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# Immunity and Tolerance to *Aspergillus* Involve Functionally Distinct Regulatory T Cells and Tryptophan Catabolism[1]

**Claudia Montagnoli,* Francesca Fallarino,* Roberta Gaziano,* Silvia Bozza,* Silvia Bellocchio,* Teresa Zelante,* Wiswanath P. Kurup,† Lucia Pitzurra,* Paolo Puccetti,* and Luigina Romani[2]***

**The inherent resistance to diseases caused by *Aspergillus fumigatus* suggests the occurrence of regulatory mechanisms that provide the host with adequate defense without necessarily eliminating the fungus or causing unacceptable levels of host damage. In this study, we show that a division of labor occurs between functionally distinct regulatory T cells (Treg) that are coordinately activated by a CD28/B-7-dependent costimulatory pathway after exposure of mice to *Aspergillus* conidia. Early in infection, inflammation is controlled by the expansion, activation and local recruitment of CD4$^+$CD25$^+$ Treg capable of suppressing neutrophils through the combined actions of IL-10 and CTLA-4 on indoleamine 2,3-dioxygenase. The levels of IFN-$\gamma$ produced in this early phase set the subsequent adaptive stage by conditioning the indoleamine 2,3-dioxygenase-dependent tolerogenic program of dendritic cells and the subsequent activation and expansion of tolerogenic Treg, which produce IL-10 and TGF-$\beta$, inhibit Th2 cells, and prevent allergy to the fungus. The coordinate activation of Treg may, however, be subverted by the fungus, as germinating conidia are capable of interfering with anti-inflammatory and tolerogenic Treg programs. Thus, regulation is an essential component of the host response in infection and allergy to the fungus, and its manipulation may allow the pathogen to overcome host resistance and promote disease.** ***The Journal of Immunology,*** **2006, 176: 1712–1723.**

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*Aspergillus fumigatus*, a termotolerant saprophyte, is associated with a wide spectrum of diseases in humans, ranging from severe infections to allergy (1, 2). The inherent resistance of immunocompetent and nonatopic subjects to *Aspergillus* diseases suggests the existence of regulatory mechanisms that efficiently oppose both inflammatory and allergic responses to the fungus. Most of the inhaled conidia are eliminated by exclusion mechanisms, through physical barriers and mediators with antimicrobial and immunomodulatory properties (3). Polymorphonuclear neutrophils (PMN)[3] are the predominant immune cells in the acute stage of the infection and are essential in the initiation and execution of the acute inflammatory response and subsequent resolution of infection. However, pulmonary pathology may be reduced under conditions of PMN deficiency (1), which suggests that PMN may act as a double-edged sword, as the excessive release of oxidants and proteases could be responsible for injury to organs and fungal sepsis. Th2 cell sensitization to fungal allergens is common in atopic subjects (2), yet respiratory exposure to inhaled conidia is a tolerogenic event in most individuals.

It is known that respiratory tolerance is mediated by lung dendritic cells (DCs) that produce IL-10 and thus induce the development of CD4$^+$ T regulatory cells (Treg) (4, 5) expressing membrane-bound TGF-$\beta$ and the forkhead family transcription factor Foxp3 (6). Different types of CD4$^+$CD25$^+$ Treg, including naturally occurring and inducible Treg, have been defined (7, 8) and so have the modes of their suppressive action, which range from the inhibitory cytokines IL-10 and TGF-$\beta$ to cell-cell contact via inhibitory CTLA-4 (9). Both natural and inducible Treg have been described in infection (10, 11), their activation occurring through Ag-specific and nonspecific mechanisms. Treg with immunosuppressive activity have also been described in fungal infections (12, 13). Consistent with the notion that signals emanating from CD28 and B7 are critical for thymic generation of Foxp3$^+$ Treg (14) and for Treg self-renewal in the periphery (15, 16), the induction of CD4$^+$CD25$^+$ Treg in candidiasis is strictly dependent on the levels of B7 costimulatory Ag expression by IL-10-producing DCs (13) and involves the IFN-$\gamma$/indoleamine 2,3-dioxygenase (IDO)-dependent pathway (17).

In the present study, we explored the contribution of Treg to the balance between resistance and immunopathology associated with *Aspergillus* infection and allergy. We evaluated the occurrence of Treg in murine models of infection and allergy to the fungus, their relative dependency on costimulatory molecules, and the contribution of IDO and fungus to their induction and functional activity.

## Materials and Methods

### *Animals*

Female BALB/c mice, 8–10 wk old, were purchased from Charles River Breeding Laboratories. Homozygous, CD28$^{-/-}$, B7-1$^{-/-}$, B7-2$^{-/-}$, B7-1$^{-/-}$/B7-2$^{-/-}$, and IFN-$\gamma^{-/-}$ mice on BALB/c background were bred under specific pathogen-free conditions in the animal facility of Perugia University (Perugia, Italy). Procedures involving animals and their care were conducted in conformity with national and international laws and policies.

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[3] Abbreviations used in this paper: PMN, polymorphonuclear neutrophil; DC, dendritic cell; Treg, regulatory T cell; BAL, bronchoalveolar lavage; IDO, indoleamine 2,3-dioxygenase; pDC, plasmacytoid dendritic cell; RC, resting conidia; SC, swollen conidia; 1-MT, 1-methyl-*dl*-tryptophan; TLN, thoracic lymph node; WT, wild type; CCFA, cell culture filtrate Ag; PAS, periodic acid-Schiff.

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### A. fumigatus, culture conditions, infection, allergy, and treatments

The *A. fumigatus* was obtained from a fatal case of pulmonary aspergillosis at the Infectious Diseases Institute of the University of Perugia (18, 19). For generation of swollen conidia (SC), resting conidia (RC) were allowed to germinate (>98% germination) by incubation in Sabouraud's broth for 6 h at 37°C. For infection, mice were anesthetized by i.p. injection of 2.5% avertin (Sigma-Aldrich) before intranasal instillation of a suspension of $2 \times 10^7$ RC or $2 \times 10^6$ SC/20 $\mu$l of saline. Quantification of fungal growth in the lungs was done by the chitin assay and results are expressed as micrograms of glucosamine per pair of lungs (18). For allergy, mice received an i.p. and s.c. injection of 100 $\mu$g of *A. fumigatus* culture filtrate extract (cell culture filtrate Ags (CCFA)) dissolved in IFA (Sigma-Aldrich) followed by two consecutive intranasal injections (a week apart) of 20 $\mu$g of CCFA. One week after the last intranasal challenge, mice received $10^7$ RC intratracheally and were evaluated 1 wk later (20). No differences were observed when mice received RC intranasally (data not shown). In either case, invasive disease after conidia challenge was not observed, as already shown (20). Cyclophosphamide (Sigma-Aldrich), 150 mg/kg/i.p., was given a day before the infection. Depletion of $CD25^+$ T cells (between 80 and 85% by FACS analysis, at 3 (infection) or 7 (allergy) days after fungal inoculum) was obtained with 100 $\mu$g of purified anti-CD25 (PC61; BD Pharmingen) or control mAb (Zymed Laboratories) injected 1 day before and the day of infection (in both models). 1-methyl-dl-tryptophan (1-MT) (Sigma-Aldrich) was dissolved in water and given in drinking water (1 mg/ml) beginning the day of the infection and continuously until the end of the experiment. Anti-IL-10R mAb (clone 1B1.3a; a gift from DNAX Research Institute, Palo Alto, CA) or isotype-matched control mAb were administered the day of infection at 100 $\mu$g/mouse intranasally. For histology, paraffin-embedded sections (3–4 $\mu$m) were stained with periodic acid-Schiff (PAS) to visualize globet cells or H&E to evaluate general morphology. Bronchoalveolar lavage (BAL) was performed by cannulating the trachea and washing the airways with $3 \times 0.5$ ml of PBS to collect the BAL fluid. Total and differential cell counts were done by staining BAL smears from allergic mice with May-Grünwald Giemsa reagents (Sigma) before analysis.

### Flow cytometry

Staining for lymphocyte Ag and costimulatory molecule expression was done as described previously (13, 21). Cells were analyzed with a FACScan flow cytofluorometer (BD Biosciences) equipped with CellQuest software. Control staining of cells with irrelevant Ab was used to obtain background fluorescence values. Data are expressed as a percentage of positive cells over total cells analyzed.

### Cell purification

Purified peritoneal $CD11b^+Gr\text{-}1^+$ PMN (>98% pure on FACS analysis) were obtained as described elsewhere (22). Lung $CD11c^+$ DCs (between 5 and 7% positive for CD8$\alpha$ and between 30 and 35% positive for Gr-1) were isolated by magnetic cell sorting with MicroBeads (Miltenyi Biotec) as described previously (19). $CD11c^+CD45R(B220)^+Ly6C^+$ plasmacytoid DCs (pDCs) were obtained from $CD11c^+$ DCs by magnetic cell sorting with MicroBeads conjugated to rat anti-mouse mPDCA-1 mAb (JF05-1C2.4.1; Miltenyi Biotec). $CD4^+$, $CD4^+CD25^+$, and $CD4^+CD25^-$ cells were separated by magnetic cell sorting from lung and thoracic lymph nodes (TLN) as described elsewhere (13). Flow cytometry was used to determine the purity of $CD4^+$ T cells (>98%) and the fractionated $CD4^+CD25^-$ (>98%) or $CD4^+CD25^+$ (>82%).

