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Fungal Proteases Induce Th2 Polarization through Limited Dendritic Cell Maturation and Reduced Production of IL-12

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Allergens are capable of polarizing the T cell immune response toward a Th2 cytokine profile in a process that is mediated by dendritic cells (DCs). Proteases derived from *Aspergillus* species (*Aspergillus* proteases; AP) have been shown to induce a Th2-like immune response when administered directly to the airway and without adjuvant or prior priming immunizations at sites remote from the lung in models of allergic airway disease. To explore mechanisms that underlie the Th2 immune response, we have investigated the effect of AP on DC function. We found that human DCs derived from $CD14^+$ monocytes from healthy donors underwent partial maturation when incubated with AP. Naive allogeneic T cells primed with AP-activated DCs proliferated and displayed enhanced production of IL-4 and reduced expression of IFN-γ as compared with naive T cells primed with LPS-activated DCs. Global gene expression analysis of DCs revealed relatively low expression of IL-12p40 in AP-activated DCs as compared with those activated by LPS, and this was confirmed at the protein level by ELISA. Exogenous IL-12p70 added to cocultures of DCs and T cells resulted in reduced IL-4 and increased IFN-γ expression when DCs were activated with AP. When the proteolytic activity of AP was neutralized by chemical inactivation it failed to up-regulate costimulatory molecules on DCs, and these DCs did not prime a Th2 response in naive T cells. These findings provide a mechanism for explaining how proteolytically active allergens could preferentially induce Th2 responses through limited maturation of DCs with reduced production of IL-12. ***The Journal of Immunology,*** **2008, 180: 6000–6009.**

Dendritic cells (DCs)[2] are class of professional APCs that are required for initiating and enhancing the immune T cell response. They differ from other APCs by their capacity to initiate in vivo primary T cell stimulation and to induce the polarization of the immune response toward a Th1 or a Th2 profile (1, 2). Several factors have been shown to influence Th polarization and recruitment in airway inflammation including the type of Ag encountered by the DC (3), the environment in which the DC has been stimulated (4), and the origin or the type of DC involved in the interaction with naive CD4 T cells (5). Furthermore, we have reported that allergens capable of inducing a Th2-like immune response may be grouped into two functional classes when administrated directly to the airway in a mouse model. One class of allergen, which includes OVA, requires priming immunizations at sites remote from the lung. In contrast, the fungal allergen AP does not require remote priming to overcome the innate resistance of the airway to Th2 immune response activation and allergic inflammation (6).

The mechanisms by which DCs differentially regulate Th1 and Th2 immune responses are not well understood. DCs express an array of costimulatory and adhesion molecules and it has been suggested that differential expression of such surface molecules by DCs influence polarization of naive T cells. For instance, level of CD86 expression appears to be more important than that of CD80 for the induction of a Th2 response (7–9). Similarly, expression of CD40, OX40 ligand, and inducible costimulatory ICOS ligand on DCs may also favor Th2 differentiation (10–12).

Functional subsets of DC have also been proposed to play a role in differentially promoting the development of Th1 and Th2 cells. In humans, DC1 derived from monocytes induce Th1 responses, whereas DC2 generated from plasmacytoid monocytes cultured in vitro with CD40L induce Th2 responses (13). Other studies have introduced additional factors that might contribute to the ability of DC subsets to elicit polarized Th cell differentiation, including maturation or activation state of DC (14), and Ag dose (15). Furthermore, it has been reported that individual Ags can stimulate myeloid DCs to induce either Th1 or Th2 immune responses dependent on the production of IL-12 (16, 17), a soluble factor that has been implicated in the development of naive precursors into Th1 cells upon activation with LPS (18) or derived signals such as CD40L costimulation (17). In addition, the level of IL-12 production by DCs can be down-regulated by microenvironmental factors such as IL-10, TGFβ, corticosteroids, vitamin D_3, or PGE_2, thus converting the DCs from Th1- to Th2-polarizing APCs (16, 19).

In the current study, we have explored mechanisms by which AP-treated DCs initiate and maintain a Th2 immune response. We found that the Th2-polarizing effect of AP may be at least partially mediated by up-regulation of DC costimulatory molecules on a background of relatively low levels of IL-12p40 production, and the proteolytic activity of AP is required for this function.

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[2] Abbreviations used in this paper: DC, dendritic cell; rh, recombinant human; AP, *Aspergillus oryzae*-derived protease; poly(I:C), polyinosinic-polycytidylic acid; C_t, threshold cycle; iDC, immature DC.

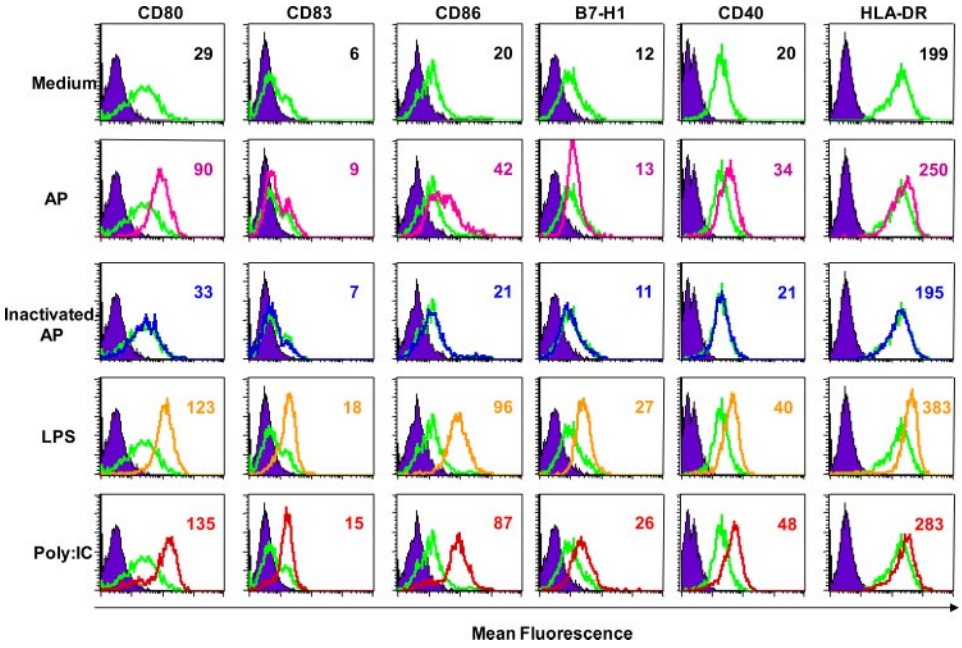

FIGURE 1. Effect of AP on DC maturation. Immature DCs were untreated (medium, green line), stimulated with active AP (20 μg/ml, purple line), treated with inactive AP (20 μg/ml, blue line), LPS (1 μg/ml, orange line), or poly(I:C) (25 μg/ml, red line). Surface marker expression was analyzed after 24 h using flow cytometry. Medium control DCs served as the reference for all other culture conditions. Numbers indicate the net mean fluorescence intensity. Results are representative of ten independent experiments.

