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ORIGINAL ARTICLE

Distinct and complementary roles for *Aspergillus fumigatus*-specific Tr1 and Foxp3$^+$ regulatory T cells in humans and mice

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Unlike induced Foxp3$^+$ regulatory T cells (Foxp3$^+$ iT$_{reg}$) that have been shown to play an essential role in the development of protective immunity to the ubiquitous mold *Aspergillus fumigatus*, type-(1)-regulatory T cells (Tr1) cells have, thus far, not been implicated in this process. Here, we evaluated the role of Tr1 cells specific for an epitope derived from the cell wall glucanase Crf-1 of *A. fumigatus* (Crf-1/p41) in antifungal immunity. We identified Crf-1/p41-specific latent-associated peptide$^+$ Tr1 cells in healthy humans and mice after vaccination with Crf-1/p41 + zymosan. These cells produced high amounts of interleukin (IL)-10 and suppressed the expansion of antigen-specific T cells *in vitro* and *in vivo*. In mice, *in vivo* differentiation of Tr1 cells was dependent on the presence of the aryl hydrocarbon receptor, c-Maf and IL-27. Moreover, in comparison to Tr1 cells, Foxp3$^+$ iT$_{reg}$ that recognize the same epitope were induced in an interferon gamma-type inflammatory environment and more potently suppressed innate immune cell activities. Overall, our data show that Tr1 cells are involved in the maintenance of antifungal immune homeostasis, and most likely play a distinct, yet complementary, role compared with Foxp3$^+$ iT$_{reg}$.
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Regulatory T (T$_{reg}$) cells have a key role for the maintenance of immune homeostasis, prevention of autoimmunity and protection against infections.[1] Besides thymus-derived naturally occurring Foxp3$^+$ nT$_{reg}$, two major subsets of induced T$_{reg}$ cells have been identified: Foxp3$^+$ regulatory T cells (Foxp3$^+$ iT$_{reg}$) and Foxp3$^-$ type-(1)-regulatory T (Tr1) cells that differ in their mode of induction, phenotype and cytokine expression but share the overall feature to suppress immune responses.[2] Foxp3$^+$ iT$_{reg}$ differentiate in the presence of sub-immunogenic doses of antigen and transforming growth factor-β (TGF-β) *in vitro* and *in vivo*,[3,4] produce high amounts of interleukin (IL)-10 and TGF-β, and protect from chronic immunopathology in response to pathogens such as *Leishmania major*, hepatitis C virus or HIV.[5–7] Tr1 cells, on the other hand, are induced through the coordinate activation of the transcription factor c-Maf by IL-27 and the aryl hydrocarbon receptor (Ahr).[8–13] Tr1 cells express latent-associated peptide (LAP) that binds TGF-β, produce high amounts of IL-10 relative to interferon gamma (IFN-γ) but no IL-4[10,14,15] and are presumed to protect from T-helper type-1(Th1)/Th17-mediated autoimmune disease in mice[16] and prevent reactions to common allergens including house dust mites[17,18] and bee venom[19] in humans.

Aspergillus fumigatus is an ubiquitous mold that can cause distinct modes of pathology: invasive aspergillosis (IA) and allergic bronchopulmonary aspergillosis (ABPA) in clinical situations such as neutropenia, immune suppression and chronic obstructive lung disease. In these cases, impaired lung immunity and subsequent fungal infections are accompanied with insufficient Th1 (IA)[20,21] and overwhelming Th2 (ABPA) responses, respectively.[22,23] Foxp3$^+$ nT$_{reg}$ as well as Foxp3$^+$ iT$_{reg}$ have been demonstrated to be essential for the induction of protective tolerance to the fungus in mice[24] and humans[25] by inhibition of overwhelming effector Th1/Th2 cell responses at late stages of experimental IA[24,26] and in ABPA patients.[25]

A clinical challenge is the induction of balanced antifungal effector T-cell responses together with T$_{reg}$-cell responses to reduce the risk for Th1/Th2-mediated immunopathology and to promote the development of a durable protective immunity to *A. fumigatus*.[23] This could be accomplished by an adoptive transfer of antifungal T cells. *A. fumigatus*-specific recombinant proteins derived from cell wall components are proposed to be the most promising immunogenic targets to induce protective antifungal immunity.[27] We[27] and others[28,29] have previously shown that epitopes derived from the

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cell wall glucanase Crf-1 induce protective antifungal Th1 responses, and more specifically, we identified an immunodominant epitope within the cell wall glucanase Crf-1 of *A. fumigatus* (Crf-1/p41, thereafter referred to p41) that induces protective Th1 responses in humans and Th1/T_{reg} in mice.[30] In the present study, we identified p41-specific Tr1 cells in the peripheral blood of healthy humans and in mice after vaccination with p41 and investigated their potential role in antifungal immunity.

RESULTS

Identification of pre-existing p41$^+$ Tr1 clones in healthy human donors

We have recently shown that the p41-peptide induces protective *A. fumigatus*-specific Th1 cell responses in humans and Th1/iT_{reg} in a mouse model of aspergillosis.[27,30] This observation let us to investigate whether p41 has the potential to induce antifungal Tr1 cells, another T_{reg}-cell subset that can be induced by inhaled antigens and regulates immune responses to these antigens. Due to the low frequency of p41$^+$ memory T cells in the peripheral blood of healthy human donors (Supplementary Figure 1), we generated p41-specific T-cell clones from *in vitro* expanded p41$^+$CD154$^+$ T cells. To ensure analysis of different T-cell clones, we determined TcR-Vβ signatures of the clones (data not shown) and excluded identical clones from subsequent analyses. Tr1 cells are characterized by their high production of IL-10 with co-production of IFN-γ in the absence of IL-4.[31] We therefore determined co-production of IL-10, IFN-γ and IL-4 by p41$^+$ T-cell clones after p41-specific restimulation by cytometric bead array. With respect to this cytokine signature, p41$^+$ T-cell clones were subdivided into a population with high and low IL-10-to-IFN-γ ratio (IL-10^{high} and IL-10^{low}) (Supplementary Table S1, Figure 1a). In contrast, none of the clones produced significant amounts of IL-4.

Next, we compared the expression of LAP and inducible T-cell costimulator (ICOS) between IL-10^{high} and IL-10^{low} p41$^+$ T-cell clones, two molecules that are expressed on Tr1 cells. LAP was specifically upregulated on p41$^+$ T-cell clones with a high IL-10-to-IFN-γ ratio upon activation (Figure 1c). In contrast, ICOS expression was upregulated on all p41$^+$ T-cell clones after restimulation. In addition, we detected transient upregulation of the T_{reg} lineage-specific transcription factor Foxp3, but not Helios,[32,33] in activated p41$^+$ T-cell clones, irrespective of their cytokine production profile (Figure 1b). However, transient Foxp3 in these clones was significantly lower compared with CD4$^+$CD25$^+$CD127^{dim} nT_{reg}. Thus, these data suggest that pre-existing IL-10-producing LAP$^+$ p41$^+$ Tr1 cells are present in the memory CD4$^+$ T-cell pool of healthy humans.