### Cocultures with Treg and CFSE labeling

Purified PMN ($2 \times 10^6$) and RC (at PMN:conidia ratio of 1:3) were added of $CD4^+CD25^+$($4 \times 10^5$) or the corresponding $CD4^+CD25^-$ ($10 \times 10^5$) at ratios mimicking the actual PMN:T cell ratios occurring in the lung early in infection. Purified DCs ($2 \times 10^5$) and RC (at DCs:RC ratio of 1:1) were added of $10^6$ $CD4^+CD25^+$ or $CD4^+CD25^-$ cells (at the optimal DCs:T cells ratio of 1:5, as by initial experiments) for 24 h before evaluation of cytokine production. Fungal overgrowth was prevented as described previously (18). Purified $5 \times 10^4$ $CD4^+CD25^+$ or $CD4^+CD25^-$ cells from lungs (at 3 days after infection) or TLN (at 10 days after infection) were incubated either alone or in combination (at the optimal $CD25^+$:$CD25^-$ ratio of 1:2, as by initial experiments) on anti-CD3$\epsilon$-coated plates (clone 145-2C11; BD Pharmingen) in the presence of 1 $\mu$g/ml soluble anti-CD28 mAb (clone 37.51; BD Pharmingen) (13) for 72 h before FACS analysis. Labeling with CFSE (Molecular Probes) was done as described elsewhere (13).

### Phagocytosis, antifungal effector activity, and cytokine production

For phagocytosis, PMN were incubated at 37°C with unopsonized RC and/or Treg for 60 min, and the percentage of internalization and conidiocidal activity was determined as described previously (18). PMN were exposed to 10 $\mu$g/ml IL-10 or 40 $\mu$g/ml CTLA-4-Ig for 5 h (19). CTLA-4-Ig consisted of a fusion protein between the extracellular domain of mouse CTLA-4 and the Fc portion of a mouse IgG3 Ab as described previously (23). For experiments involving the fusion protein, control treatment consisted of native IgG3 produced as previously described (23). PMN production of reactive oxidant intermediates was done as described elsewhere (17) and the results are expressed as nanomoles $O_2^-$ per $10^6$ cells. For cytokine production, Treg were incubated on anti-CD3$\epsilon$-coated plates in the presence of soluble anti-CD28 mAb for 24 h. IL-10 and CTLA-4 blockade was obtained by incubating PMN and Treg with 10 $\mu$g/ml hamster anti-mouse CTLA-4 (4F10; BD Pharmingen) and anti-IL-10R mAb.

### Adoptive transfer of cells

Recipient $CD28^{-/-}$ (infection model) or CCFA-sensitized wild-type (WT) mice (allergy model) were infused i.v., 1 day before the infection, with $10^6$/0.5 ml of PBS of early (from $B7\text{-}1^{-/-}$ lung) or late (from $B7\text{-}2^{-/-}$ TLN) $CD4^+CD25^+$ or the corresponding $CD4^+CD25^-$ cells.

### Hydroxyproline assay

Total lung collagen levels were determined as previously described (20). Hydroxyproline concentrations were calculated from a standard curve of hydroxyproline.

### Determination of serum IgE

The total IgE in the serum samples was measured by ELISA (24).

### Western Blotting for IDO and kynurenine assay

IDO expression was performed by immunoblotting with rabbit polyclonal IDO-specific Ab on whole cell lysates or on PMN and DCs from uninfected mice exposed in vitro to IFN-$\gamma$ (200 U/ml) for 5 h followed by heat-inactivated (65°C, 30 min) RC or SC (at PMN:fungi ratio of 1:3) for an additional 12 h (17). The positive control consisted of IDO-expressing $MC_{24}$ transfectants and the negative control of mock-transfected $MC_{22}$ cells. IDO functional activity was measured, in supernatants of cells from lung or TLN, in terms of ability to metabolize tryptophan to kynurenine measured by HPLC (17).

### Cytokine and ELISPOT assays

The levels of cytokines in pooled lung homogenates and culture supernatants were determined by specific ELISAs (BD Pharmingen and R&D Systems). Cytokine-producing cells were enumerated by ELISPOT assay on purified $CD4^+$ cells as described previously (17). Results are expressed as the mean number of cytokine-producing cells ($\pm$SE) per $10^4$ cells, calculated using replicates of serial 2-fold dilutions of cells.

### RT-PCR

Total RNA was extracted (TRIzol; Invitrogen Life Technologies) from cells at 3 (lung) or 10 (TLN) days after infection. Synthesis and PCR of cDNA were done as described elsewhere (18). The sequences of gene-specific primers were as follows: *FoxP3:* forward, CAG CTG CCT ACA GTG CCC CTA G; reverse, CAT TTG CCA GCA GTG GGT AG; GATA3*:* forward, GAA GGC ATC CAG ACC CGA AAC; GATA3: reverse, ACC CAT GGC GGT GAC CAT GC; IFN-$\gamma$: forward, TGA ACG CTA CAC ACT GCA TCT TGG; reverse, CGA CTC CTT TTC CGC TTC CTG AG; and CCR7: forward, GAG ACT CGA GAG AGC ACC ATG GAC CCA GG; reverse, GAG AGA ATT CCT ACG GGG AGA AGG TTG TGG. Amplification efficiencies were validated and normalized against GAPDH.

### Statistical analysis

The log rank test was used for paired data analysis of the Kaplan-Meier survival curves. Student's *t* test or ANOVA and Bonferroni's test were used to determine the statistical significance of differences in organ clearance and in vitro assays. Significance was defined as $p < 0.05$. The data reported are either from one representative experiment of three independent experiments or pooled from three to five experiments, otherwise $n = 8$. The in vivo groups consisted of six to eight mice per group.

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## Results

### *Distinct Treg subsets are activated in mice exposed to* A. fumigatus

WT, B7-1$^{-/-}$, B7-2$^{-/-}$, and CD28$^{-/-}$ mice were infected intranasally with *Aspergillus* conidia and monitored for CD4$^+$CD25$^+$ T cell number and function in the lung and TLN. Although no major differences were observed in terms of the absolute numbers of total cells recovered among the different mice, the numbers of CD4$^+$CD25$^+$ T cells were different among uninfected mice and were modified by infection (Table I). A population of CD4$^+$CD25$^+$ T cells was present in the lung of uninfected B7-1$^{-/-}$ mice and increased early in infection to decline thereafter. In contrast, a population of CD4$^+$CD25$^+$ T cells appeared late in the lung and TLN of B7-2$^{-/-}$. Similar to B7-1$^{-/-}$ mice, CD4$^+$CD25$^+$ T cells increased early within lung and late in TLN in WT mice, but were neither detected nor increased after infection in CD28$^{-/-}$ (Table I). The finding that CD4$^+$CD25$^+$ T cells were not detected in double-deficient B7-1$^{-/-}$/B7-2$^{-/-}$ mice also (data not shown), argues against the role of other costimulatory molecules in CD4$^+$CD25$^+$ T cell expansion (5). Cytofluorometric analysis revealed that CD4$^+$CD25$^+$ T cells detected in the lungs of B7-1$^{-/-}$ and WT mice were CD45RB$^{low}$, CTLA-4$^+$, and CD62L$^-$ in the early phase but not in the late phase where positivity was only observed for the activation marker CD69 (Fig. 1*A*). In TLN of B7-2$^{-/-}$ and WT mice, CD4$^+$CD25$^+$ T cells detected early were mainly CD69$^+$, whereas those detected late were CD45RB$^-$, CTLA-4$^+$, and CD62L$^+$. Early CD4$^+$CD25$^+$ cells in the lungs were also partially TCR$\gamma\delta^+$ (Fig. 1*A*). Further analysis revealed that *FoxP3* was expressed on CD4$^+$CD25$^+$ T cells obtained from lung at the early phase or from TLN at the late phase (Fig. 1*B*) but not on cells from lung at the late phase or TLN at the early phase (data not shown). Neither *FoxP3*$^+$CD25$^+$ population expressed IFN-$\gamma$ or the Th2-specific transcription factor GATA3, being therefore distinct from Th1 and Th2 cells. Of interest, IFN-$\gamma$ was expressed in CD4$^+$CD25$^-$ T cells from TLN but GATA3 was expressed in CD4$^+$CD25$^-$ T cells from lung, which is compatible with a sequential pattern of Th2 and Th1 cell activation early and late in infection, respectively (Fig. 1*B*).

Because CCR7 is a key molecule in the lymphocyte return from peripheral tissues to lymph nodes (25), we speculated that the levels of CCR7 expression would predict the migratory capacity of circulating T cells once seeded into the lungs. The results, reported in Fig. 1*C,* clearly show that early CD4$^+$CD25$^+$T cells in the lungs were CCR7$^-$, as opposed to CD4$^+$CD25$^+$ T cells appearing late in the lungs and TLN that were CCR7$^+$. This pattern of chemokine receptor expression is therefore consistent with the proposed model in which circulating T cells remain in the lungs or migrate through afferent lymph to draining lymph nodes depending on their level of CCR7 expression (26).