Materials and Methods

Reagents and Abs

Recombinant human IL-4 (rhIL-4), and GM-CSF (rhGM-CSF) were purchased from R&D Systems. Purified LPS (*Salmonella abortus* equi; <1% protein; 10^6 EU/mg) and *Aspergillus oryzae*-derived proteases (AP; Lot no. 102K0989, 3.7 U/mg solid) were obtained commercially (Sigma-Aldrich) and reconstituted to 0.5 mg/ml and 10 mg/ml in water and PBS, respectively. IL-12p70 (rhIL-12p70) and FITC-, PE-, and PE-Cy5-, or APC-conjugated mAbs to HLA-DR, CD1α, CD80, CD83, CD86, B7-H1, CD40, CD11c, CD4, IL-4, IL5, IL-13, and IFN-γ were purchased from BD Bioscience.

Preparation of inactivated AP

Metalloproteinase activity of AP was inhibited >96% by repeated addition of phosphoramidon (1 mM; Roche Applied Science), 1, 10-phenanthroline (1 mM; Sigma-Aldrich), EDTA (1 mM; Sigma-Aldrich), and arphamenine (10 μM; Sigma-Aldrich) for 2 h at room temperature followed by overnight dialysis against PBS (15). Endotoxin content of AP was evaluated (0.96 EU corresponding to 20 μg/ml AP) with QCL-1000 chromogenic *Limulus* amoebocyte lysate assay (Bio-Whittaker) according to the manufacturer's instructions. The total amount of protease was determined using quenched fluorescein-casein substrate (Molecular Probes). Enzymatic release of fluorescent signals was quantified by a microplate fluorometer BMG Fluostar Galaxy V4.30.0 (BMG Labtechnologies) according to the manufacturer's instructions and data was expressed as percentage of inhibition (6).

Generation of cultured human DC

Monocyte-derived DCs were generated as previously described (20). Human PBMCs were separated from heparinized buffy coats from healthy donors (Gulf Coast Regional Blood Center, Houston, Texas) by standard gradient centrifugation with Ficoll-Hypaque (Amersham-Pharmacia). The low density PBMCs were harvested and $CD14^+$ cells were positively selected using CD14-microbeads and AutoMacs equipment (Miltenyi Biotec). To induce DC differentiation, 10^6 cells/ml $CD14^+$ monocytes were cultured in complete medium (RPMI 1640-10% FBS), 500 IU/ml human rGM-CSF (R&D Systems), and 400 IU/ml human rIL-4 (R&D Systems) at 37°C under 5% CO2. On day 2 or 3, the DC cultures received an additional dose of GM-CSF and IL-4. On day 5, nonadherent DCs were harvested by gentle pipetting and recultured with or without indicated stimuli in the presence of 1 μg/ml LPS, 25 μg/ml polyinosinic-polycytidylic acid (poly(I:C)) (Sigma-Aldrich), and 0.2–2-20 μg/ml AP. Aliquots of DC culture supernatants were assayed for cytokine release by ELISA (R&D Systems).

Analysis of cell surface markers

Surface expression of DC maturation markers was analyzed using multicolor flow cytometry. DCs (either untreated or stimulated for 24 h with LPS, poly(I:C), or AP) were harvested, washed, and suspended in cold PBS containing 5% FCS and 0.05% NaN_3. They were then incubated with saturating concentrations of FITC-, PE-, and PE-Cy5-, or APC-conjugated mAbs. Stained cells were analyzed using a FACSCalibur flow cytometer equipped with CellQuest software (BD Biosciences).

DC-T cell cocultures

Allogeneic $CD4^+$ T cells were enriched using the CD4 T cell isolation kit II (Miltenyi Biotec) according to the manufacturer's instructions. DCs activated for 24 h were washed and cocultured with allogeneic naive $CD4^+$/$CD45RA^+$ T cells (10^5cells/well) in complete RPMI 1640 culture medium. Cell proliferation was quantified by pulsing the cell for 16 h with 1 μCi of [^{3}H]thymidine (Amersham Biosciences) before being collected and assessed for radioactive incorporation. To analyze T cell polarization, naive CD4 T cells were cocultured with allogeneic DCs that had been stimulated with LPS, poly(I:C), or AP (proteolytically-active or -inactivated AP, see above). The cells were incubated in a 96-well plate at a DC:T ratio of 1:4.

Table I. *The effect of AP and other treatments on DC maturation*

Marker	Medium	AP	Inactivated AP	LPS	Poly(I:C)	P Value[a] (n = 10)
CD80	39.3 ± 2.5	78.1 ± 7.4	41.6 ± 2.4	128.1 ± 8.1	113.7 ± 10.3	0.001
CD83	16.1 ± 2.8	26.9 ± 3.3	16.2 ± 2.4	40.8 ± 3.8	42 ± 4.4	0.052
CD86	24.7 ± 2.6	64.2 ± 7.4	26.4 ± 2.7	129.9 ± 24.3	126 ± 21.4	0.001
CD40	25.8 ± 2.4	41.3 ± 4	25.8 ± 2.8	56.3 ± 6.1	57.9 ± 4.3	0.05
B7-H1	13 ± 1.5	23.3 ± 5.7	13.9 ± 2	31.2 ± 6.3	32 ± 6.2	0.074
HLA-DR	331.6 ± 57.6	669.2 ± 164.3	331.9 ± 54.1	924.3 ± 219.7	894.2 ± 214.6	0.001

[a] Statistical analyses were performed for costimulatory molecules, self-peptide HLA-DR, and maturation markers expression in treated iDCs using two-way ANOVA analysis in combination with Bonferroni post test. The mean fluorescence intensity values ± SEM for the indicated marker are provided.

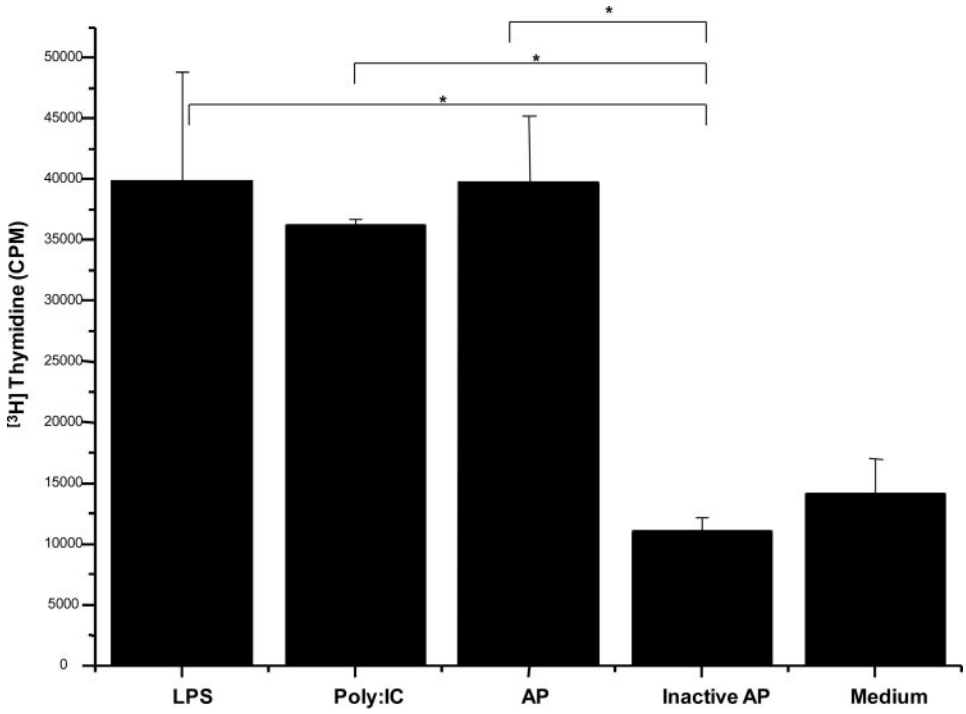

FIGURE 2. AP induces allo-stimulation activity of DCs. DCs (2.5×10^4) untreated (medium), treated with AP or inactive-AP (20 μg/ml), LPS (1 μg/ml), or poly(I:C) (25 μg/ml) were incubated together with allogeneic naive T cells for 7 days. T cell proliferation was measured by [^{3}H]thymidine incorporation after 16 h of culture. Results are given as mean cpm $\pm$ SD triplicate cultures and are representative of three independent experiments from different healthy donors (*, $p < 0.001$).