Human p41$^+$ Tr1 clones exert a suppressive activity against CD4$^+$ T cells

We next addressed the question whether p41$^+$ Tr1 clones are able to suppress proliferation of autologous conventional CD4$^+$ T cells (T_{conv}) in *in vitro* coculture assays. p41$^+$ Tr1 clones significantly suppressed proliferation of CD4$^+$CD25$^-$ T_{conv} (31 ± 2%; Figure 2a). This effect was specific for p41$^+$ Tr1 clones as T_{conv} proliferation was not suppressed but rather increased in the presence of p41$^+$ T_{eff} clones, most likely referred to their high IL-2 production (data not shown). Of note, p41$^+$ Tr1 clones also significantly suppressed *in vitro* expansion of p41-specific CD4$^+$ T cells (51 ± 5% suppression) in an antigen-specific manner (Figure 2b, upper panel and lower left). This suppression was contact-independent, as expansion of p41$^+$ T cell was still reduced by 57 ± 5% after separation from p41$^+$ Tr1 clones by a semi-permeable membrane (Figure 2b lower right). Collectively, these data show that p41$^+$ Tr1 clones are capable of suppressing the proliferation of T_{conv} and expansion of p41$^+$ T cells in a contact-independent manner.

Human p41$^+$ Tr1 clones are not artificially induced during *in vitro* expansion

Tr1 cells can be induced under different polarizing conditions *in vitro*.[34] To exclude that CD154$^+$ T-cell pre-selection or addition of IL-7 and IL-15 during *in vitro* culture affect polarization of p41$^+$ Tr1 cells, we generated p41$^+$ T-cell clones from peripheral blood mononuclear cells (PBMCs) exclusively in the presence of IL-2. Consistent with our previous data, p41$^+$ Tr1 clones generated in the absence of polarizing conditions exhibited a significantly increased IL-10-to-IFN-γ cytokine profile (Supplementary Figure 2A) without producing considerable amounts of IL-4 (data not shown), suppressed proliferation of T_{conv} by 36 ± 1% (Supplementary Figure 2B) and expansion of p41$^+$ T cells by 46 ± 5% (Supplementary Figure 2C).

Further evidence that p41$^+$ Tr1 clones are not artificially induced *in vitro* comes from analyses of cytomegalovirus (CMV)-specific CD4$^+$ T-cell clones. The analysis of memory CMVpp65-specific CD4$^+$ T-cell clones (donor 1) or, more restricted, CMVpp65$_{283\text{-}297}$-specific CD4$^+$ T-cell clones (donor 2)[35] demonstrated that these clones could not be subdivided in terms of their IL-10-to-IFN-γ cytokine profile (Table 1). In fact, all clones produced high amounts of IL-4. Furthermore, these clones did not differentially upregulate LAP but exhibited a Foxp3 expression pattern comparable with p41$^+$ T-cell clones (Supplementary Figure 3A, B). Consequently, CMV-specific T-cell clones failed to suppress *in vitro* proliferation of autologous T_{conv} cells (Table 1). Overall, these data demonstrate that p41$^+$ Tr1 cells were not artificially induced as a consequence of pre-selection or cytokine-mediated polarization *in vitro* but were rather *in vitro* expanded from *in vivo*-induced p41$^+$ Tr1 cells.

Intranasal vaccination with p41 and zymosan induces Tr1 cells in mice

We next asked whether Tr1 cells can be induced *in vivo* by the p41-peptide. To this end, we vaccinated mice with the p41-peptide and zymosan or CpG-oligodeoxynucleotide (ODN), two adjuvants that have shown to support IL-10 production by murine T cells *in vitro* (data not shown). Animals that received anti-CD3 monoclonal antibody (mAb) intranasally served as positive control for Tr1 cell induction.[36,37] We comparatively analyzed lungs of control anti-CD3-treated mice and of mice treated with p41 + zymosan or p41 + CpG-ODN for the presence of LAP$^+$, Foxp3$^+$ and IL-10$^+$ cells by immunofluorescence staining. Similar to mice treated with anti-CD3 mAb, mice that had received p41 + zymosan revealed the presence of IL-10$^+$ and LAP$^+$, but not Foxp3$^+$, cells in the lung parenchyma (Figure 3a). In contrast, IL-10$^+$ and Foxp3$^+$, but not LAP$^+$, cells were present in the lungs of mice treated with p41 + CpG-ODN. Neither LAP$^+$ nor Foxp3$^+$ cells were induced in the lungs of mice treated with zymosan + control C22 peptide. We also used flow cytometric analysis to confirm the expansion of LAP$^+$ cells (both CD4$^+$ and CD4$^-$) in the lungs (Figure 3b) and thoracic lymph nodes (TLN) (Figure 3c) of mice treated with p41 + zymosan or control anti-CD3 mAb but not p41 + CpG-ODN (Figures 4b and c). LAP$^+$ cells also expressed ICOS and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) (data not shown).

To further confirm whether the LAP$^+$IL-10$^+$ phenotype correlates with a Tr1-type function, we next analyzed their cytokine expression pattern and suppressive capacity. LAP$^+$ lung cells isolated from mice treated with p41 + zymosan and anti-CD3 mAb stained positive for