To evaluate the pattern of cytokine production by early (lung) and late (TLN) CD4$^+$CD25$^+$ T lymphocytes, cells from either uninfected or infected WT and B7-1$^{-/-}$ (lung) or WT and B7-2$^{-/-}$ (TLN) mice were assessed for Th1 (IFN-$\gamma$), Th2 (IL-4), or Treg (IL-10/TGF-$\beta$) production after stimulation with anti-CD3 and soluble anti-CD28 mAbs. Although levels of IFN-$\gamma$ and IL-4 were consistently negligible, production of IL-10 and TGF-$\beta$ increased in either type of cells after infection, early CD25$^+$ cells being high producers of IL-10 and late CD25$^+$ cells of TGF-$\beta$ (Fig. 1*C*). Production of IL-10 and TGF-$\beta$ was low by late CD4$^+$CD25$^+$ T cells from lung or early CD4$^+$CD25$^+$ T cells from TLN (data not shown).

Taken together, these data indicate the occurrence of phenotypically distinct CD4$^+$CD25$^+$ T cell populations in mice exposed to *A. fumigatus*, each population being distinct from Th1 and Th2 effectors, producing IL-10 and TGF-$\beta$ and expressing a "combination code," in terms of chemokine receptor expression, that is necessary for effective suppression (9). The finding that Th1 cell reactivity was concomitantly down-regulated in the presence of early CD25$^+$ T cells and promoted in the presence of late CD25$^+$ T cells, suggests that the capacity of early CD25$^+$ T cells (early Treg) to produce anti-inflammatory IL-10 may predict a central role in dampening inflammation, while the capacity of late CD25$^+$ T cells (late Treg) to produce TGF-$\beta$, known to be pivotally involved in tolerance to aeroallergens (6), may predict a tolerogenic activity in allergy to the fungus.

### *Early and late Treg determine susceptibility to* A. fumigatus *infection and allergy*

To verify whether early Treg suffice to dampen the inflammatory response and late Treg the allergic response to the fungus, we characterized parameters of inflammatory and adaptive immunity to *Aspergillus* in experimental models of fungal infection and allergy in the different types of mice. In infection, although all intact mice survived microbial challenge, fungal burden was higher and the inflammatory tissue pathology milder in WT or B7-1$^{-/-}$ than in B7-2$^{-/-}$ or CD28$^{-/-}$ mice (Fig. 2*A*). Signs of severe parenchymal destruction associated with foci of inflammatory cells were present in the lungs of B7-2$^{-/-}$ and, to a lesser extent, CD28$^{-/-}$ but not WT or B7-1$^{-/-}$mice, despite the fact that the early influx of inflammatory cells was not significantly different among the different mice (~7- to 8-fold increase of Gr1$^+$CD11c$^-$ PMN in each group without significant variations of F4/80$^+$ macrophages). However, although no differences were observed in the handling of the fungus by PMN from the different types of uninfected mice, the expression of local antifungal effector activity was different among the different mice upon infection and inversely correlated with fungal growth (data not shown). Further studies in mice treated

Table I. *Occurrence of CD4$^+$CD25$^+$ T cells in mice exposed to* A. fumigatus

| Mice[a] | Lung | | | TLN | | |
|---|---|---|---|---|---|---|
| | Control | Early | Late | Control | Early | Late |
| BALB/c | 4 ± 1 (40 ± 2) | 14 ± 3[b] (71 ± 4) | 12 ± 3[b] (52 ± 4) | 4 ± 2 (15 ± 3) | 7 ± 3 (16 ± 2) | 14 ± 2[b] (20 ± 3) |
| B7–1$^{-/-}$ | 10 ± 2 (38 ± 3) | 25 ± 4[b] (72 ± 3) | 12 ± 3 (41 ± 3) | 6 ± 3 (19 ± 2) | 12 ± 3 (15 ± 4) | 12 ± 4 (13 ± 4) |
| B7–2$^{-/-}$ | 5 ± 1 (35 ± 4) | 4 ± 2 (61 ± 3) | 15 ± 4[b] (60 ± 5) | 7 ± 2 (15 ± 2) | 17 ± 4[b] (17 ± 3) | 25 ± 3[b] (22 ± 2) |
| CD28$^{-/-}$ | 2 ± 1 (39 ± 2) | 3 ± 1 (59 ± 4) | 3 ± 1 (48 ± 4) | 3 ± 1 (17 ± 3) | 4 ± 2 (16 ± 6) | 5 ± 3 (14 ± 3) |

[a] Mice were infected with $2 \times 10^7$ *Aspergillus* RC intranasally and the numbers of CD4$^+$CD25$^+$ cells were evaluated in lung and TLN 3 (early) and 10 (late) days after the infection by flow cytometry. Control indicates uninfected mice. Numbers refer to percentages (mean ± SE) of double-positive cells over total cells analyzed. In parentheses, the absolute numbers ($\times 10^5$ ± SE) of total cells recovered. The results shown represent one of three independent experiments.

[b] $p < 0.05$, infected vs uninfected.

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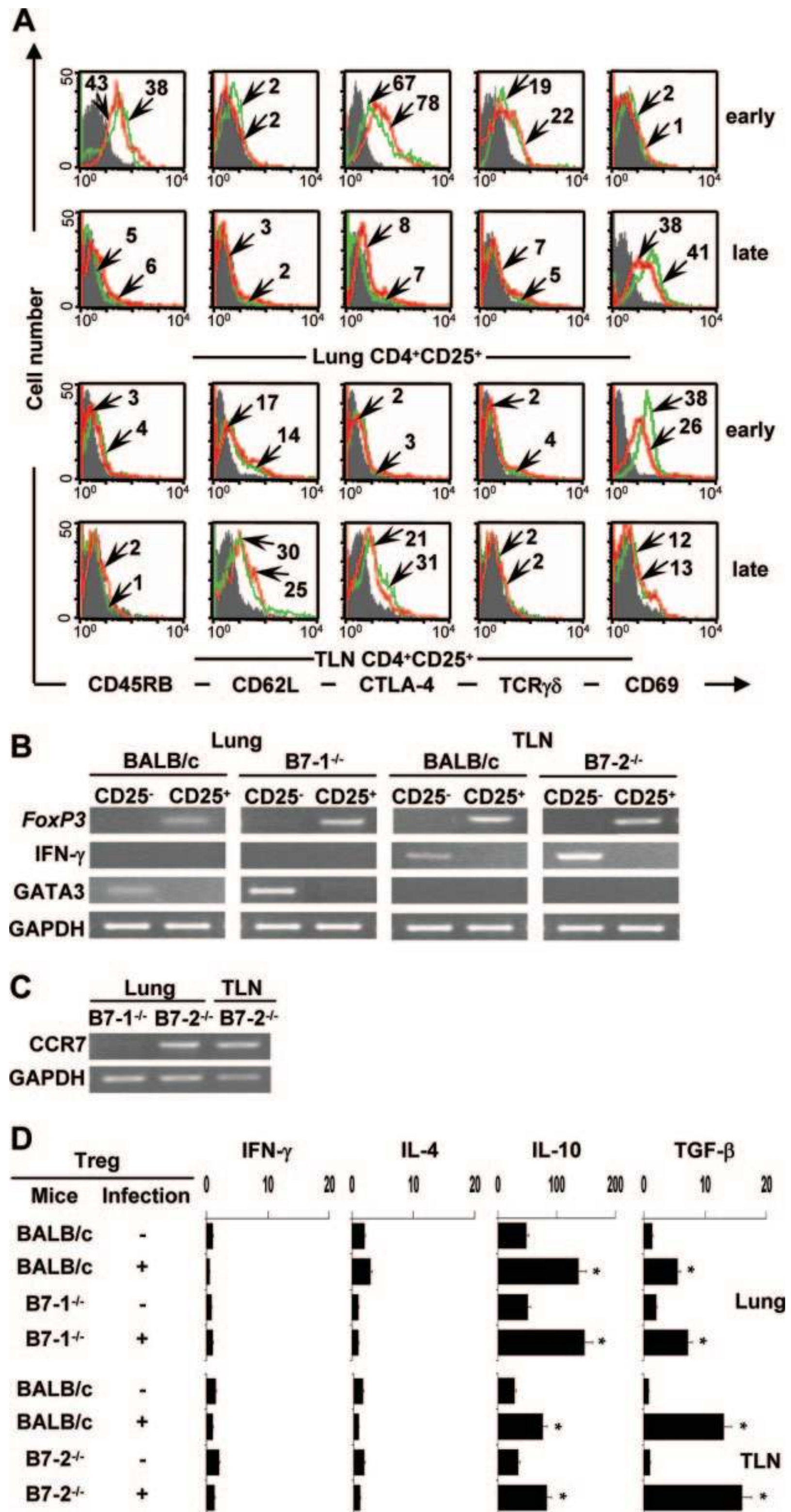