In some experiments, naive CD4 T cells were cultured with immobilized anti-CD3 (10 μg/ml, UCHT1; BD Biosciences), anti-CD28 mAbs (1 μg/ml, CD28.2; BD Biosciences), and rIL-2 (50 IU/ml; BD Biosciences) in the presence of either rIL-4 (50 ng/ml; BD Biosciences) and anti-human IL-12 mAb (p40/p70, 10 μg/ml, C8.6; BD Biosciences) for Th2-polarizing conditions, or rIL-12 (50 ng/ml; BD Biosciences) and anti-human IL-4 mAb (10 μg/ml, MP4–25D2; BD Biosciences) for Th1 polarizing condition. LPS- and poly(I:C)-activated DCs were harvested, washed, and used for priming naive CD4 T cells into Th-1 polarized T cells. In addition, neutralizing anti-IL-4 and anti-IL-12 mAbs with or without recombinant human IL-12 (rhIL-12, p70 heterodimer; BD Biosciences) were added at the beginning of the DC-T cell coculture to generate a strong Th1 and Th2 polarization response, respectively. Quantification of cytokine production (IL-6, IL-8, IL-10, IL-12p40, TNF-α, MIP-1β, and GM-CSF) was performed using R&D DuoSet ELISA development kits (R&D Systems).

Analysis of cytokine production and intracellular staining

In primary culture, naive CD4 T cells were cocultured with allogeneic stimulated DCs or in presence of cytokines and Abs polarizing Th1 (IL-12 and anti-IL-4 mAb) or Th2 (IL-4 and anti-IL-12 mAb) conditions for 7

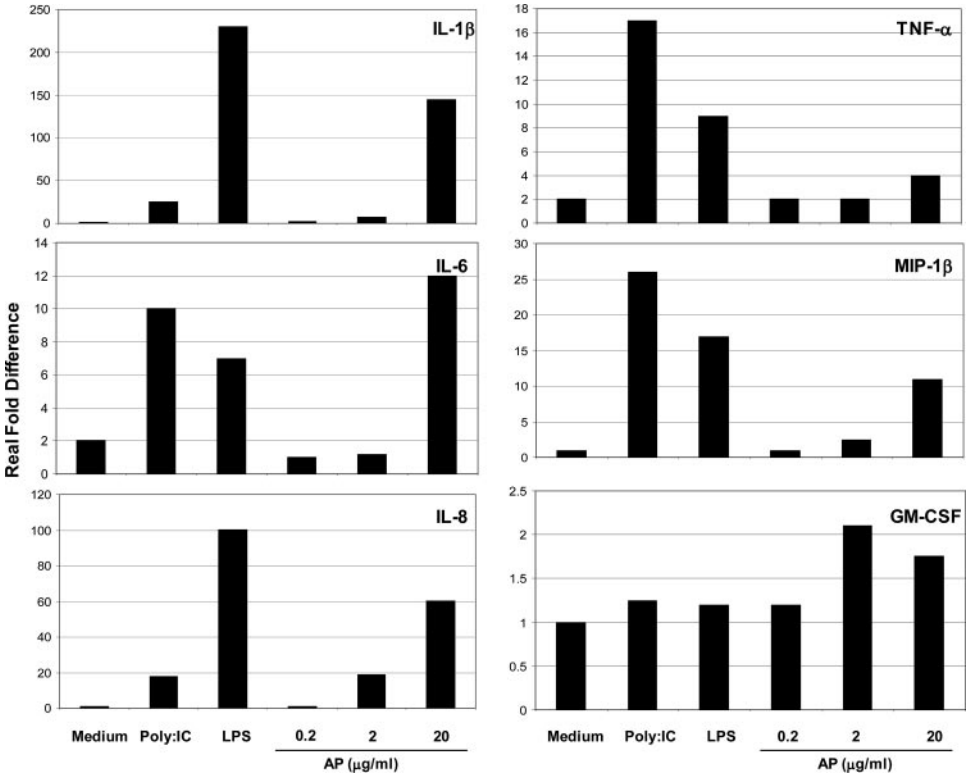

FIGURE 3. AP induced low up-regulation of proinflammatory cytokine genes. Immature DCs (4×10^6) were untreated or treated with increasing concentrations of AP, LPS (1 μg/ml), or poly(I:C) (25 μg/ml). Total mRNA was collected 5 h later and subjected to quantitative PCR analysis for the cytokines IL-1β, IL-6, IL-8, TNF-α, MIP-1β, and GM-CSF mRNA expression. Data are expressed as real fold difference, as described in methods and are representative of four independent experiments.