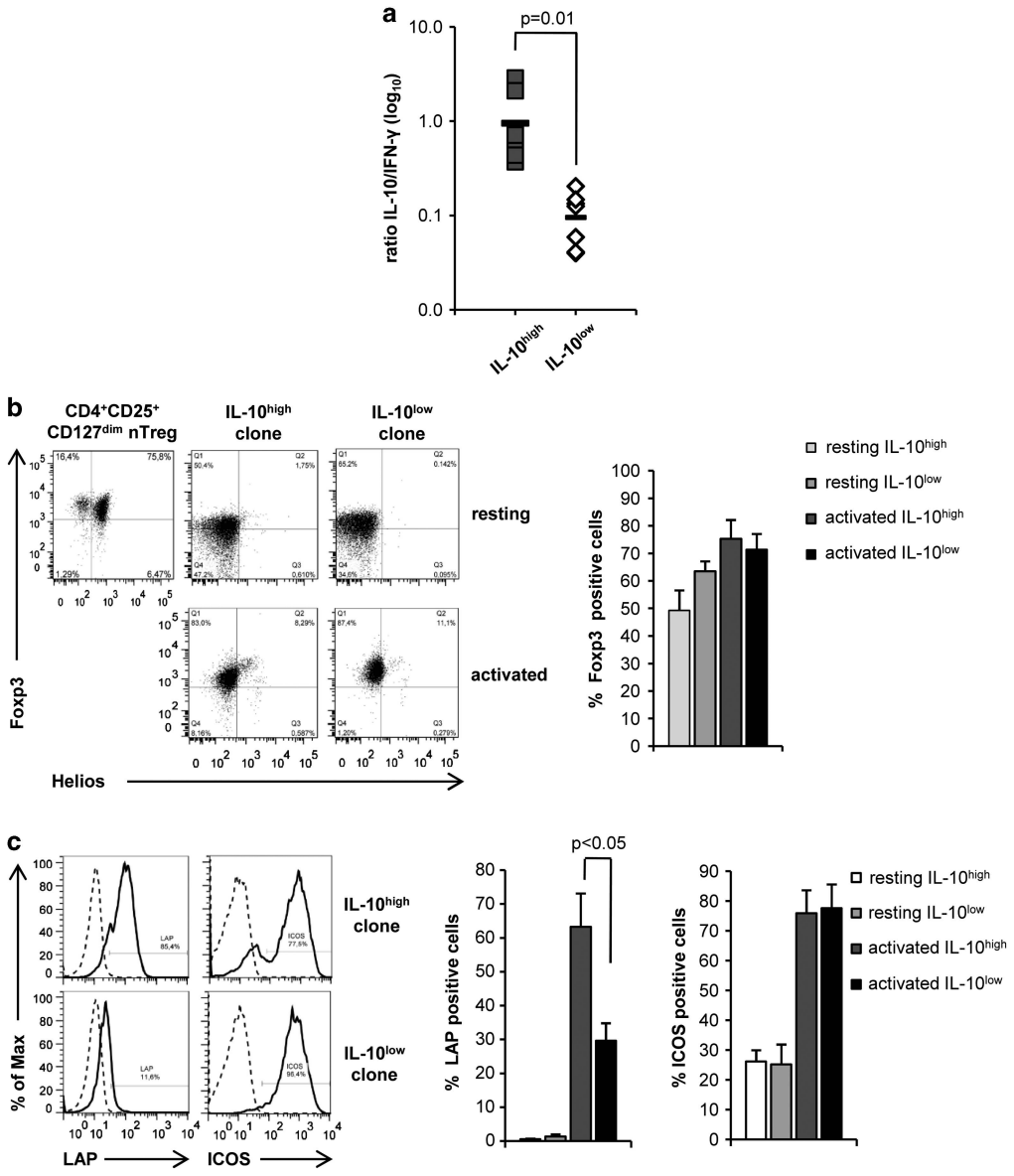


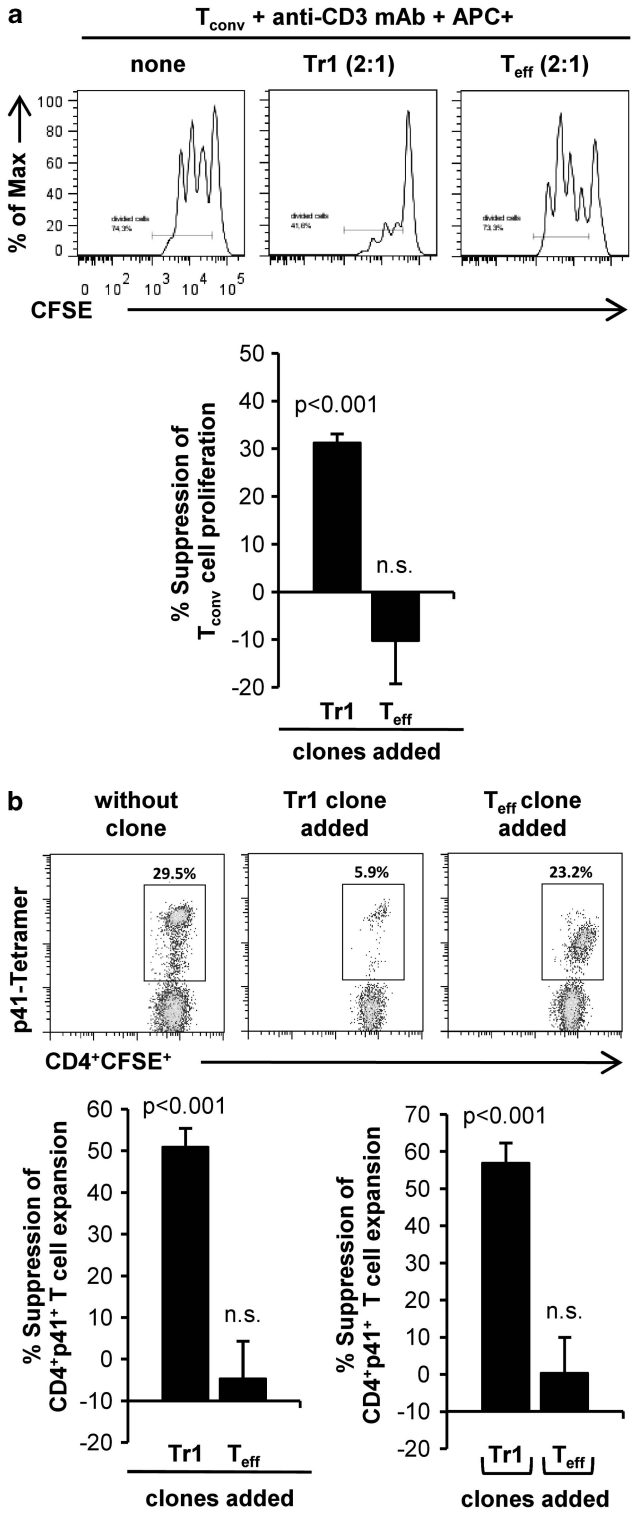
Figure 1 Identification of human p41$^+$CD4$^+$ Tr1 cell clones in the peripheral blood of healthy human donors. (**a**) CD4$^+$p41$^+$ T-cell clones were restimulated with p41-pulsed DC for 48 h prior analysis of IL-10 and IFN-γ secretion from culture supernatants by Cytometric Bead Array. The diagram summarizes the ratio of IL-10-to-IFN-γ release ± s.d. of p41$^+$CD4$^+$ T-cell clones ($n=9$ per group; three different donors). (**b**) Flow cytometric analysis of the Foxp3 and Helios expression in resting CD4$^+$CD25$^+$CD127^{dim} nT$_{reg}$ and in one representative CD4$^+$ T-cell clone with high (IL-10^{high}) and low (IL-10^{low}) IL-10-to-IFN-γ ratio (upper panel) and after stimulation with autologous p41-pulsed DC for 24 h (ratio 10:1, lower panel). The diagram summarizes the percentage of Foxp3$^+$expression in resting and activated IL-10^{high} and IL-10^{low} p41$^+$CD4$^+$ T-cell clones ± s.d. ($n=7$ per group; three different donors). (**c**) Histograms show expression of LAP and ICOS of one representative activated IL-10^{high} and IL-10^{low} p41$^+$CD4$^+$ T-cell clone (solid lines) compared with isotype controls (dashed lines) and diagram summarizes percentage of LAP and ICOS expression ± s.d. on resting and activated p41$^+$CD4$^+$ T-cell clones, respectively ($n=7$ per group; three different donors).

IL-10 but not IFN-γ (Figure 4a), a finding confirmed by the ability of T cells isolated from the lungs of these animals (and similarly from TLN, data not shown) to secrete increased amounts of IL-10 but not IFN-γ upon anti-CD3 stimulation *in vitro* (Figure 4c). In addition, IL-10-secreting cells, isolated from lungs of p41 + zymosan-treated mice, suppressed proliferation of responder CD4$^+$ T cells in a dose- and IL-10-dependent manner as shown by titration of Tr1 cells and addition of a neutralizing mAb, respectively (Figures 4b and d). Neutralization of TGF-β had a minor effect on this suppressive capacity. Since p41 + zymosan administered subcutaneously failed to induce Tr1 cells in the lungs (data not shown), our data show that LAP$^+$ IL-10$^+$ Tr1 cells are induced upon intranasal exposure to fungal antigens in the presence of zymosan, whereas intranasal administration of fungal antigens + CpG-ODN induced Foxp3$^+$ iT$_{reg}$, as already reported.[27,30]

p41-induced Tr1 cells decrease inflammatory T cells in murine aspergillosis

We next asked for the role Tr1 cells might have in the pathogenesis of IA. To this purpose, mice, vaccinated as described above, were subsequently intranasally infected with *A. fumigatus* conidia and analyzed for their susceptibility to fungal infection in terms of fungal

growth and inflammatory pathology. Vaccination of mice with p41 + zymosan did not significantly affect fungal growth in the lung (Figure 5a) but decreased cellular infiltrates (Figure 5b) and neutrophils (Figure 5c) in the lung and bronchoalveolar lavage (BAL) (Figure 5b insets and numbers). Consistent with previous observations,[27,30] under conditions of Foxp3$^+$ iT$_{reg}$ induction by p41 + CpG-ODN treatment, both fungal burden and inflammation were decreased.