**FIGURE 1.** Distinct Treg populations are activated by the exposure *to A. fumigatus* resting conidia. *A,* Phenotypic and gene-expression analysis of $CD4^+CD25^+$ cells (*B* and *C*) isolated from lung or TLN early (3 days) or late (10 days) of BALB/c (red line), B7-$1^{-/-}$ (green line, *upper panels*), or B7-$2^{-/-}$ (green line, *lower panels*) mice intranasally infected with $2 \times 10^7$ resting conidia. Purified $CD4^+CD25^+$ cells were reacted with FITC-conjugated mAbs (numbers represent the percentage of positive cells over total cells analyzed) or assessed, along with the corresponding $CD4^+CD25^-$ cells, for gene expression by RT-PCR. No mRNAs were detected in cells from uninfected mice, except for *Foxp3* that was detected in $CD4^+CD25^+$ cells from uninfected B7-$1^{-/-}$ mice. *C,* $CD4^+CD25^+$ T cells are used. *D,* Cytokine production by $CD4^+CD25^+$ isolated from either uninfected or infected mice as above and cultured on anti-CD3ϵ-coated plates in the presence of soluble anti-CD28 mAb for 24 h. Cytokines (picograms per milliliter) were assessed by ELISA. *, $p < 0.05$, infected vs uninfected. Bars, SE. The results shown represent one representative experiment of three (FACS and RT-PCR) or three independent experiments (cytokine production).

with cyclophosphamide, known to deplete natural Treg (27), confirmed that early Treg act by limiting the inflammatory response at the expenses of fungal growth. Cyclophosphamide treatment reduced the number of $CD4^+CD25^+$ T cells in the lungs, decreased survival of WT and B7-$1^{-/-}$ but not of B7-$2^{-/-}$ or $CD28^{-/-}$ mice and exacerbated the inflammatory pathology, despite significant reduction of fungal growth (Fig. 2*B*). Because depletion of PMNs as by treatment with Gr-1 mAb rendered all mice equally susceptible to the infection (data not shown), cyclophosphamide unmasks the role of early Treg in infection. As a matter of fact, similar to cyclophosphamide, CD25 depletion decreased survival, fungal load and IL-10 production in B7-$1^{-/-}$ but not B7-$2^{-/-}$ mice (Fig. 2*C*).

In allergy, the number of eosinophils in the BAL fluid, levels of serum IgE, and lung hydroxyproline (hallmarks of hypersensitivity to *A. fumigatus* (20, 24)) were all increased upon allergen challenge in *Aspergillus*-sensitized WT, B7-$1^{-/-}$ and, less, $CD28^{-/-}$,

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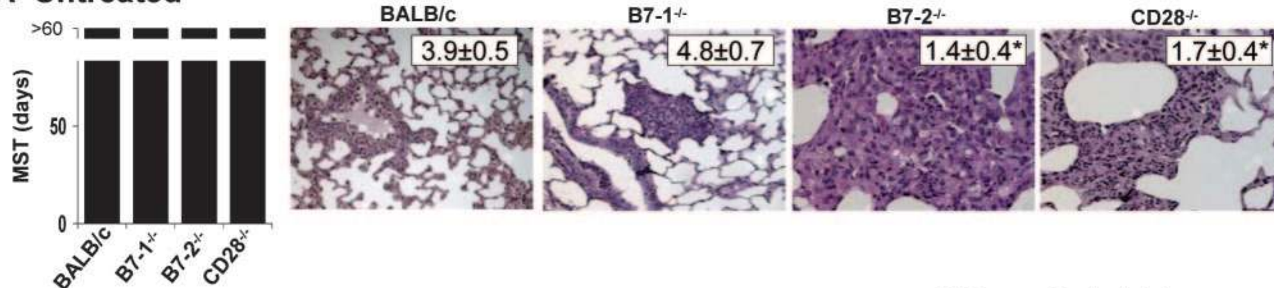


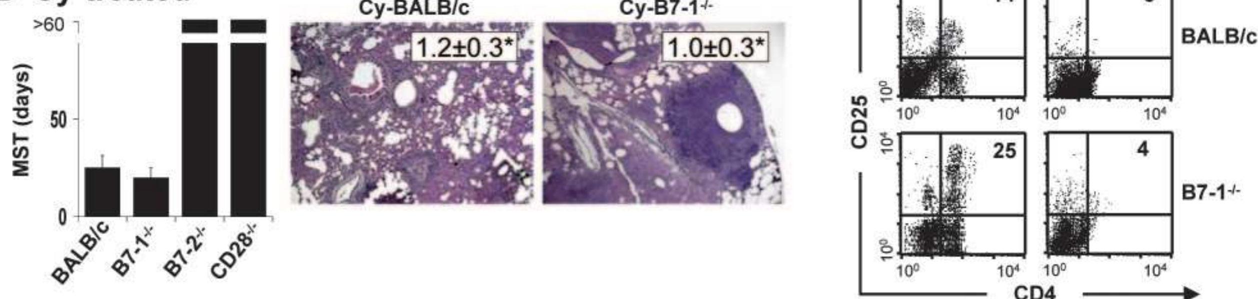


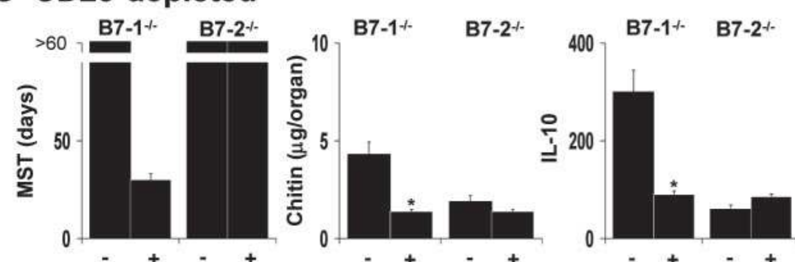


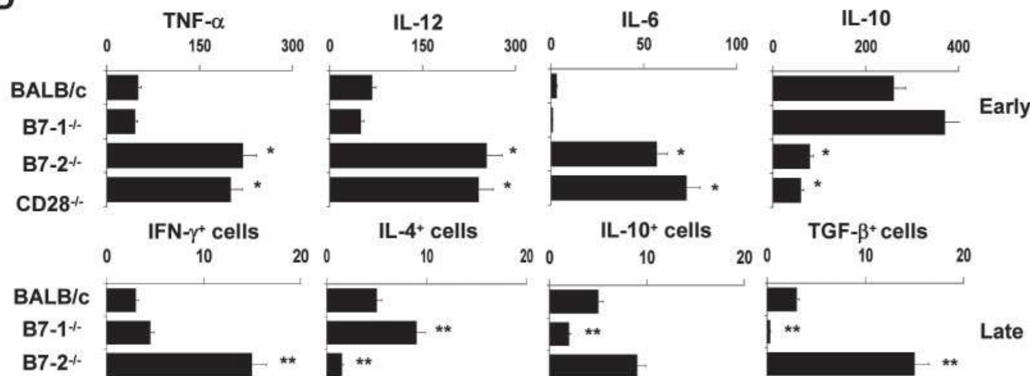


**FIGURE 2.** The occurrence of early Treg correlates with decreased inflammation in mice with infection. *A* Mice injected with $2 \times 10^7$ resting conidia intranasally were assessed for survival (median survival time (MST), days), histopathology (PAS staining), and chitin content (glucosamine ± SE, microliters per organ, values in boxes). Original magnification, ×200 (BALB/c and B7-1$^{-/-}$) and ×400 (B7-2$^{-/-}$ and CD28$^{-/-}$). Results of one of three independent experiments. *, $p < 0.05$, B7-2$^{-/-}$/CD28$^{-/-}$ vs B7-1$^{-/-}$/BALB/c mice. *B,* Mice were treated with cyclophosphamide (Cy) or not (none) a day before the infection as in *A*. FACS analysis was done on lung cells 3 days after the infection. Numbers refer to percent positive cells. Original magnification, ×25. *, $p < 0.05$, Cyclophosphamide-treated vs untreated mice. *C,* Mice received the day before and the day of the infection the anti-CD25 mAb (+) or control mAb (−) and were assessed for survival, chitin content, and IL-10 production (pg/ml) 3 days after the infection. (D) Cytokine production in lung homogenates 3 days (early) after the infection and frequencies of cytokine-producing CD4$^+$ cells in TLN 10 days after the infection (late). Results of one of three independent experiments. Cytokine levels (picograms per milliliter) were below the detection limits of the assay in lung homogenates from uninfected mice. Frequencies (mean ± SE per $10^4$cells, ELISPOT assay) of cytokine-producing cells in uninfected mice were below the detection limits of the assay, except for IFN-γ-producing cells in B7-2$^{-/-}$ mice (4 ± 1). *, $p < 0.05$, B7-2$^{-/-}$/CD28$^{-/-}$ vs B7-1$^{-/-}$/BALB/c mice. **, $p < 0.05$, B7-2$^{-/-}$ and B7-1$^{-/-}$ vs BALB/c mice.

but not B7-2$^{-/-}$ mice, unless they were CD25$^+$T cell depleted (Fig. 3*A*). Histopathology confirmed the induction of extensive peribronchial infiltrates of lymphocytes and eosinophils (with bilobed nuclei, *inset*s of Fig. 3*B*), globet cell hyperplasia, and mucus deposition in the airways of allergic but not B7-2$^{-/-}$ mice (Fig. 3*B*). Interestingly, an inverse relationship was observed between the occurrence of allergy and the ability to restrict fungal growth (data not shown).