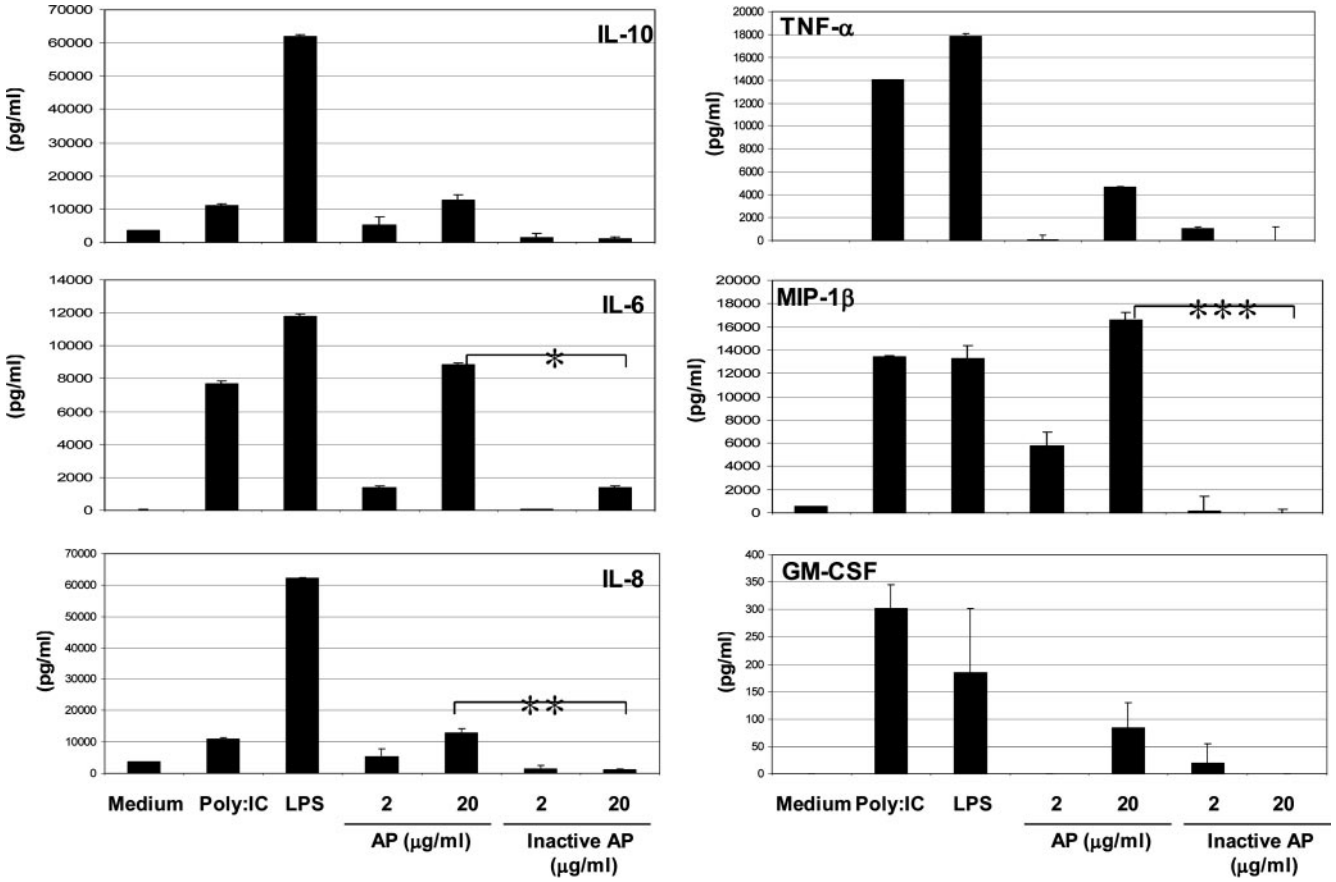

FIGURE 4. Effect of DCs that have been matured with LPS, poly(I:C) and AP. Immature DCs (4×10^6) were untreated or treated with increasing concentrations of AP, LPS (1 μg/ml), or poly(I:C) (25 μg/ml). Whole conditioned medium was collected 24 h later and subjected to an ELISA quatification for IL-10, IL-6, IL-8, TNF-α, MIP-1β, and GM-CSF cytokine production. Data are given as mean (pg $\pm$ SD) of triplicate cultures and are representative of three independent experiments from three different healthy donors (*, $p < 0.005$; **, $p < 0.03$; ***, $p < 0.001$).

days. Expanded T cells were then washed and stimulated again with immobilized anti-CD3 and anti-CD28 mAbs for 48 h. Culture supernatants were collected and cytokine production was assessed by ELISA for IL-2, IL-4, IL-5, IL-13, IFN-γ, and TNF-α (R&D Systems). After one week of culture, T cells were stimulated again with PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (500 ng/ml; Sigma-Aldrich) for 6 h. To prevent cytokine secretion, Brefeldin A (1:1000, eBioscience) was added for the final 2 h. Intracellular cytokine staining was performed using PE-conjugated mAbs to IL-4, IL-5, IL-13, and FITC-conjugated IFN-γ (all from BD Biosciences) as described (21).

RNA isolation and quantitative RT-PCR

Total RNA samples from DCs that were stimulated for 5 h were isolated using the RNeasy kit including DNase digestion (Qiagen). The cDNA templates were synthesized from RNA samples using GeneAmp RNA PCR kit (Applied Biosystems). Oligonucleotide primers were selected from the nucleotide sequences using Primer Express 2.0 (Applied Biosystems). Real-time quantitative PCR was performed with the ABI Prism 7900 (Applied Biosystems) sequence detection system, using TaqMan reagents, according to the manufacture's instructions. Equal amounts of each RNA sample were used as PCR templates in reactions to obtain the threshold cycle (C_t). The C_t was normalized using the known C_t from GAPDH RNAs to obtain ΔC_t. To compare the relative levels of gene expression in different cells, ΔC_t values were calculated by using the ΔC_t values associated with the lowest expression levels as the basis. ΔC_t values were then transformed to the real fold increase in expression by $2^{\Delta Ct}$.

Microarray analysis

Total RNA was isolated from human immature DCs using the RNeasy Mini kit according to the manufacturer's instruction (Qiagen). Biotinylated cDNA probes were generated and hybridized to the Affymetrix Genechip Human Genome U133 set (HG-U133) according to the manufacturer's protocol (Affymetrix). Scanned images of hybridized chips were aligned and analyzed using the Affymetrix GeneChip software MAS 5.0, according to the manufacturer's directions. Signal intensity for IL-12p35 and IL-12p40 cDNA was calculated as the difference of filtered perfect match probes minus mismatch probes. The signal intensities were normalized to the mean intensity of all the genes represented on the array, and global scaling (scaling to all probe sets) was applied before comparison analysis.

Statistical analysis

The paired Student *t* test was used to compare proliferation levels and cytokine production between AP and each individual other DC treatments. Statistical significance of AP's effect as compared with LPS and poly(I:C) treatments on CD80/CD83/CD86/CD40/B7-H1 and HLA-DR up-regulation was calculated with two-way ANOVA analysis in combination with Bonferroni post test (Graph Pad Prism version 4.00 for Windows). Values of $p \leq 0.05$ were considered statistically significant.

Results

Characterization of AP-treated human DCs

Isolated $CD14^+$ monocytes were cultured for 5 days with GM-CSF and IL-4, and then analyzed for the expression of DC markers CD1α, CD11c, and HLA-DR to confirm that they displayed a profile characteristic of immature DCs (iDCs). The cultured monocytes thus treated expressed high levels of HLA-DR, CD1α, and CD11c and low levels of costimulatory molecules (CD80 and CD86) (data not shown). To determine the effect of AP on the maturation and function of human DCs, iDCs from healthy donors were treated with AP for 24 h. Exposure to AP induced moderate up-regulation of costimulatory molecules (CD80/CD86/CD40) and self peptide HLA-DR expression in iDCs, whereas other maturation markers (CD83, B7-H1) remained unchanged (Fig. 1). In