Quantification of the production of important effector cytokines in antifungal immune resistance[23] by lung infiltration cells revealed significantly increased IL-10 but decreased IFN-γ levels under conditions of Tr1 cell induction by p41 + zymosan (Figure 5d), whereas tumor necrosis factor-alpha and IL-17A production was not affected in these mice. In contrast, significantly increased levels of IFN-γ accompanied by decreased levels of IL-17A and tumor necrosis factor-alpha were observed in mice vaccinated with p41 + CpG-ODN. In addition, an increase in IL-27, a dendritic cell (DC)-derived cytokine required for the differentiation of Tr1 cells *in vivo*,[13] was observed in p41 + zymosan but not p41 + CpG-ODN-treated mice (Figure 5d). These data suggest that the occurrence of Tr1 cells is associated with increased IL-10 and IL-27 levels, but decreased IFN-γ levels in infection, while an induction of Foxp3$^+$ iT$_{reg}$ is associated with high levels of IL-10 and IFN-γ and concomitant inhibition of tumor necrosis factor-alpha and IL-17A.

Next, we analyzed Th lineage-specific transcription factors in CD4$^+$ T cells from TLN to receive further insight into T-cell function

Table 1 Functional characterization of cytomegalovirus-specific CD4$^+$ T-cell clones

Donor #	*Clone*	*CMV epitope*	*Cytokine production (pg m^{-1})*			*% T$_{conv}$ cell proliferation in the presence of the clone (T$_{conv}$:clone2:1)*
			IL-10	*IL-4*	*IFN-γ*	
1	6A5	pp65pool	43	484	1309	146
	9F9		4	959	755	89
	1F5		18	402	1259	115
	9B10		25	590	1164	147
2	6B6	pp65$_{283-297}$	222	626	876	88
	2G12		212	1333	1509	119
	10A6		841	994	974	86

Abbreviations: CMV, cytomegalovirus; IL, interleukin.
CMVpp65$^+$ CD4$^+$ T-cell clones were restimulated with peptide-pulsed DC (Donor 1: CMV peptide pool; Donor 2: CMVpp65$_{283-297}$) for 48 h. Thereafter, production of the cytokines IL-10, IL-4 and IFN-γ was determined from culture supernatant by CBA. Proliferation of T$_{conv}$ in response to platebound anti-CD3 monoclonal antibody plus irradiated CD2$^-$ stimulator cells was analyzed in the presence of the indicated clone by carboxyfluorescein diacetate, succinimidyl ester (CFSE) dilution (T$_{conv}$ without clone = 100% proliferation).

Figure 2 Human p41$^+$ Tr1 cell clones suppress expansion of CD4$^+$ T cells. (**a**) T-cell clones were cocultured together with CFSE-labeled autologous CD4$^+$CD25$^-$ T cells (T$_{conv}$) (1:2 ratio) and irradiated p41-pulsed CD2-depleted APC (1:2:4 (clone:T$_{conv}$:APC)) in round bottom plates, coated with 0.5 μg ml^{-1} anti-CD3 mAb. After three days, CFSE dilution was determined by flow cytometry. The histograms show CFSE dilution of T$_{conv}$ in the absence and presence of one representative T$_{eff}$ and Tr1 clone. Diagrams summarize % ± s.d. suppression of T$_{conv}$ proliferation in the presence Tr1 and T$_{eff}$ clones (n = 9 per group; three different donors) at a 1:2:4 ratio (clone:T$_{conv}$:APC). (**b**) CFSE-labeled PBMCs were cultured together with CellVue-Claret-labeled p41-specific T-cell clones at 2:1 ratio. After 24 h of culture and every second day, IL-2 were added to the culture. After seven days, the T cells were restimulated with p41-pulsed autologous DC at a ratio of 10:1 and cultured for additional six days in the presence of IL-7 and IL-15. Afterward, expansion of CFSE$^+$CD4$^+$p41$^+$ T cells was analyzed by flow cytometry using a p41-specific PE-labeled tetramer. Dotplot analysis shows one experiment in the absence (left) and presence of a representative Tr1 clone (middle) and T$_{eff}$ clone (right). Diagrams summarize % ± s.d. suppression of CD4$^+$CFSE$^+$p41$^+$ T-cell expansion in contact-dependent presence of Tr1 and T$_{eff}$ clones (left) (n = 6 per group; three different donors) as well as in contact-independent cocultures in transwell systems (right) (n = 5 per group; three different donors).