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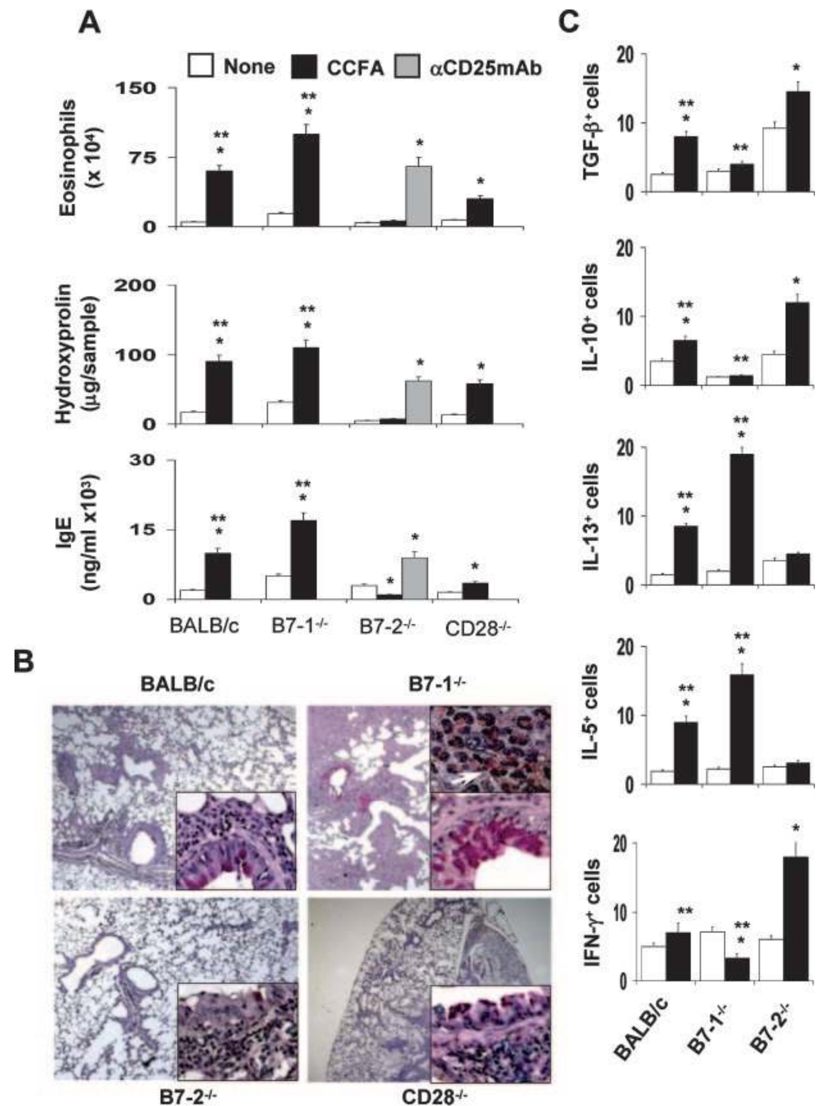


**FIGURE 3.** The occurrence of late Treg correlates with decreased inflammation in mice with allergy. Mice were sensitized with *A. fumigatus* culture filtrate extract (CCFA), challenged with $10^7$ resting conidia intratracheally, and assessed for absolute number of eosinophils in the BAL, lung hydroxyproline, and total serum IgE (*A*), histology (*B*), and (C) Th/Treg immunity 1 week later (*C*). Anti-CD25 mAb was given 1 day before and the day of the infection. Lung sections were PAS stained to visualize globet cells (original magnification, ×25 and ×400 in the *inset*s) or H&E stained to evaluate eosinophils (arrows in the *inset* of B7-$1^{-/-}$ mice at ×1000 magnification). Results of one of two independent experiments. Frequencies (mean ±SE per $10^4$cells, ELISPOT assay) of cytokine-producing $CD4^+$ cells in TLN of uninfected mice were below the detection limits of the assay, except for IFN-γ-producing cells in B7-$2^{-/-}$ mice (7 ± 2). *, $p < 0.05$, CCFA-sensitized vs unsensitized (none) mice. **, $p < 0.05$, B7-$1^{-/-}$ and BALB/c vs B7-$2^{-/-}$ mice.

The analysis of the pattern of inflammatory, Th1/Th2/Treg cytokine production in infection and allergy revealed that early in infection, the balance between proinflammatory (TNF-α, IL-12p70, and IL-6) and anti-inflammatory (IL-10) cytokine production was skewed toward the anti-inflammatory state in WT and B7-$1^{-/-}$ mice and toward the inflammatory state in B7-$2^{-/-}$ and $CD28^{-/-}$ mice (Fig. 2*D*). In contrast, late in infection and similarly in allergy, the number of IFN-$\gamma^+CD4^+$ cells was lower in WT and B7-$1^{-/-}$ than in B7-$2^{-/-}$ mice, and the opposite was true for the number of IL-4-(late infection) or IL-5- and IL-13 (allergy)-producing $CD4^+$ cells (Figs. 2*D* and 3*C*). In terms of Treg, the number of IL-10- or TGF-β-producing $CD4^+$ cells was elevated in B7-$2^{-/-}$ and, to some extent, in WT mice with either infection or allergy but not in B7-$1^{-/-}$mice (Figs. 2*D* and 3*C*). The frequencies of Th2 cells were particularly low in $CD28^{-/-}$ mice with infection or allergy (data not shown), a finding consistent with the importance of CD28 signaling for Th2 cell activation (16). These results, combined with those of Fig. 1*B*, clearly indicate the occurrence of a distinct pattern of Th1/Th2 cell activation associated with the concomitant occurrence of distinct Treg populations capable of dampening inflammation (early Treg) or allergy (late Treg) to the fungus.

*Distinct functional activities of early and late Treg*

Consistent with the notion that Treg are capable of directly affecting effector Th cells and inhibiting innate immune cells through inhibitory cytokines (28) and IDO (21), we evaluated early (from lung of B7-$1^{-/-}$) and late (from TLN of B7-$2^{-/-}$) Treg from either uninfected or infected mice for inhibition of Th and innate effector cells in vitro or upon adoptive transfer in vivo. On evaluating the effect on the proliferative activity of the corresponding $CD4^+CD25^-$ cells, we found that late more than early $CD25^+$ cells greatly inhibited the proliferation of the corresponding $CD25^-$ population and neither type of $CD25^+$ cells proliferated when compared with $CD25^-$ cells (Fig. 4*A*). As the levels of IL-4 were concomitantly decreased in conditions of inhibition by late $CD25^+$ cells (data not shown), this suggests that Th2 cells were inhibited.

The suppressive activity on innate effector functions was assessed on PMN and DCs, because of their central role in the inductive and effector pathways of antifungal immunity (29) and because of the presence in these cells of a functional program that is regulated by the CTLA-4/IDO-dependent axis (17). Phagocytosis, fungicidal activity, and respiratory burst of WT PMN exposed to *Aspergillus* conidia were also assessed in the presence of

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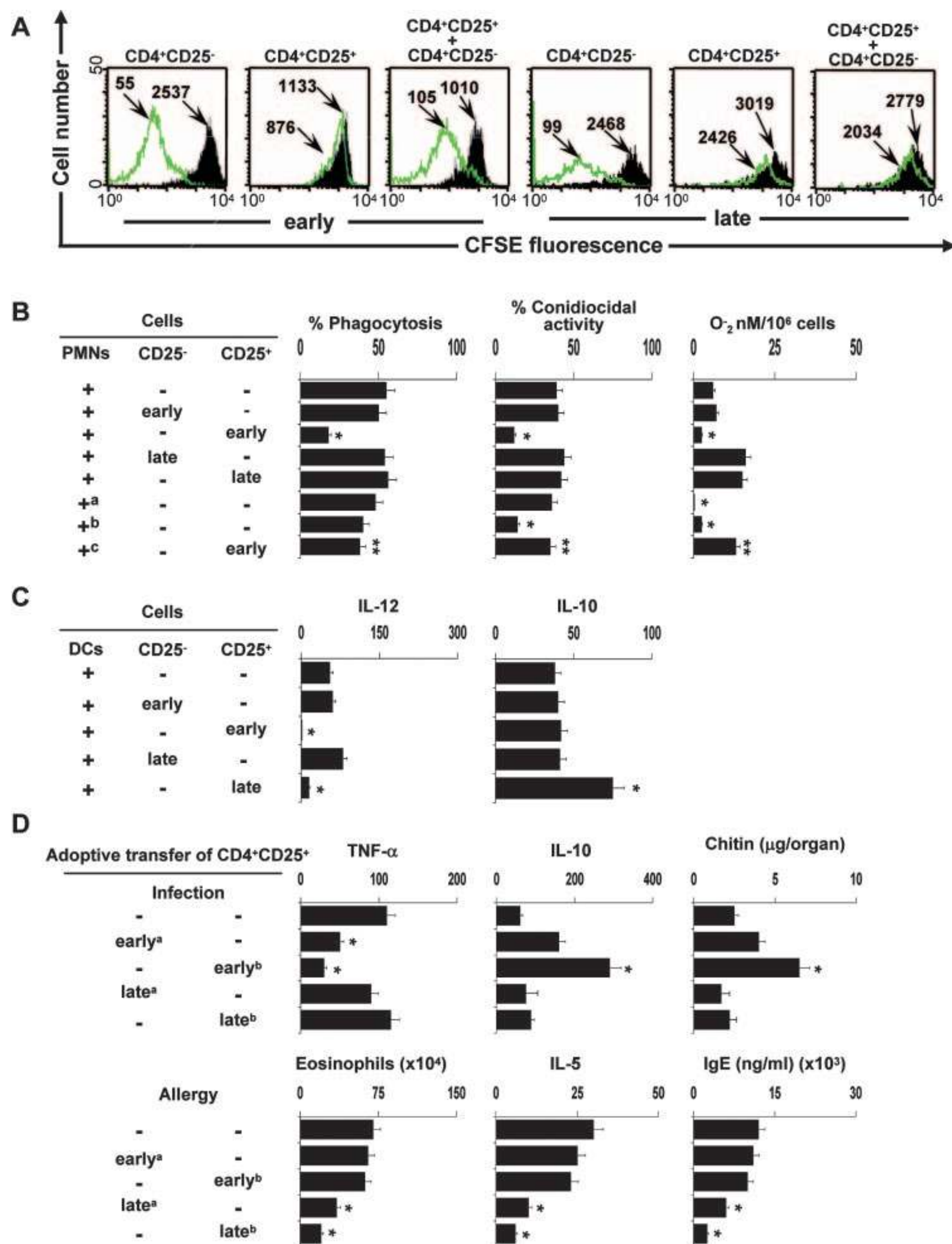