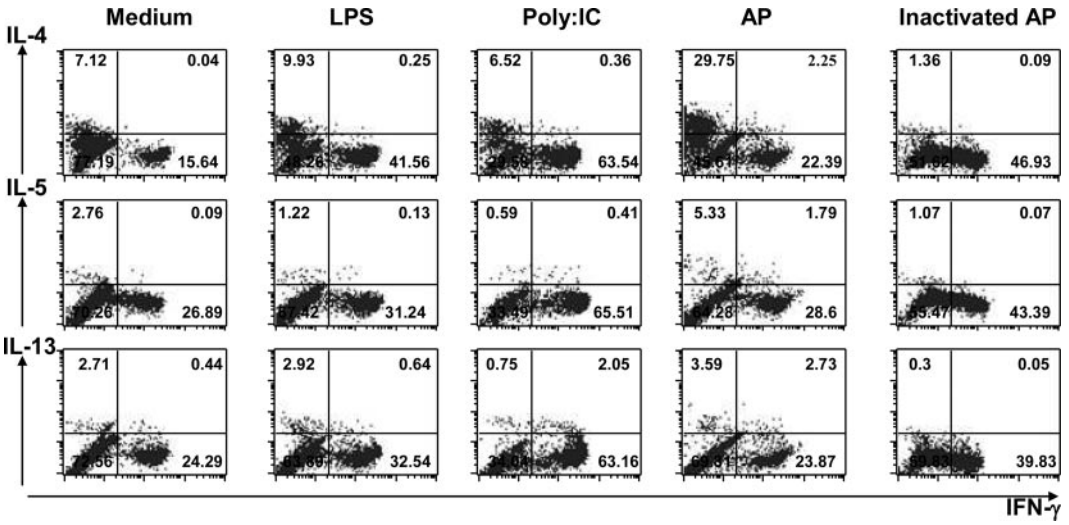

FIGURE 5. Analysis of cytokine expression by DC-primed naive CD4 T cells. Purified naive CD4 T cells were cultured with allogeneic untreated iDCs (medium alone) or iDCs treated with, AP (20 μg/ml), inactivated AP (20 μg/ml), LPS (1 μg/ml), or poly(I:C) (25 μg/ml) for 7 days. The expanded cells were stimulated again with PMA plus ionomycin for intracellular cytokine expression analysis. Data represent one of three independent experiments from one of three donors with similar results. Numbers within the quadrants indicate the percentage of the expanded cells that stained positive for each cytokine (IL-4, IL-5, IL-13, or IFN-γ).

contrast, when iDCs were stimulated with either LPS or poly(I:C), all maturation markers and HLA-DR expressions were highly up-regulated (Fig. 1). The effect of AP on CD80/CD86 and HLA-DR up-regulation as compared with LPS or poly(I:C) treatment was significantly reduced ($p < 0.001$), but for CD40 this difference was at the limit of significance ($p = 0.05$), whereas other markers (CD83, B7-H1) showed no significant difference (Table I). To evaluate whether the protease activity of AP is required for the modulation of costimulatory and self peptide HLA-DR expression, DCs were treated with AP whose protease activity had been chemically inactivated using protease inhibitors (see *Materials and Methods*). Inactivation of protease activity abolished the ability of the AP to up-regulate costimulatory molecules and self-peptide HLA-DR expression (Fig. 1). The change in surface marker expression appeared to correlate with allostimulatory capacity of DCs. AP-treated DCs when incubated with naive allogeneic $CD4^+CD45RA^+$ T lymphocytes induced a significant T cell proliferative response as compared with DCs treated with inactivated AP ($p < 0.001$) (Fig. 2).

To analyze the effect of AP on cytokine mRNA expression, DCs were incubated for 5 h in the presence of varying amounts of AP. There was a dose-dependent increase in the expression of proinflammatory cytokines IL-1β, IL-6, IL-8, and MIP-1β mRNA as analyzed by real-time quantitative PCR (Fig. 3). The secretion of IL-6, IL-8, and MIP-1β cytokine as assessed by ELISA was also significantly increased in a dose-dependent manner after 24 h of

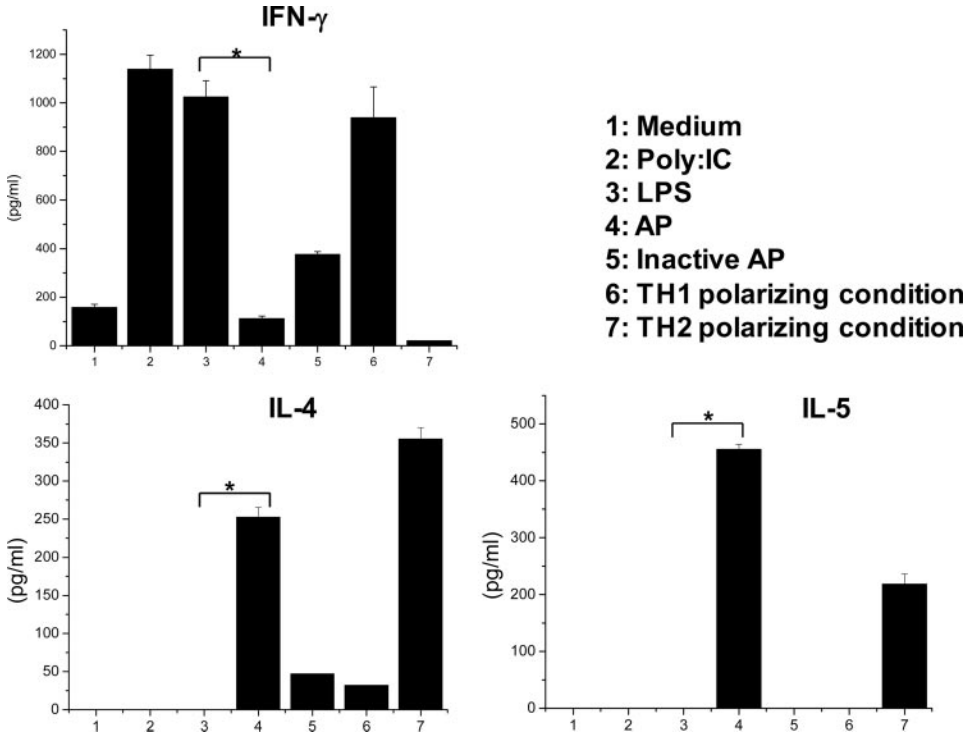

FIGURE 6. Analysis of cytokine production by primed naive CD4 T cells. Purified naive CD4 T cells were cultured with allogeneic untreated (medium) or treated iDCs for 7 days (AP:4 (20 μg/ml), or inactive AP:5 (20 μg/ml), or LPS:3 (1 μg/ml), or poly(I:C):2 (25 μg/ml)). The expanded cells were collected and stimulated again with anti-CD3/CD28 mAbs during 48 h for measurement of IFN-γ, IL-4, and IL-5 cytokines in the culture supernatants by ELISA. Data are given as mean (pg $\pm$ SD) of triplicate cultures and are representative of three independent experiments from three different healthy donors (*, $p < 0.007$).