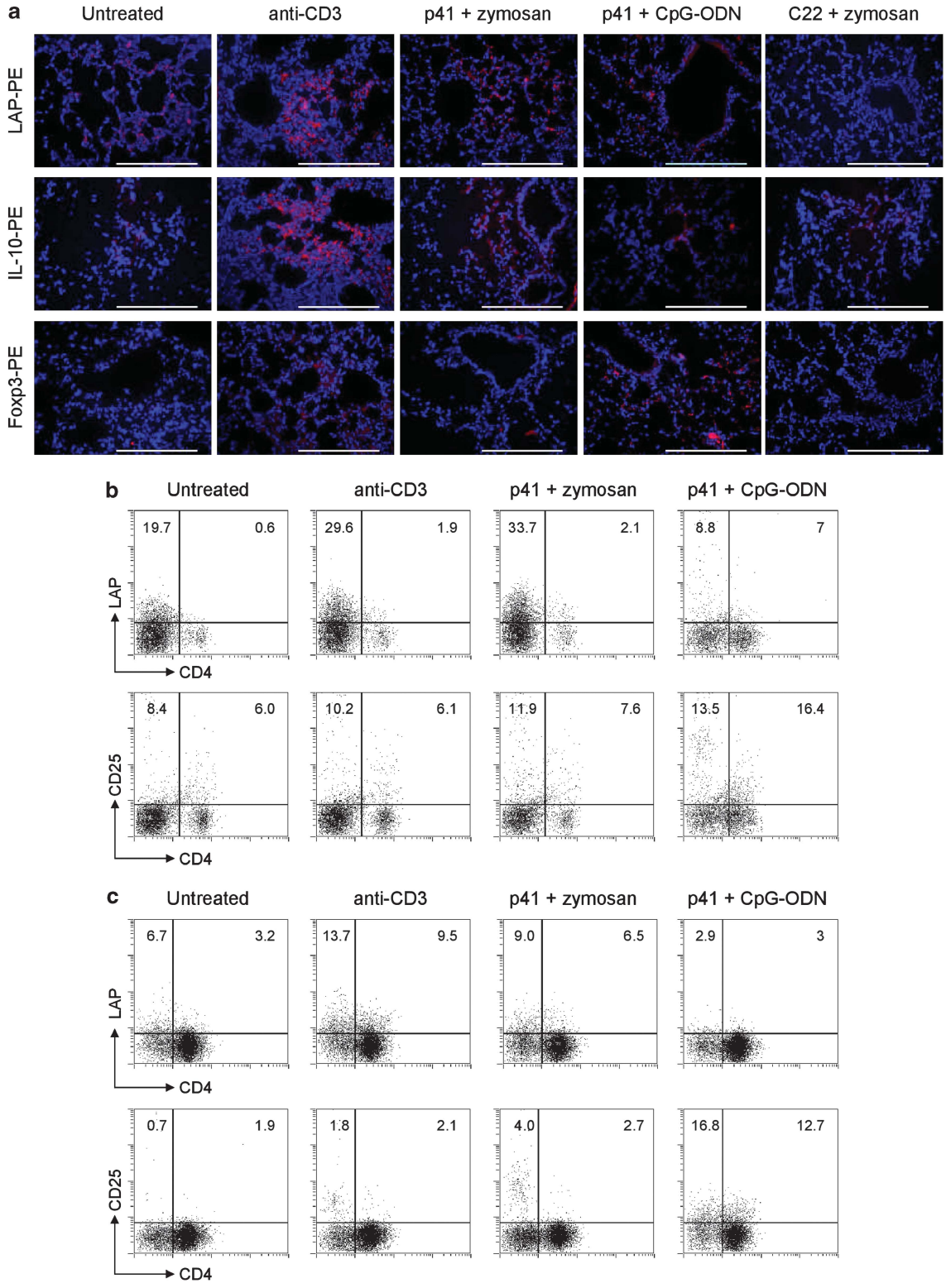


Figure 3 p41 induces murine Tr1/T_{reg} *in vivo* depending on adjuvant. Immunofluorescence staining of lungs (**a**) and flow cytometry of lungs (**b**) and TLN (**c**) from C57BL/6 mice intranasally treated with anti-CD3, the p41-peptide or the control C22 peptide along with zymosan or CpG-ODN. Lungs were stained with anti-LAP-PE, anti-IL-10-PE or anti-Foxp3 followed by PE secondary antibody and 4',6-diamidino-2-phenylindole for nuclei for Tr1/T_{reg}-cell assessment at the end of each treatment. Representative images of two independent experiments were acquired with a $\times 40$ objective. Scale bars, 200 μm. Total cells were stimulated *in vitro* with anti-CD3 + CD28 for 24 h before surface staining with anti-CD4-FITC (fluorescein), anti-CD25-PE and anti-LAP-PE. Numbers refer to % positive cells.

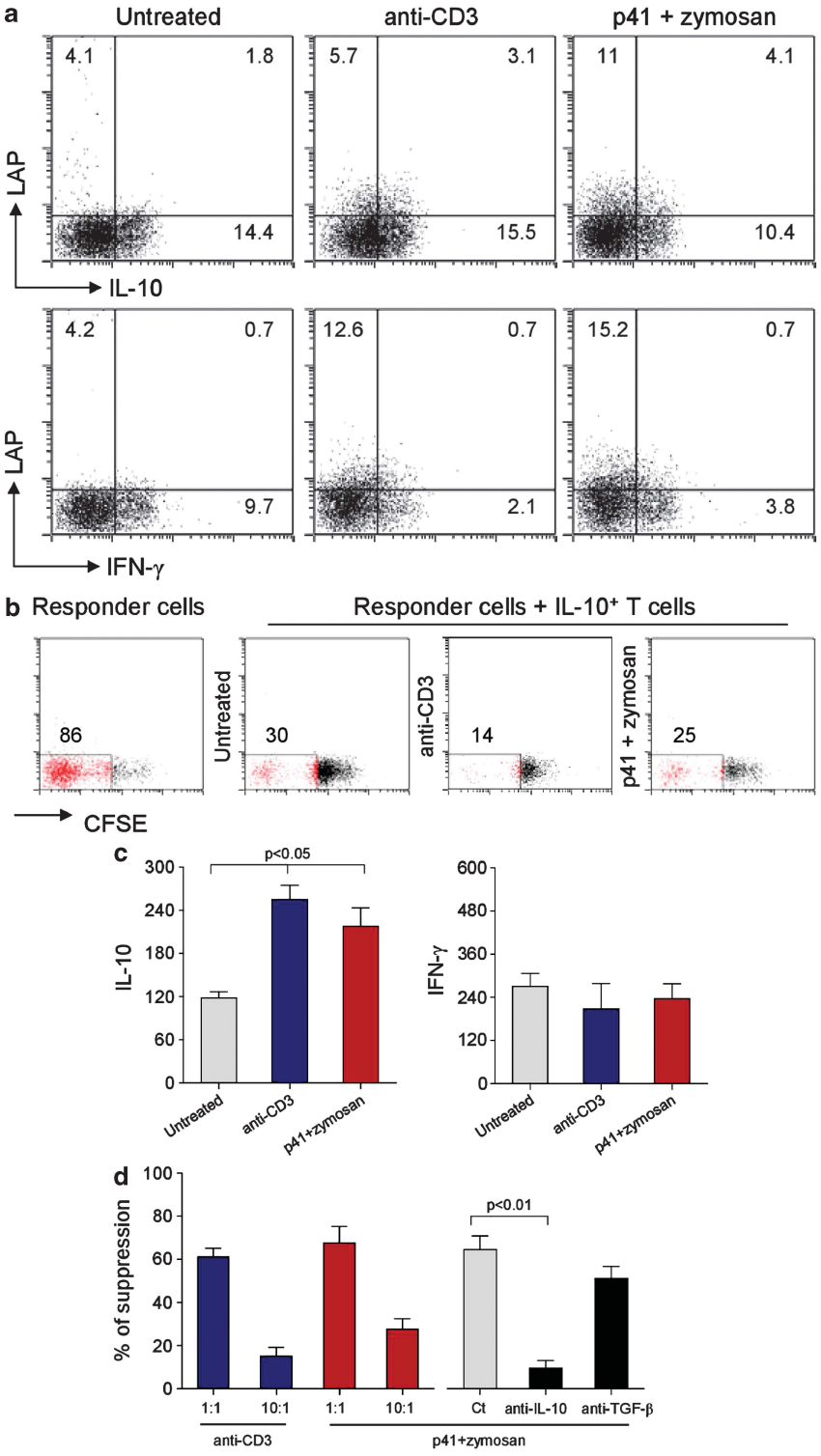


Figure 4 Mouse Tr1 cells are IL-10$^+$IFN-γ^- and suppress T-cell proliferation. Mice were intranasally treated as decribed previously in Figure 3. (**a**) Total lung cells were stimulated *in vitro* with anti-CD3 + CD28 for 24 h before surface stained with anti-LAP-PE and, once permeabilized, stained for intracellular IL-10 or IFN-γ. Numbers refer to % positive cells. (**b**) Suppression of proliferation of CD4$^+$CD25$^-$CFSE-labeled responder T cells by IL-10$^+$ T cells (1:1 ratio) from lungs of treated mice. Cells were anti-CD3 + CD28 stimulated for 72 h. The CFSE signal was analyzed by flow cytometry. The percentages of proliferating cells (red) are shown. (**c**) Cytokine levels (pg ml^{-1}, enzyme-linked immunosorbent assay) in the supernatants of total lung cells from naive mice (untreated) or mice treated as above. Cells were stimulated *in vitro* with anti-CD3 + CD28 for 24 h. Data are mean ± s.d. $P<0.05$, one-way analysis of variance Bonferroni post-test. (**d**) Suppression of proliferation of CD4$^+$CD25$^-$CFSE-labeled responder T cells by purified T cells from lungs of treated mice. Cells were anti-CD3 + CD28 stimulated for 72 h. Anti-IL-10 or anti-TGF-β neutralizing antibodies (50 μg ml^{-1}) were added to cocultures at 1:1 ratio. Suppression (%, as indicated) was expressed as the relative inhibition of the percentage of CFSE cells ($100 \times$(1% CFSE^{low}CD4$^+$CD25$^-$ T cells in coculture/% CFSE^{low} CD4$^+$CD25$^-$ T cells alone)) for CFSE-based measurement of proliferation. Data are mean values ± s.d.. *P*-value derived from two-way analysis of variance test, $P<0.01$. Ct, isotype-matched antibody.