**FIGURE 4.** Distinct functional activities of early or late Treg in vitro and in vivo. *A,* Proliferation of early and late (3 and 10 days postinfection, respectively) $CD4^+CD25^+$ or $CD4^+CD25^-$ cells, either alone or in combination, obtained from lungs of B7-$1^{-/-}$ (early) or TLN of B7-$2^{-/-}$ (late) infected mice. Cells were labeled with CFSE and incubated (green histograms) or not (black histograms) on anti-CD3ε-coated plates in the presence of soluble anti-CD28 mAb for 72 h. The numbers represent the mean channel fluorescence intensity. Antifungal effector activity of PMN (*B*) and cytokine production by DCs (*C*) in the presence of $CD4^+CD25^+$ or $CD4^+CD25^-$ cells obtained as above. Peritoneal PMN or lung DCs were exposed to resting conidia in the presence of T cells for 60 min (for phagocytosis and oxidant production) or 24 h for cytokine production. PMN exposed to CTLA-4-Ig (*a*) or to IL-10 for 5 h (*b*) or to Treg (*c*) in the presence of anti-IL-10R + anti-CTLA-4 mAbs. Anti-IL-10R + anti-CTLA-4 mAbs alone had no effects. *C,* No cytokine production was observed in cells exposed to conidia in the absence of DCs. ∗, $p < 0.05$, unexposed vs Treg-exposed PMN or DCs. ∗∗, $p < 0.05$, Treg + anti-IL-10R + anti-CTLA-4 mAbs vs Treg alone. *D,* Adoptive transfer of $CD4^+CD25^+$ or $CD4^+CD25^-$ cells ($10^6$ cells, i.v., 1 day before the infection) from naive (*a*) or infected (*b*) mice into recipient $CD28^{-/-}$ mice (infection) or CCFA-sensitized BALB/c mice (allergy). Cytokines (picograms per milliliter in lung homogenates), lung chitin content, absolute number of eosinophils in BAL, and total serum IgE were assessed at 3 (infection) or 7 (allergy) days after fungal inoculum. ∗, $p < 0.05$, with and without adoptively transferred cells.

CTLA-4-Ig, a soluble CTLA-4 agonist, or IL-10. Early but not late $CD25^+$ or $CD25^-$ T cells suppressed PMN functional activities (Fig. 4*B*) including TNF-α production (data not shown). In line with previous observations (17), CTLA-4-Ig greatly impaired the oxidant production, leaving phagocytosis and fungicidal activity largely unaffected (Fig. 4*B*), a finding suggesting that the inhibitory activity of $CD4^+CD25^+$ cells on PMN likely occurs through both cell-cell, contact-dependent and -independent mechanisms. As a matter of fact, the exposure of PMN to IL-10 impaired both fungicidal activity and oxidant production, and the inhibitory activity of Treg was abrogated under conditions of both CTLA-4 and IL-10 blockade (Fig. 4*B*). Therefore, the suppressive functions of early $CD25^+$ cells on innate cells occur through CTLA-4 and IL-10. With respect to DCs, both early and late $CD25^+$ cells inhibited

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IL-12 production of lung DCs in response to conidia but, upon coculturing fungus-pulsed DCs with late $CD25^+$ cells, high levels of IL-10 were also produced (Fig. 4*C*), a finding suggesting that, irrespective of the relative contribution of each type of cells to cytokine production, a bidirectional influence may occur between DCs and Treg, as suggested (21).

For the assessment of functional activity in vivo, early or late $CD25^+$ cells from naive or infected mice were adoptively transferred into $CD28^{-/-}$ mice with infection or, because of the low frequency of Th2 cells in $CD28^{-/-}$ mice, in WT mice with allergy. Upon adoptive transfer in $CD28^{-/-}$ mice with infection, early but not late $CD25^+$ cells decreased TNF-$\alpha$ production and increased IL-10 and fungal load, an effect greater with Treg from infected than naive mice (Fig. 4*D*). In allergy, late but not early $CD25^+$ cells dampened parameters of inflammation, an effect maximally observed with cells from infected mice (Fig. 4*D*). All together, these data suggest that the capacity of early Treg to inhibit aspects of innate immunity is central to their anti-inflammatory activity, while the capacity of late Treg to inhibit aspects of adaptive Th2 immunity predicts a tolerogenic activity to the fungus. In addition, the functional activity of each Treg is inducible in infection.

*Treg induction and function are dependent on IDO*

To elucidate mechanisms through which the exposure to the fungus results in the coordinate activation of distinct Treg capable of selectively inhibiting aspects of innate and adaptive immunity, we assessed IDO expression and functional activity in infection, because this enzyme is crucially involved in the regulation of innate and adaptive antifungal immunity, through an activity on both PMN and DCs that is strictly dependent on costimulatory molecule expression (17, 21, 30). IDO protein expression was induced early in infection in the lungs (both PMN and DCs) and TLN of WT mice and further increased thereafter. Interestingly, IDO expression was decreased after cyclophosphamide treatment, a finding linking IDO activity to early Treg functioning (Fig. 5*A*). IDO expression correlated with functional enzyme activity, as shown by the elevated levels of local kynurenine production, known to reflect IDO functional activity (21), in the lungs and TLN of infected WT mice (Fig. 5*B*). Because kynurenine production were particularly elevated in TLN of B7-$2^{-/-}$ mice late in infection or in allergy (Fig. 5*B*), these data suggest that activation of IDO occurs progressively in infection, particularly under conditions of B7-2 deficiency. As a matter of fact, B7-1 expression progressively increased in both lungs and TLN in infection while B7-2 expression only increased early, particularly in lungs, and declined thereafter (Fig. 5*B*). Therefore, IDO functional activity positively correlated with expression of B7-1, known to mediate strong IDO activation upon CTLA-4 engagement (23).

The relative importance of IDO in the generation or functioning of early or late Treg was further addressed in studies where IDO blockade was achieved by treatment in vivo with 1-MT, which inhibits enzyme activity via tryptophan competition (17). Treatment with 1-MT, known to be associated with reduced kynurenine production (17), increased both the fungal burden and proinflammatory cytokine production in WT mice, without affecting the survival. However, survival was significantly reduced upon concomitant blockade of both IDO and Treg, a finding suggesting that the two treatments act additively (Fig. 5*C*). Interestingly, 1-MT failed to increase the fungal burden and to promote inflammation in B7-$1^{-/-}$ mice, both effects being induced by IL-10 blockade (Fig. 5*C*). Therefore, IDO is not required for early Treg generation but contributes, along with IL-10, to Treg suppressive activity. Consistent with previous studies (31), IDO blockade exacerbated allergy and resulted in increased levels of IgE/IL-5 and decreased production of IL-10/TGF-$\beta$ (Fig. 5*C*), a finding that suggests loss of tolerogenic Treg. Together, these results indicate that induction of IDO serves a crucial role in *Aspergillus* infection and allergy and is involved in both Treg functioning and induction (late Treg).

IFN-$\gamma$ is one major activating signal for IDO (21, 23, 30). To correlate levels of IFN-$\gamma$ produced early in infection with the expression of early innate antifungal resistance and the activation of IDO-dependent tolerogenic Treg, parameters of infection and allergy were evaluated in IFN-$\gamma^{-/-}$ mice. Concomitantly with the impaired IDO expression and functional activity as seen in IFN-$\gamma^{-/-}$ mice with candidiasis (data not shown and Ref. 17), resistance but not the occurrence of Treg was impaired early in infection as opposed to allergy where symptom exacerbation was concomitant with the lack of occurrence of tolerogenic Treg (Fig. 5*D*). Together, these data suggest that low levels of IFN-$\gamma$ production early in infection are associated with defective activation of tolerogenic Treg, which links inflammatory events occurring at the early stages of the infection to subsequent allergic responses to the fungus through IFN-$\gamma$/IDO.