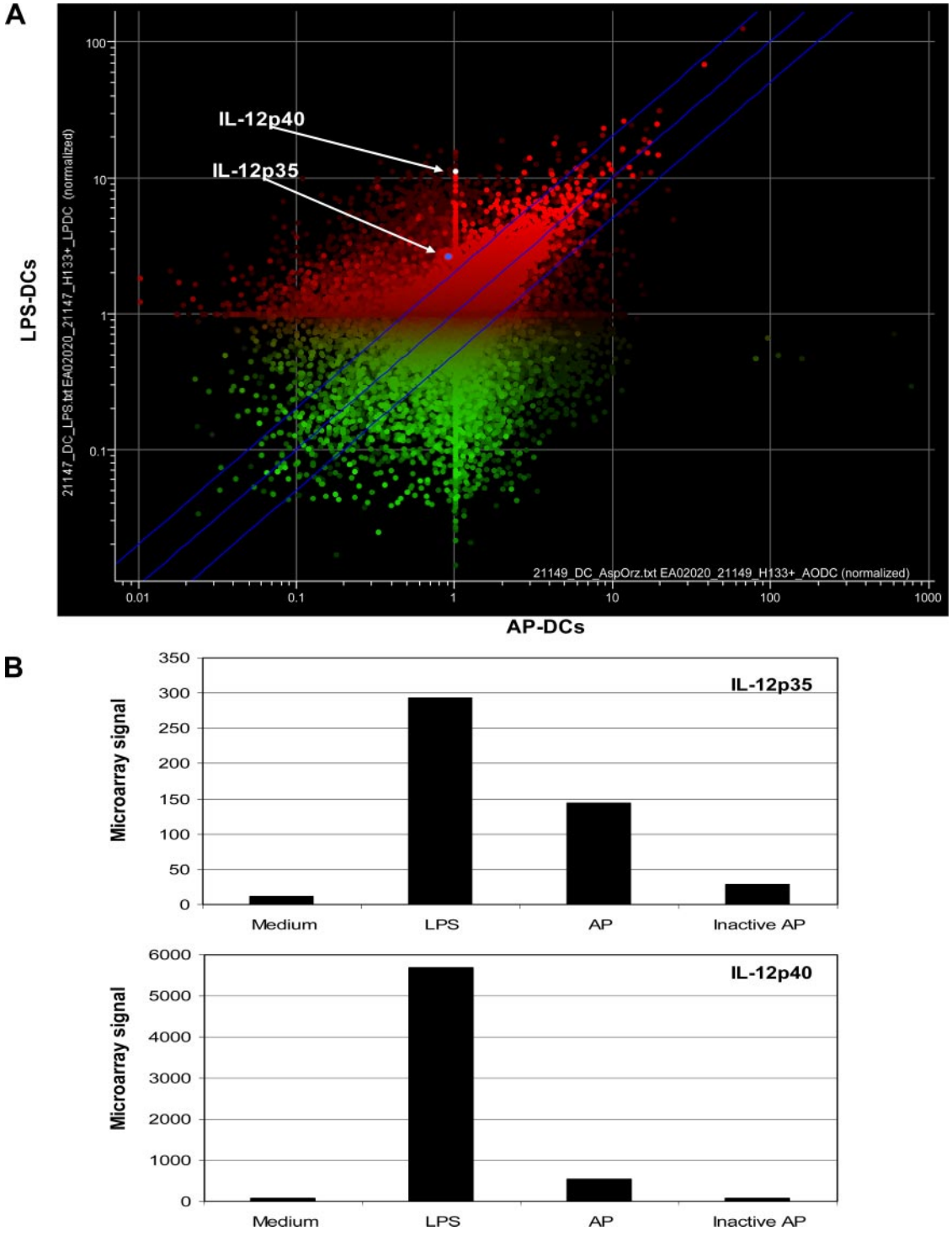

FIGURE 7. Differential expression of IL-12 genes by LPS and AP stimulated DCs. Total RNA samples from immature DCs that were untreated (medium) or 5 h treated with LPS (1 μg/ml), AP (20 μg/ml), or inactive AP (20 μg/ml), were subjected to microarray analysis. *A*, Scatter plot for visualization of relative expression of ~53000 genes and ESTs between two treatments (LPS- or AP-stimulated DCs). IL-12p35 and IL-12p40 are labeled by blue dot plot and white dot blot colors respectively. The solid blue lines indicate a 2-fold difference between LPS- and AP-treated DC samples. *B*, Fold differences of IL-12p35 and IL-12p40 mRNA expression by DC following AP or LPS treatment. Data are representative of two independent experiments.

culture with AP-activated DCs as compared with DCs treated with inactivated AP ($p < 0.005$, $p < 0.03$, and $p < 0.001$, respectively, Fig. 4). However, the secretion of IL-10, TNF-α, and GM-CSF in AP-activated DCs was also increased but at lower levels as compared with LPS- and poly(I:C)-treated DCs (Fig. 4). Inactivation of the protease activity of AP markedly inhibited the induction of cytokine secretion (Fig. 4). These results suggest that AP induces partial maturation of DCs and moderate production of proinflammatory cytokines and this function is at least in part due to the proteolytic activity of AP.

AP treatment shifts DC polarizing capacity to Th2

Studies with murine models of asthma have identified a variety of proteases that can induce a potent human atopy phenotype. With

these allergens, proteolytic activity appears to be required for the development of inflammation and a Th2 immune response (6). These findings prompted us to analyze the phenotype of primary T cell responses induced by human DCs matured in the presence of AP. Although naive allogeneic T cells primed with LPS- or poly(I:C)-activated DCs differentiated into Th1 lymphocytes with characteristic production of large amounts of IFN-γ and undetectable levels of IL-4, IL-5, and IL-13 (Figs. 5 and 6), naive allogeneic T cells primed with AP activated DCs displayed a significantly reduced capacity to induce IFN-γ-producing Th1 like cells and an enhanced capacity to induce IL-4-producing Th2-like cells (Fig. 5 and 6, $p < 0.007$).

Proteolytic inactivation of AP completely abolished its capacity to induce IL-4 production from T cells via treated DCs as analyzed by intracellular cytokine staining. However, IFN-γ expression was not decreased (Fig. 5). Consistent with the intracellular staining results, IL-4 and IL-5 cytokine secretion was also significantly increased in T cells primed with AP-treated DC, whereas IFN-γ protein secretion was higher from T cells primed with LPS- or poly(I:C)-treated DC (Fig. 6). Thus, AP-activated DCs can prime naive CD4 T cells into Th2 cytokine-producing cells and this phenomenon appears to be dependent on proteolytic activity of AP.

AP-activated DCs produce reduced levels of IL-12

Several factors are known to influence the differentiation of Th2 cells including: strength of TCR signal (22), nature and level of surface-expressed costimulatory molecules (23), promotion of IL-4 production (23), and reduction of IL-12 production (24). To understand the mechanism by which DCs may initiate Th2 differentiation, we performed global gene expression analyses of DCs treated with AP, or LPS using an Affymetrix U133 microarray. Among the differentially expressed genes, IL-12 is highly induced in LPS-treated DCs. The expression of IL-12 p40 subunit mRNA in AP-treated cells is ~10-fold lower as compared with DCs treated with LPS; whereas IL-12 p35 subunit expression was ~2-fold lower (Fig. 7, *A* and *B*). This pattern of expression was confirmed by real time PCR (data not shown). AP had a much lower capacity to induce IL-12(p40) release from DCs as compared with LPS (Fig. 8). This difference was not due to cytotoxic effects of AP as determined by proliferation assay (Fig. 2) or trypan blue cell counting (data not shown).