after vaccination with p41. Expression of *c-maf*, a Tr1-specific transcript,[8,12] was specifically increased after treatment with p41 + zymosan and anti-CD3 mAb (Figure 5e). As expected, *Foxp3* transcripts were increased after p41 + CpG-ODN treatment. Of note, Th1-related *Tbet* and Th17-specific *Rorc* transcripts were decreased after vaccination with p41 + zymosan but increased after p41 + CpG-ODN treatment. Together, these data demonstrate differential expression of Th1/Th17-related transcription factors under Tr1- or Foxp3$^+$ iT$_{reg}$-inducing condition, suggesting that these cells differently control inflammatory Th1 and Th17 cell responses in experimental aspergillosis.

Mouse Tr1 cells are activated through Ahr and require IL-10 to exert their suppressive function

Studies in humans and mice show that differentiation of Tr1 cells *in vivo* requires AhR[11,36] in addition to IL-27[13] but not IL-10.[38] We therefore investigated Tr1 cell activation and function in *Ahr*$^{-/-}$ and *Il10*$^{-/-}$ mice. Neither LAP$^+$ nor IL-10$^+$ cells could be induced in the lungs of untreated *Ahr*$^{-/-}$ mice or after treatment with p41 + zymosan (Figures 6a and b). In contrast, LAP$^+$ cells were present in *Il10*$^{-/-}$ mice (Figures 6c and d). In addition, we detected a higher degree of lung inflammation in *Ahr*$^{-/-}$ and *Il10*$^{-/-}$ mice (Figures 6e and f) that could not be controlled by treatment with p41 + zymosan. The treatment did not modify fungal burden (data not shown). To determine the role of IL-10 in the regulatory activity of Tr1 cells and to define the potential of TGF-β[16] to this activity, mice were treated with p41 + zymosan together with neutralizing IL-10 or TGF-β mAb at the time of infection. Neutralization of IL-10 and, to a lesser extent, TGF-β increased the lung inflammatory pathology (Figure 6g). These data suggest that AhR rather than IL-10 is involved in the induction of Tr1 cells, whereas the suppressor activity of these cells is mainly mediated by IL-10.

DISCUSSION

There is emerging evidence that Foxp3$^+$ T$_{reg}$ are involved in the regulation of antifungal immune responses. Animal and clinical studies of IA and ABPA had shown that these cells suppress overwhelming inflammatory Th1 and Th2 responses and support the generation of long-term antifungal T-cell memory.[24] However, it remains unclear whether Tr1 cells, that control the maintenance of immune homeostasis to inhaled allergens,[25,39] might also be involved in maintenance of a protective antifungal immunity. In the present study, we identified for the first time *A. fumigatus*-specific Crf-1/p41$^+$ Tr1 cells in mice and humans and demonstrated that these cells possess the overall features of Tr1 cells by the means of high IL-10 production, LAP expression in the absence of Foxp3 and the capacity to suppress T-cell activation *in vitro* and *in vivo*.

We identified human p41-specific Tr1 cells within the memory CD4$^+$ T-cell pool of healthy donors after *in vitro* expansion under distinct culture conditions. These cells showed the characteristics of Tr1 cells in terms of their LAP expression, high IL-10 production in the absence of significant amounts of IL-4,[31] and their ability to suppress *in vitro* proliferation of CD4$^+$ T cells in an antigen-independent as well as p41-dependent manner. On the other hand, human p41$^+$ memory Tr1 cells differed from previously described primary-induced Tr1 cells with respect to their transient upregulation of Foxp3 expression. However, our observation that all analyzed p41$^+$ T-cell clones as well as CMV-specific T-cell clones upregulated Foxp3 upon restimulation, irrespective of their differentiation state and, more strikingly, CD4$^+$CD25$^+$CD127^{dim} nT$_{reg}$ expressed higher amounts of Foxp3, strongly suggests that this expression is related to the memory state of the T-cell clones without affecting their function.[40]

It has recently been shown that *A. fumigatus*-specific Foxp3$^+$ Helios$^+$ nT$_{reg}$ can be identified in healthy humans after enrichment of CD137$^+$ T cells activated by *A. fumigatus* lysates.[41] Although we cannot exclude that p41-specific Foxp3$^+$Helios$^+$ T$_{reg}$ exist in the peripheral blood of healthy humans, these cells were undetectable under our *in vitro* culture conditions, indicating that p41-peptide predominantly induces Tr1 cells in healthy humans. Therefore, these data support our notion that p41$^+$ Tr1 cells might have a crucial role in maintenance of *A. fumigatus*-specific immune homeostasis. However, it would be of interest to determine whether p41-specific can be identified after distinct pre-selection strategies such as CD137$^+$ enrichment.

Besides the characterization of human p41$^+$ Tr1 cells, our study provided the opportunity to analyze the induction and function of p41$^+$ Tr1 in a mouse model of aspergillosis. Analysis of p41 + zymosan-vaccinated mice revealed the induction of LAP$^+$ IL-10$^+$ Tr1 cells *in vivo*, accompanied by an increase in IL-27 production by lung-infiltrating cells and c-Maf expression in CD4$^+$ T cells isolated from TLN. As vaccination of *Ahr*$^{-/-}$ mice failed to induce LAP$^+$ IL-10$^+$ Tr1 cells, our data suggest that p41$^+$ Tr1 cell differentiation is mediated via the activation of c-Maf by IL-27 in cooperation with AhR, as previously reported.[8–11,13]

In summary, our data show that antifungal IL-10-producing LAP$^+$ Tr1 cells exist in the peripheral blood of healthy humans and can be induced in mice via cooperative activation of c-Maf via IL-27 and AhR. Direct comparison of p41$^+$ Tr1 cells with Foxp3$^+$ iT$_{reg}$ of the same specificity showed that these T$_{reg}$-cell subsets differ in their mode of induction and, in part, in their suppressive function. Given the ability of the p41-peptide to induce protective antifungal Tr1 cells as well as Th1/Foxp3$^+$ iT$_{reg}$ cells,[30] we suggest that this peptide provides a versatile tool to restore protective *A. fumigatus*-specific immunity in clinical settings of invasive aspergillosis and ABPA.