*Manipulation of IDO by the fungus*

Because the inflammatory response to the fungus is dependent on fungal morphotype (17, 32), we hypothesized that the robust inflammatory response seen against germinating SC more than RC may unmask the ability of the fungus to subvert Treg induction and functioning. To test this hypothesis, we assessed RC and SC for their ability to interfere with IDO expression in vitro in tolerogenic DCs and PMN as well as with Treg functioning in vivo. We found that RC or SC had opposite effects on IDO induction in PMN and lung pDCs exposed to IFN-$\gamma$. Similar to IFN-$\gamma$, RC induced IDO expression while SC impaired the IFN-$\gamma$-induced IDO expression in both types of cells (Fig. 6*A*). In vivo, the production of TNF-$\alpha$ was significantly increased while that of IL-10 decreased in WT and B7-$1^{-/-}$ mice infected with SC as compared with RC, while no differences were observed in $CD28^{-/-}$ mice. Inflammation caused by SC was associated with decreased production of local kynurenins and, interestingly, with a decreased number of lung $CD4^+CD25^+$ T cells (Fig. 6*B*). Therefore, SC may counteract Treg activity through an inhibitory action on IDO activation in PMN and DCs.

## Discussion

This study shows that distinct Treg populations capable of mediating anti-inflammatory or tolerogenic effects are coordinately induced by the exposure of mice to *Aspergillus* conidia, an event to which costimulation, IDO, and the pathogen contribute. Early in infection, a population of Treg, expressing the same phenotype as those that control intestinal inflammation and autoimmunity (28, 33), suppresses lung inflammatory responses to the fungus. Late in infection and, similarly in allergy, a population of Treg of the same phenotype as those controlling graft-vs-host disease (34) or diabetes (33) develops with the ability to control allergic inflammatory response to the fungus.

The control of inflammation early in infection is mainly exerted at the level of PMN that are essential in initiation and execution of the acute inflammatory response to the fungus (29). Early Treg inhibited the antifungal effector and proinflammatory activities of PMN, an activity occurring through both contact-dependent (CTLA-4/IDO) and -independent (IL-10) mechanisms. These results confirm previous evidence on the occurrence of PMN capable of exhibiting suppressive antifungal effector activity through the CTLA-4/IDO-dependent mechanism (17, 22) as well as the suppressive activity of IL-10 against the fungus (35). Moreover, the discovery of Treg as an early source of IL-10 may contribute to

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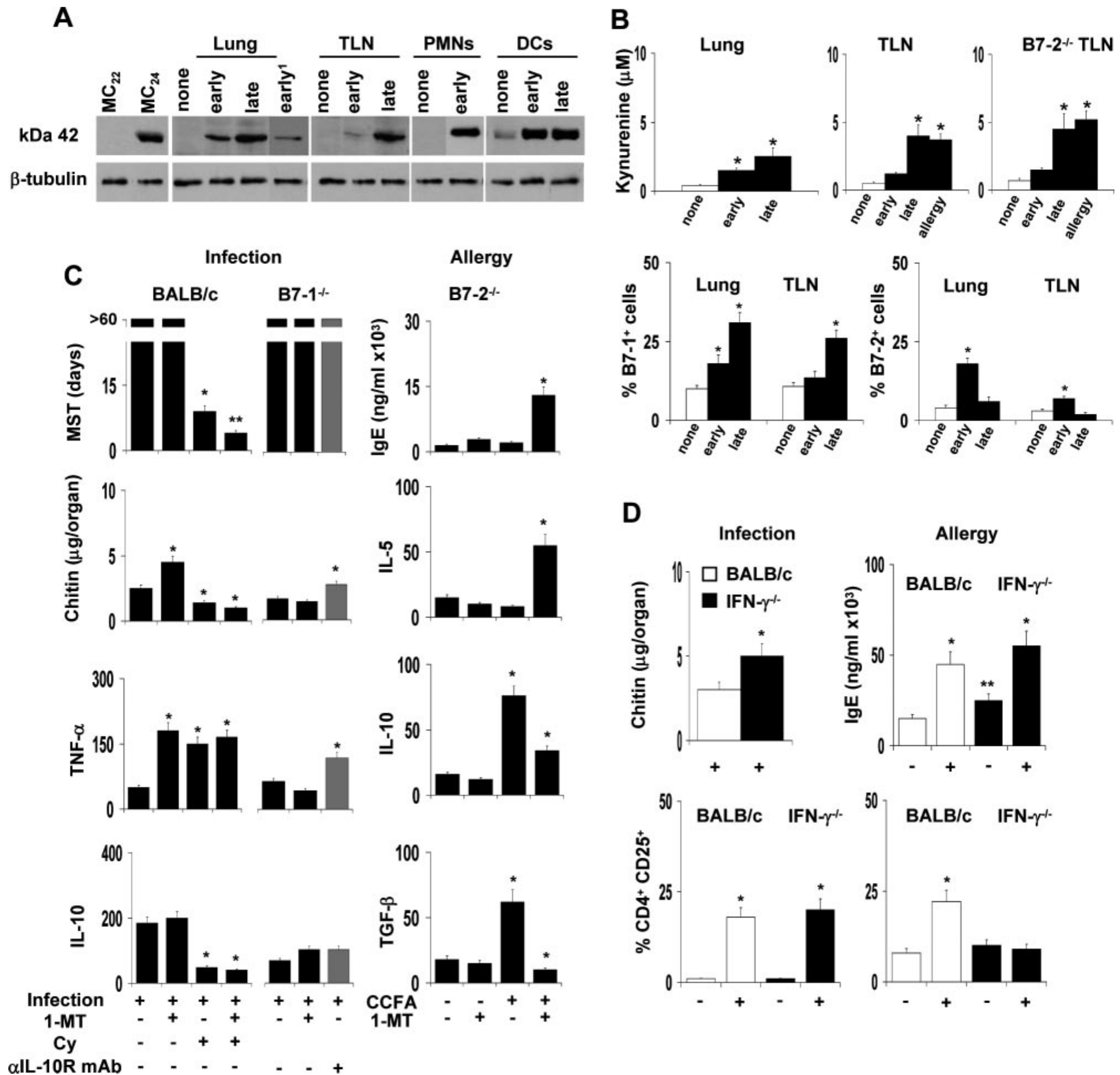


**FIGURE 5.** Treg induction and function are dependent on IDO. *A,* IDO expression in BALB/c mice with aspergillosis, either untreated or treated with cyclophosphamide ([1]). Infected mice were assessed for IDO protein expression by Western blotting on whole lung or TLN lysates, lung PMN, and DCs at 3 (early) or 10 (late) days after infection. None, Uninfected mice. $MC_{24}$ and $MC_{24,}$ positive and negative IDO controls. *B,* Kynurenins were measured in the supernatants of cells from lungs or TLN of BALB/c or TLN of B7-$2^{-/-}$ mice 3 (early) or 10 (late) days after infection or 7 days after fungal challenge in allergy. $*$, $p < 0.05$, infected vs uninfected (none) mice. Percentages of B7-$1^+$ or B7-$2^+$ cells in lung or TLN of uninfected (none) or infected, 3 and 10 days earlier, BALB/c mice. Percent positive over total cells analyzed on FACS analysis. $*$, $p < 0.05$, infected vs uninfected mice. The results shown represent one representative experiment of three. *C,* IDO blockade impairs resistance to infection and allergy to *Aspergillus*. 1-MT (1 mg/ml in drinking water) was given the day of infection and continued until death or throughout the experiment. Cyclophosphamide (Cy) was administered as described in the legend to Fig. 2. Anti-IL-10R mAb was administered intranasally the day of the infection. Cytokines (picograms per milliliter in lung homogenates), lung chitin content, and total serum IgE were assessed at 3 (infection) or 7 (allergy) days after the fungal challenge. $*$, $p < 0.05$, treated mice vs untreated mice. $**$, $p < 0.05$, 1-MT + Cy-treated vs either treatment alone. *D,* Infection and allergy in IFN-$\gamma^{-/-}$ mice. Lung chitin content, total serum IgE, and number of lung (infection) or TLN (allergy) $CD4^+CD25^+$ T cells were assessed at 3 (infection) or 7 (allergy) days after the fungal inoculum. $*$, $p < 0.05$, infected (+) vs uninfected (−) mice. $**$, $p < 0.05$, IFN-$\gamma^{-/-}$ vs BALB/c mice.

explain both the significance of variable levels of IL-10 as well as the variable efficacy of IL-10 blockade observed in different infection settings (36).