AP activated DCs polarize naive CD4 T cells to a Th2 phenotype

We next asked whether the polarization by AP-DCs shifted T cells toward a Th2 development profile as the result of diminished IL-12 production. Naive allogeneic T cells primed by AP-treated DCs differentiated into T lymphocytes which expressed higher amounts of IL-4, IL-5, and IL-13 (Fig. 9, *A*, *C*, and *D*) but much lower IFN-γ as compared with T cells primed by LPS-treated DC (Fig. 9, *A* and *C*) ($p < 0.008$), thus resembling a Th2 phenotype. Addition of exogenous IL-12(p70) at the beginning of AP-DC-T cell coculture (Fig. 9, *A*, *C*, and *D*) primed naive $CD4^+$ T cells toward a partial Th1 immune response, indicating that the observed Th2 polarization may be at least partly a consequence of low IL-12p40 production by AP-activated DCs. Addition of IL-12 did not restore the response completely to a Th1 profile, suggesting that there are a likely other Th1-driving mediators in this experimental system. In contrast, when IL-4-neutralizing Abs were added at the beginning of the AP-DC-T cell coculture (Fig. 9*B*) induction of Th2 cells was almost completely abrogated, demonstrating that the Th2-polarizing effect of AP-activated DCs was dependent on IL-4 as well as IL-12 levels. Collectively, these results suggest that AP-DC stimulation did not support the development of IFN-γ-producing T cells (i.e., Th1) but rather favored the development of Th2-like IL-4- producing cells.

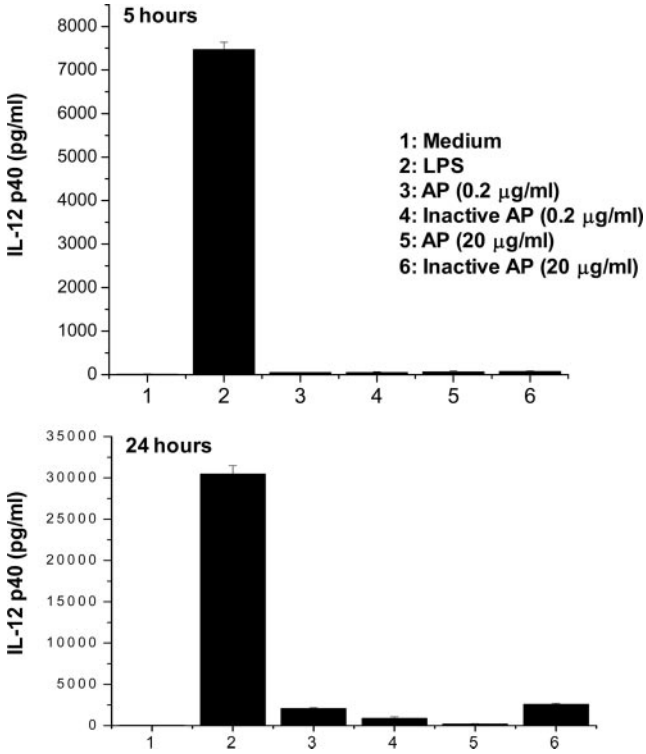

FIGURE 8. AP-stimulated DCs produce less IL-12. DCs were treated with either LPS (1 μg/ml) or varying concentrations of AP or inactivated AP. IL-12p40 concentrations were determined in culture supernatants by ELISA after 5 h and 24 h. Results are given as mean-pg $\pm$ SD for triplicate cultures and are representative of four independent experiments.

Discussion

The molecular mechanisms by which allergens interact with and influence the immune system are not fully understood. We demonstrate here that monocyte-derived DCs treated with fungal AP drive a Th2-like immune response. In recent years, considerable attention has been focused on identifying allergens that might be associated with allergy and asthma. In the case of fungal allergens, the major associated allergic diseases are asthma, allergic rhinitis, allergic broncho-pulmonary mycosis, and hypersensitivity pneumonitis (25). Moreover, purified, natural, and extracted fungal allergens are valuable tools for the study of the involvement of T cells in the allergic processes (26). In our previous published work we had revealed a mechanism by which the proteolytic activity of the fungal AP might bias Th cell subset development in favor of a Th2 immune response in the murine experimental model of asthma (6). To refine this study, we demonstrate here that the proteolytic activity of AP induces a limited immune-modulation of human DCs leading to partial maturation as characterized by selective up-regulation of costimulatory molecules (CD80 and CD86), HLA-DR (Fig. 1) and relatively low production of proinflammatory cytokines IL-10, TNF-α, and GM-CSF (Fig. 4). We believe that this partial maturation might be a mechanism by which AP-treated DCs promote a Th2-like immune response of naive allogeneic T cells as compared with the Th1-like response that is induced by LPS- or poly(I:C)-treated DCs (Fig. 5). In addition, a recent study demonstrated that along with the addition of maturation stimuli, the kinetics of activation can also influence the capacity of DCs to induce different types of T cell responses through