METHODS

Peptides

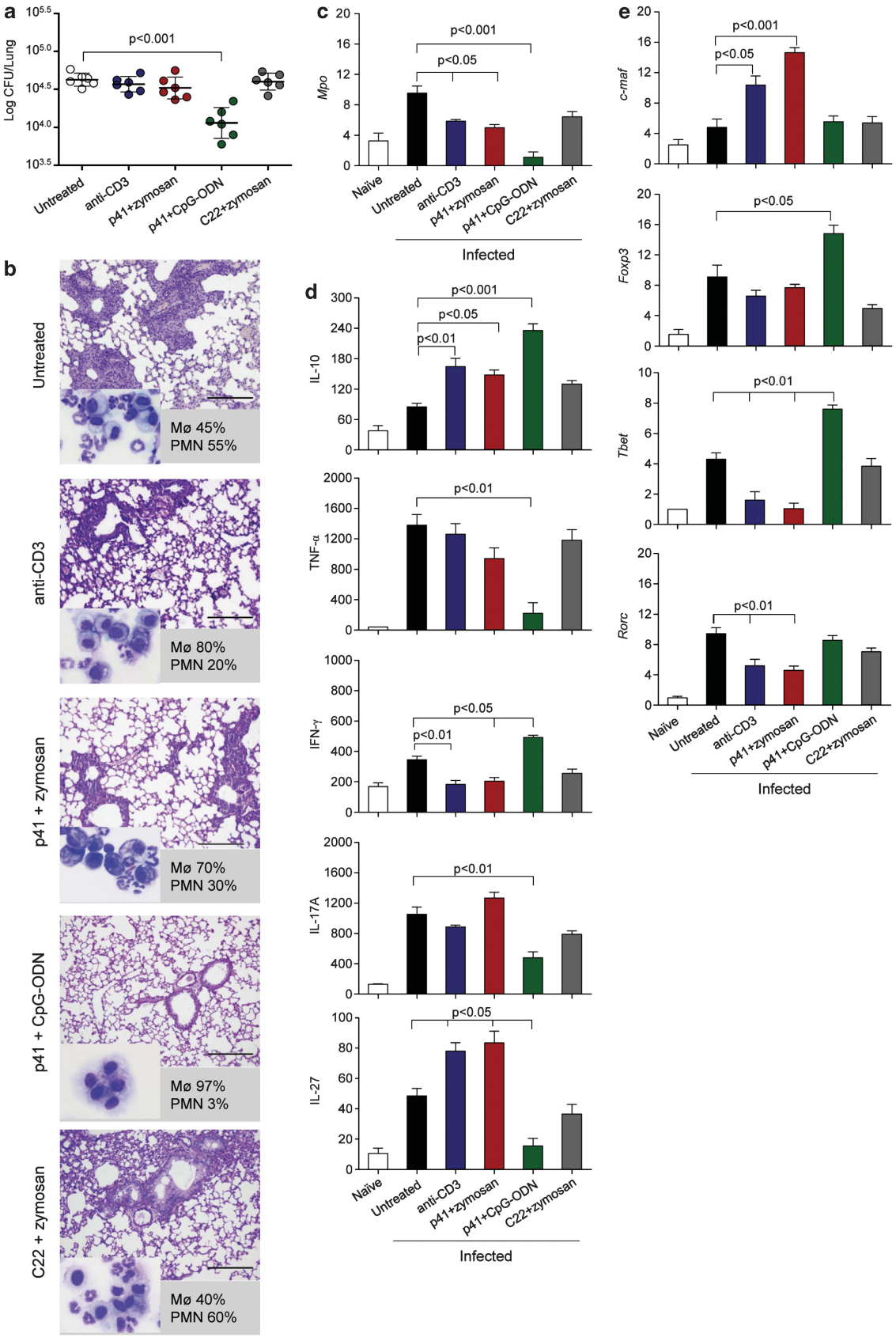
The *A. fumigatus*-derived Crf-1/p41-peptide (FHTYTIDWTKDAVTW), Crf-1/p22 peptide (TDFYFFFGKAEVVMK) andCMV-derived peptide CMVpp65$_{283\text{-}297}$ (KPGKISHIMLDVAFT) were purchased from ProImmune (Oxford, UK). The CMVpp65 peptide pool was obtained from JPT (Berlin, Germany).

Ethics statement

Human studies were performed after written informed consent of the study participants, in accordance with the Declaration of Helsinki, and were approved by the institutional review board of the University hospital Würzburg (#214/12). Mouse experiments were performed according to the guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS No. 123) and the Italian Approved Animal Welfare Assurance A–3143–01. Legislative decree 157/2008-B regarding the animal license was obtained by the Italian Ministry of Health lasting for 3 years (2011–2014). The protocol was approved by Perugia University Ethics Committee. Infections were performed under avertin anesthesia and all efforts were made to minimize suffering.

Human blood donors and cell isolation

Blood was obtained from healthy HLA-DRB01*04-typed donors after informed consent. PBMCs were isolated by density gradient centrifugation using Ficoll/Hypaque (Biochrom, Berlin, Germany). CD4$^+$ T cells and CD2$^-$ antigen-presenting cells (APCs) and monocytes were selected by magnetic microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the


a
Log CFU/Lung
p<0.001
Untreated
anti-CD3
p41+zymosan
p41+CpG-ODN
C22+zymosan
b
Untreated
Mø 45%
PMN 55%
anti-CD3
Mø 80%
PMN 20%
p41 + zymosan
Mø 70%
PMN 30%
p41 + CpG-ODN
Mø 97%
PMN 3%
C22 + zymosan
Mø 40%
PMN 60%
c
Mpo
p<0.001
p<0.05
Naive
Untreated
anti-CD3
p41+zymosan
p41+CpG-ODN
C22+zymosan
Infected
d
IL-10
TNF-α
IFN-γ
IL-17A
IL-27
e
c-maf
Foxp3
Tbet
Rorc

manufacturer's instructions. Briefly, $CD4^+$ T cells were enriched by depletion of non-$CD4^+$ T cells, $CD2^-$ APC were enriched by depletion of $CD2^+$ cells and monocytes were positive selected by enrichment of CD14+ cells. Monocyte-derived DCs were generated as described previously.[42]

Generation of human p41-specific T-cell clones

p41-specific T cells were expanded from $CD154^+$ p41-specific T cells as described previously[43] or from PBMCs activated by 5 μg ml^{-1} p41 in the presence of 5 U ml^{-1} IL-2 (Chiron; Tuttlingen, Germany). At day 7, cultures were restimulated with p41-pulsed monocytes at 5:1 and expanded for additional 7 days. $p41^+$ T cells were purified using a HLA-DRB01*04 Phycoerythrin (PE)-labeled major histocompatibility class II tetramer (Beckman Coulter, Marseille, France) and anti-PE MicroBeads (Miltenyi Biotech). T-cell cloning was performed as described before.[44]

Flow cytometric characterization of T-cell clones

For phenotypic analysis, the following anti-human mAb were used: CD4 (Horizon V500, clone: RPA-T4), Foxp3 (AlexaFluo 647 or PE, 259D/C7; all BD Pharmingen, San Diego, CA, USA), ICOS (PE-Cy7, ISA3; eBioscience, San Diego, CA, USA), LAP (PE, TW4-2F8), and Helios (Pacific Blue, 22F6, BioLegend, San Diego, CA, USA). For analysis of surface markers, cells were incubated with mAbs at 4 °C for 20 min. For staining of intracellular molecules fixation/permeabilization buffers (eBiosciences) were used. Cells were analyzed by FACS-Canto II flow cytometer (BD Biosciences, Heidelberg, Germany) and FlowJo software (Tree Star, Ashland, OR, USA). IFN-γ and IL-10 production by T-cell clones was determined from culture supernatants after 48 h of restimulation with p41-pulsed DC by cytometric bead array according to the manufacturer's instructions (CBA, BD Biosciences). Quantification of cytokine production was analyzed using FCAP Array v2.0 Software (SoftFlow, Pécs, Hungary).