Despite production of IL-10 and CTLA-4 expression, late Treg failed to inhibit PMN inflammatory responses. Since CCR7 and CD62L expression defines the "combination code" for homing of T cells to lymph nodes more than inflammatory tissues (25), this may explain the failure observed after adoptive transfer and at the same time highlights that specific homing represents an essential feature of cells committed to suppress (9). However, the failure to exert effects in vitro suggests that levels of IL-10 production and CTLA-4 expression (incidentally lower than those seen with early Treg) are insufficient to inhibit PMN. The suppressive activity of late Treg consisted in a marked inhibition of Th2 cell responses, an

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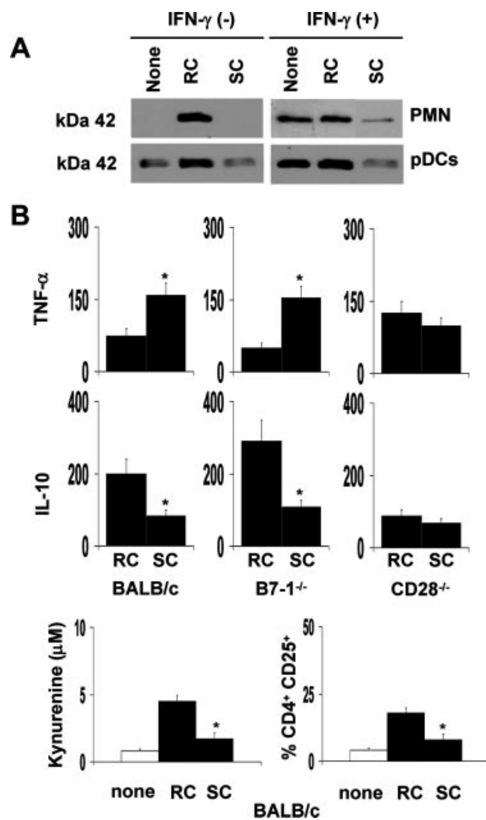


**FIGURE 6.** Manipulation of IDO by the fungus. *A,* IDO protein expression (Western blotting) in PMN and lung pDCs exposed (+) or not (−) to IFN-$\gamma$ (200 U/ml) and/or resting (RC) or swollen (SC) heat-inactivated conidia (at PMN:fungi ratio of 1:3) in vitro. None, Cells alone. *B,* Mice were infected intranasally with $2 \times 10^7$ RC or $2 \times 10^6$ SC before the assessment of cytokine (picograms per milliliter by ELISA) and kynurenine productions in lung homogenates or number of lung $CD4^+CD25^+$ T cells by FACS, 3 days later. *, $p < 0.05$, SC- vs RC-infected mice. Cytokine and kynurerine levels in uninfected mice were below the detection limits of the assay. None, Uninfected mice.

action that occurs at the level of responding $CD4^+CD25^-$ Th2 cells. Although respiratory tolerance is mediated by a spectrum of adaptive Treg (4–6, 37, 38), we found that tolerance to fungal allergens is achieved by Treg that produce TGF-$\beta$ more than IL-10. Without disproving the role of IL-10 as natural suppressor of inflammation in allergy to the fungus (39, 40) and as capable of activating the tolerogenic program on DCs (41), the finding that an increase in TGF-$\beta$ production was observed upon adoptive transfer of late Treg in allergic mice (our unpublished data) suggests that Treg require TGF-$\beta$ in vivo to carry out their suppressive activity in allergy (42). Recently, reverse signaling through B7 expressed on effector T cells has been shown to represent a novel mechanism by which CTLA-$4^+$ Treg specifically suppress activated T cells (43). Although CCR7 expression predicts that late Treg effectively colocalize with effector Th2 cells in TLN, where antifungal Th2 responses are generated upon DC migration (19), the relative contribution of TGF-$\beta$, IL-10, and reverse signaling in the suppressive activity of tolerogenic Treg awaits clarification.

Although eosinophils have been shown to contribute to the Th balance in allergy through IDO (44), lung $CD11c^+$ DCs are necessary and sufficient for Th2 stimulation in airway allergy (45), whereas lung pDCs prevent it (46). Therefore, an action on pDCs may have major effects on tolerogenic Treg induction. In this regard, the anti-inflammatory activity of early Treg on DCs whose IL-12 production in response to the fungus was greatly reduced is of interest. First, it could account for the skewing of the antifungal Th responses toward the Th2 type, resulting in fungal persistence and increased susceptibility to fungal allergy. Second, consistent with the notion that the IFN-$\gamma$/IDO-dependent axis contributes to the induction of Treg in response to fungi (17), early Treg, by affecting IFN-$\gamma$ production, indirectly exert a fine control over the induction of tolerogenic Treg. These effects were all seen in mice after the exposure to *Aspergillus* conidia and were reminiscent of those obtained with certain viral infections that substantially and persistently altered lung DC functioning by a mechanism that involved IFN-$\gamma$ (47). In the case of *Aspergillus*, not only was the occurrence of early Treg inversely correlated with susceptibility to allergy, but tolerogenic Treg were not induced by IDO blockade or in conditions of IFN-$\gamma$ deficiency. This finding ruled out a major role for B7-1 requirement per se in the generation of late Treg. Thus, a unifying mechanism linking natural Treg to tolerogenic respiratory Treg in response to the fungus is consistent with the revisited "hygiene hypothesis" of allergy in infections (48) and may provide at the same time mechanistic explanations for the significance of 1) the augmented allergic response following fungal pneumonia seen in the absence of IFN-$\gamma$ (49); 2) the variable level of IFN-$\gamma$ seen in allergic diseases and asthma (50); and 3) the paradoxical worsening effect on allergy of Th1 cells (50, 51). The scenario would suggest that defects of pDCs may predispose to fungal allergy, as already observed in children with atopic asthma (52). However, the lower number of pDCs in nonallergic B7-$2^{-/-}$ as compared with allergic B7-$1^{-/-}$ mice (our unpublished observations) also points to the importance of qualitative defects of pDCs in fungal allergy. In this regard, as Treg induce IDO on DCs trough CTLA-4/B7-1 binding (21), a control of quality of tolerogenic DCs is also exerted by nearby Treg, an "infectious tolerance" effect greatly promoted in conditions of superior availability of B7-1 molecules such as in tolerant B7-$2^{-/-}$ mice.

Together, these results suggest that regulatory mechanisms operating in the control of inflammation and allergy to the fungus are different but interdependent as the level of the inflammatory response early in infection may impact on susceptibility to allergy in conditions of continuous exposure to the fungus. IDO has a unique and central role in this process because it participates in the effector and inductive phases of early and tolerogenic Treg. This may explain the beneficial effect on fungal allergy of CpG oligodeoxynucleotides (24) known to induce IDO that is found to inhibit experimental asthma (31) and to have increased activity in asymptomatic atopy (53). However, the finding that IDO expression was also modified by the fungus makes IDO squarely placed at the host-fungus interface where its perturbation by the fungus is a unique and efficient mechanism by which Treg induction and activity could be subverted. Thus, IDO inhibition may account for the ability of SC to overcome host resistance mechanisms and promote inflammation in both infection (through an action on PMN) and allergy (through an action on pDCs). The unique ability of the fungus to subvert Treg induction in a morphotype-dependent fashion further adds to the notion that recognition of Ags derived from pathogens is an essential step in the induction and function of Treg (10, 11).

The data of the present study are compatible with a scenario in which a division of labor occurs between functionally distinct Treg populations that are coordinately activated upon the exposure to *Aspergillus* (Fig. 7). Early in infection, anti-inflammatory $CCR7^-$ Treg, requiring B7-2/CD28 for generation, suppress through the combined action of IL-10 and CTL4–4 acting on IDO. The concurrent engagement of Treg and effector cells at inflammatory sites allows immune responses to be vigorous enough to provide adequate host defense, without necessarily eliminating the fungus or

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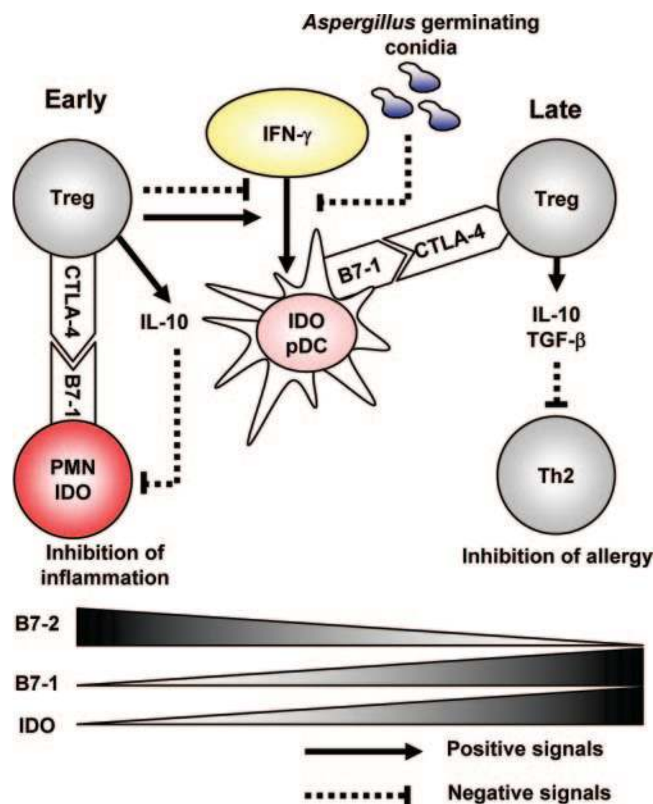


**FIGURE 7.** The central role of the IFN-γ/IDO-dependent pathway in immunity and tolerance to *Aspergillus* and its subversion by the fungus. The production of IFN-γ is squarely placed at the host-pathogen interface where IDO activation exerts a fine control over the inductive and effector pathways of immunity and tolerance to *A. fumigatus* infection and allergy. At the bottom, the levels of IDO and costimulatory molecule expression in infection are shown.

causing an unacceptable level of host damage. Indeed, the levels of IFN-γ produced in this early phase set the subsequent adaptive stage by conditioning the tolerogenic program of DCs through IDO. This results in the occurrence of tolerogenic $CCR7^+$ Treg producing IL-10 and TGF-β and inhibiting allergic Th2 cells upon migration to draining lymph nodes. The coordinate activation of Treg is subverted by fungus, as the germinating conidia are capable of interfering with both the anti-inflammatory and tolerogenic regulatory programs.

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## Disclosures

The authors have no financial conflict of interest.

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