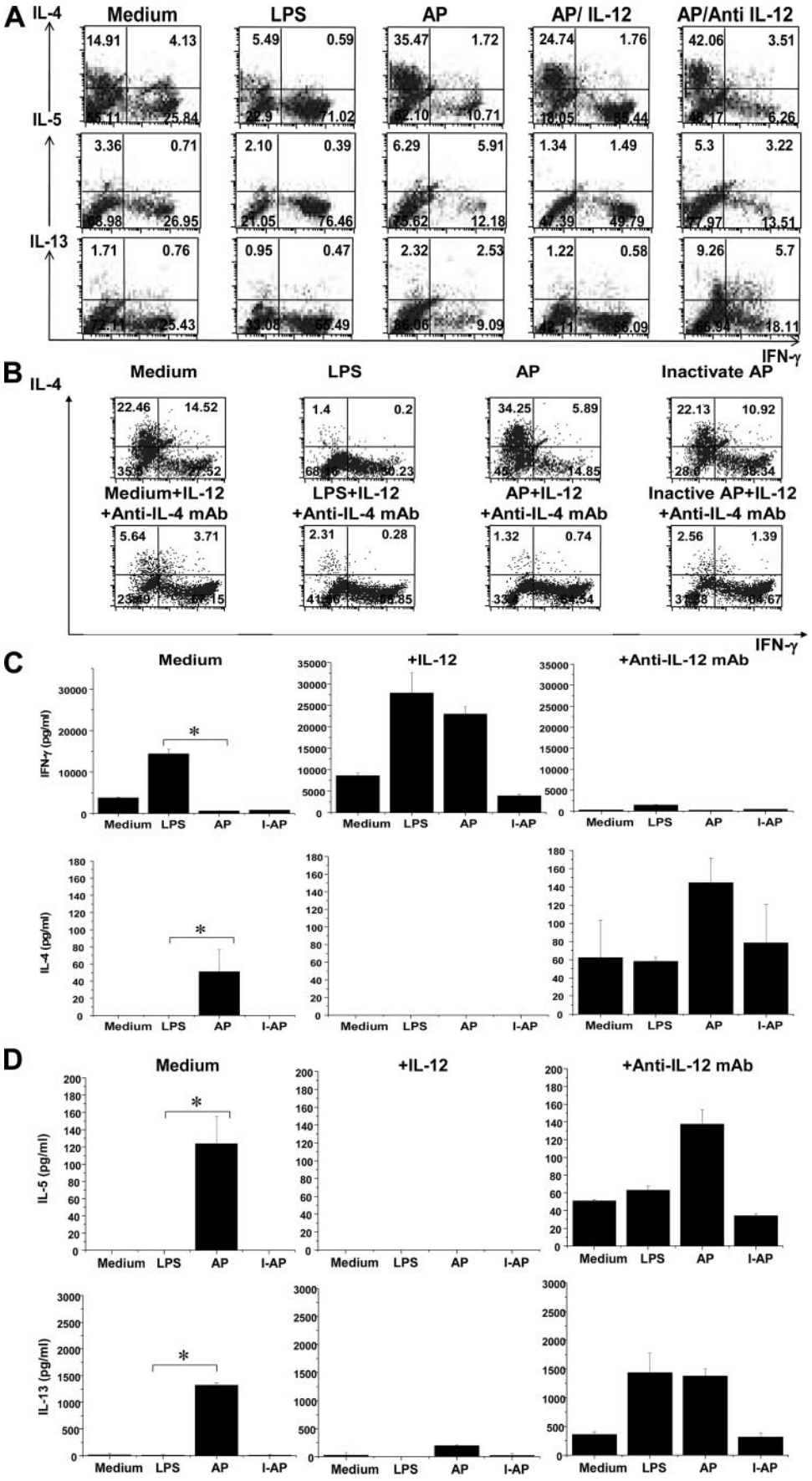

FIGURE 9. AP activated DCs polarize naive CD4 T cells to Th2 profile by default. DCs were treated with LPS, AP, and inactivated AP. After 24 h cells were washed and cultured with naive allogeneic T cells (DC/T cell ratio 1:4) that were expanded for 7 days. Relative T cell polarization was determined by analyzing intracellular IFN-γ and IL-4 accumulation via flow cytometry after PMA-ionomycin re-stimulation with PMA and ionomycin in the presence of brefeldin A treatment. *A*, To assess the effect of IL-12 on AP-dependent Th2 polarization, human rIL-12 (50 ng/ml) or neutralizing mAb anti-IL-12 (10 μg/ml) was added at the beginning of the culture. *B*, To explore the role of IL-4 in Th2 polarization induced by AP-treated DCs, IL-4 neutralizing Ab (10 μg/ml) was added at the beginning of the DC/T cell culture. Numbers within the quadrants indicate the percentage of the expanded cells that stained positive for each respective cytokine (IL-4, IL-5, IL-13, or IFN-γ). *C* and *D*, Analysis of cytokine production by allogeneic DC-primed naive CD4 T cells. Expanded cells were collected and re-stimulated with anti-CD3/CD28 mAbs and IFN-γ, IL-4, IL5, and IL-13 concentrations in the culture supernatants were determined by ELISA. Data represent one of three experiments. The results in *A–D* are representative of three independent experiments (*, $p < 0.008$).

an exhaustion of cytokine production (2). However, in the present study we did not observe this phenomenon with AP-matured DCs, suggesting the existence of a unique pathway controlling Th2 polarization. Among the soluble factors that might influence Th1/Th2 balance, we found two candidate cytokines, IL-1β and IL-6 that were significantly up-regulated (Fig. 3 and 4) in AP-treated DCs. Interestingly, these two cytokines are thought to promote Th2 immune responses, either through the requirement of a costimulatory signal transduced via the IL-1 receptor (27) or through the activation of NF of activated T cells member c2 (NFATc2; Refs. 28, 29). Also, IL-6 produced by APCs has been shown to induce IL-4 production by naive $CD4^+$ T cells and promote their differentiation into Th2 effector cells while inhibiting Th1 differentiation (29, 30). AP treatment induced a dose-dependent activation of proinflammatory cytokines (Fig. 4) but with greatly diminished release of IL-12p40 (Fig. 8) and IL-12p70 (data not shown) from human DCs relative to cells stimulated with LPS or poly(I:C). Reduced IL-12 production was confirmed at the mRNA level, demonstrating that IL-12 gene regulation occurred predominantly at the p40 subunit level rather than the p35 subunit level (Fig. 7*B*). Decreased IL-12 production by these AP-activated DCs might enable them to direct the differentiation of allo-Ag-primed naive human $CD4^+$ T cells toward a Th2 cytokine profile as suggested by prior studies (31, 32). We showed that DCs matured in the presence of AP induce less IFN-γ and more IL-4, IL-5, and IL-13 production by CD4 T cells as compared with DCs matured with LPS or poly(I:C) (Fig. 9*A*, *C*, *D*). Taken together, our data suggest that AP-DCs can polarize naive human $CD4^+$ T cells toward a Th2 immune response through up-regulation of costimulatory molecules on the surface of DC in the absence of high levels of IL-12.

Interestingly, protease-inactive AP promoted production of IFN-γ-producing T cells (Figs. 5 and 6) despite inducing markedly diminished DC production of IL-12 (Figs. 7 and 8). This finding is consistent with our prior observation that AP induces both Th1 and Th2 cells in vivo, but only Th2 cells are recruited to sites of AP challenge (33, 34). The current findings indicate that induction of Th1 cells by AP may occur independently of IL-12, but the precise mechanism of this response requires further study.

The addition of exogenous IL-12 to the AP-DCs culture during priming of naive $CD4^+$ T cells was not able to completely restore a Th1 immune response (Fig. 9*A*). This suggests the existence of other factors in addition to low levels of IL-12 production that might induce Th2 differentiation. The relatively high level of production of IL-6 by AP-DCs may play an intermediate role in inducing IL-4 production from primed $CD4^+$ T cells as it was described previously (29, 30). However neutralization of IL-6 did not alter IL-4 and IFN-γ secretion by T cells in response to LPS-, AP-, and inactivated AP-treated DCs (data not shown). These findings further indicate that AP induces both Th1 and Th2 responses through novel mechanisms, although the AP-dependent Th2 response clearly requires IL-4.

Maturation stimuli differ in their ability to induce mature tolerogenic (i.e., semimature) or mature immunogenic (i.e., fully mature) DCs by their capacity to produce cytokines, despite high expression of MHC II and costimulatory molecules (35). It has been also found in other studies that fully mature DCs produce large amounts of the proinflammatory cytokines IL-12p40 and TNF-α (36), similar to our observation when immature DCs were treated with LPS. Signals that induce cytokine production by matured-DCs include microbial recognition through pathogen-associated molecular patterns and/or pattern recognition receptors (37) or indirect recognition through complement and/or complement receptors (38, 39). The mechanism by which AP induces partial maturation of DCs and the subsequent polarization of naive $CD4^+$ T cells toward IL-4 producing cells is the subject of ongoing investigation, but it is unlikely to involve TLRs or complement proteins. For instance, the activation of DCs with AP in the presence of additional C3a or C5a complement proteins did not modulate the maturation of these cells when compared with AP treatment alone (data not shown).

Collectively, our data provide compelling evidence for the role of the proteolytic activity of AP in the maturation of human monocyte-derived DC. Our data suggest that monocytes-derived DCs that have been conditioned by the proteolytic activity of AP will provide the initial driving force to prime allogeneic CD4 cells to develop Th2 like immune response upon re-stimulation and low IL-12 production will serve to further amplify this process. Additional studies are required to define the specific proteolytic activity of AP, which induces Th2 polarization, and the molecular mechanisms by which the proteolytic signal is transduced by DCs; such analyses may provide additional insight into common allergic diseases such as asthma.

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Disclosures

The authors have no financial conflict of interest.

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