eosinophil activation in a  $\beta_2$  integrin-dependent manner (46). However, depletion of C3bi or heat activation of serum in culture medium did not affect the eosinophils' response to *A. alternata* organisms nor did Fc $\gamma$ RII blocking Ab (Fig. 5). Thus, the  $\beta$ -glucan molecules on the *A. alternata* cell wall are likely recognized directly by eosinophil CD11b, although the potential involvement of unknown serum-derived intermediate molecules cannot be excluded.

Interestingly, there were clear differences in the various anti-CD11b mAbs used to block eosinophil and neutrophil responses to  $\beta$ -glucan. An anti-I-domain Ab (clone 2LPM19c) prevented the activation of both eosinophils and neutrophils stimulated with purified  $\beta$ -glucan. In contrast, an anti-CD11b mAb (clone M1/70), which blocks the lectin-domain function of CD11b, and another lectin-domain blocking mAb (clone 5C6) (data not shown) failed to block eosinophil activation by  $\beta$ -glucan, but the clone M1/70 did inhibit neutrophil activation by more than 70% (Fig. 7B). Both clones, 2LPM19C and M1/70, bound similarly to eosinophils and neutrophils (Fig. 7A). Then, how can we explain the different inhibitory effects of anti-CD11b blocking mAbs, in particular the differential effects of the Abs, which block lectin-domain function on eosinophils and neutrophils? In eosinophils and neutrophils, CD11b may have different carbohydrate modifications or different surface interacting molecules or both. For example, CD11b may be physically associated with a low affinity IgG receptor, Fc $\gamma$ RIII (CD16), which is expressed abundantly on neutrophils but shows limited expression on eosinophils (47, 48). In contrast, we found recently that a GPI-anchored protein, CD66b, is constitutively associated with CD11b and plays a critical role in eosinophil activation (49). Therefore, while CD11b is expressed by both eosinophils and neutrophils, it may be involved differently in the effector functions of these cell types. In general, the lectin-domain of CD11b is considered essential for glucan recognition by phagocytic cells (28–30). Therefore, a major question still remains: how does the CD11b on eosinophils recognize and/or interact with  $\beta$ -glucan without the involvement of a lectin-domain?

Fungi, including *A. alternata*, are multicellular organisms and express various biological molecules during different stages of

their lives. Therefore, the mechanisms involved in the immunological and inflammatory responses against fungi are likely complex. Furthermore, fungi are genetically highly diverse; thus, the immunological responses to them may also be diverse. For example, *Candida* spores are small and phagocytosable (50); in contrast, *A. alternata* spores are much larger (~50  $\mu\text{m}$ ) than the size of leukocytes. This study shows for the first time that eosinophils demonstrate antifungal immune responses with intact *A. alternata*. The potential clinical implications of this observation and the further elucidation of the pathophysiology of human diseases are likely substantial. Apparently, the innate immunity exerted by human eosinophils may reach beyond parasitic helminths and may extend to other nonphagocytosable organisms such as filamentous fungi. Furthermore, recent studies suggest that the fundamental pathogenesis of asthma and allergic responses may be dysregulated immune responses to chitin-encased insects and fungi (15, 16), which were evolutionally developed to protect against chitin-encased parasites. The immunological responses of eosinophils to both parasitic helminths and fungi fit perfectly with this model and lead us to speculate that eosinophils may play an important role in such dysregulated immune responses. Several epidemiological studies also implicate exposure or sensitization to *Alternaria* in the development and exacerbation of allergic airway diseases (17–19). Thus, dysregulated immune responses of eosinophils to *A. alternata*, other filamentous fungi, and potentially other chitin-encased insects, such as mites and cockroaches, may play a pivotal role in chronic inflammation and the pathology of the airways in human disease, such as asthma.

## Acknowledgments

We thank Diane L. Squillace, James L. Checkel, and Gail M. Kephart for technical assistance, Cheryl R. Adolphson for editorial assistance, and LuRaye S. Eischens for secretarial help.

## Disclosures

The authors have no financial conflict of interest.

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