Suppression assays with human T-cell clones

Responder $CD4^+$ T cells were labeled with 1 μM carboxyfluorescein diacetate, succinimidyl ester (CFSE) (Invitrogen, Darmstadt, Germany) for 10 min at room temperature prior coculture with T-cell clones and p41-pulsed irradiated $CD2^-$ cells (30 Gy) at a 2:1:4 ratio in 96-well round bottom plates coated with 1 μg ml^{-1} anti-CD3 mAb as described previously.[45] After 3 days, CFSE dilution was analyzed by flow cytometry. For antigen-specific suppression, PBMCs were labeled with CFSE and T-cell clones with CellVue-Claret (Polysciences, Eppelheim, Germany) according to the manufacturer's instructions. CFSE-labeled PBMCs were cocultured with CellVue-Claret-labeled T-cell clones at 3:1 in the presence of 5 μg ml^{-1} p41-peptide and 5 U ml^{-1} IL-2. After 7 days, the cultures were restimulated with p41-pulsed DC at 10:1 and expanded for additional 6 days in the presence of 10 ng ml^{-1} IL-7 and IL-15. For transwell experiments, PBMCs and T-cell clones were separated by 0.4-μm membranes (Costar Corning Life Science, Lowell, MA, USA). Expansion of $CFSE^+CD4^+p41^+$ T cells was determined by flow cytometry using the p41 tetramer.

Animals

Female C57BL/6 mice, 8–10-week old, were purchased from Charles River (Calco, Italy). Homozygous *Ahr*$^{-/-}$ and *Il10*$^{-/-}$ mice on C57BL/6 background were bred under specific pathogen-free conditions at the Animal Facility of Perugia University, Perugia, Italy.

Infection and treatments

Mice were anesthetized by intraperitoneal injection of 2.5% avertin (Sigma-Aldrich, St Louis, MO, USA) before the intranasal instillation of a suspension of 2×10^7 viable conidia from the *A. fumigatus* AF293 strain. Mice were monitored for fungal growth ($\log_{10}$ colony-forming unit per lung, mean ± s.d.), histopathology (periodic acid-Schiff) and lung immunofluorescence (see below). BAL fluid was collected by cannulating the trachea and washing the airways with 3 ml of phosphate-buffered saline. Total and differential cell counts were done by staining BAL smears with May-GrünwaldGiemsa reagents (Sigma-Aldrich) before analysis. At least 200 cells per cytospin preparation were counted and the absolute number of each cell type was calculated. Histology sections and cytospin preparations were observed using a BX51 microscope (Olympus, Milan, Italy) and images were captured using a high-resolution DP71 camera (Olympus). Mice were treated with a total of 60 μg of anti-IL-10 (JES5.2A5) or TGF-β (1D11) mAbs (Bioceros BV, Utrecht, The Netherlands) at the time of infection. Control mice received either a rat IgG1 mAb isotype or a murine IgG1 mAb isotype with similar effects. Mice received intranasally 0.5 μg CD3-specific antibody (clone 145-2C11, Bioceros BV, Utrecht, Netherlands) for five consecutive days, 20 μg of p41 or C22 control peptide along with 10 μg CpG ODNODN 1862) (Invitrogen, Srl, Milan, Italy) or zymosan (Sigma-Aldrich) administered at 14, 7, 3 days before the infection. Assays were done three days after the infection or a day after the end of each treatment.

Immunofluorescence of mouse sections

Lung sections were deparaffinized and stained with PE-LAP (TGF-β1, BioLegend, Campoverde Srl, Milan, Italy), PE-IL-10 (eBioscience) and polyclonal Foxp3 (abcam, Cambridge, UK) followed by goat anti-rabbit PE secondary antibody (BioLegend).

Preparation of mouse lung and thoracic lymph node cells

For isolation of lung and TLN cells, lungs and TLN were aseptically removed and cut into small pieces in cold medium. The dissected tissue was then incubated in Hank's balances salt solution without Ca and Mg (Lonza Verviers Belgium) medium containing collagenase XI (0.7 mg ml^{-1}; Sigma-Aldrich) and type IV bovine pancreatic DNase (30 μg ml^{-1}; Sigma-Aldrich) for 30–45 min at 37 °C. The action of the enzymes was stopped by adding 10 ml of medium, and digested lungs and TLNs were further disrupted by gently pushing the tissue through a nylon screen. The single cell suspension was then washed, centrifuged at 200 *g* and resuspended in phosphate-buffered saline containing 0.5% fetal bovine serum. $CD4^+$ T cells (both $CD25^+$ and $CD25^-$) were isolated with the CD4 MicroBeads (Miltenyi Biotech). Naïve $CD4^+$ $CD25^-$ T cells were isolated from lung by magnetic sorting with the $CD4^+$ $CD25^+$ Regulatory T Cell Isolation Kit, (Miltenyi Biotech). $IL\text{-}10^+$ cells were isolated with the Mouse IL-10 Secretion Assay Cell Enrichment and Detection Kit (Miltenyi Biotech) from non-adherent lung cells cultured on anti-CD3-coated plates (clone 145-2C11; BD PharMingen) in the presence of 2 μg ml^{-1} soluble anti-CD28 mAb (clone 37.51; BD PharMingen) overnight at 37 °C.

Coculture experiments with mouse Tr1 and CFSE labeling

Naïve $CD4^+CD25^-$ cells were labeled with CFSE (10 mM in dimethylsulfoxide; Molecular Probes, Eugene, OR, USA) and stimulated in 96-well flat bottom plates (Corning Incorporated, New York, NY, USA) with anti-$CD3^+$/$CD28^+$ at 1×10^5 cells per well in the presence of $IL\text{-}10^+$ cells, at 1:1 responder:Tr1 ratio or in the presence of purified T cells at 1:1 or 10:1 ratios. Anti-IL-10 or anti-TGF-β neutralizing antibodies (50 μg ml^{-1}) were added to

Figure 5 p41-induced murine Tr1 cells decrease inflammatory T cells in murine aspergillosis. C57BL/6 mice were treated as decribed previously in Figure 3 and infected intranasally with live *Aspergillus* conidia (six mice per group). Mice were assessed for (**a**) lung fungal growth ($\log_{10}$ colony-forming unit per organ ± s.d.), (**b**) lung histopathology (periodic acid-Schiff staining) and BAL cellular morphometry (indicated as % of mononuclear (Mø) or polymorphonuclear (PMN) cells in the inset (May–GrünwaldGiemsa staining)). Representative images of two independent experiments were acquired with a ×40 objective. Scale bars, 200 μm. (**c**) *Mpo* expression (reverse transcriptase-PCR) on total lung cells. (**d**) Levels of cytokines (pg ml^{-1}) by specific enzyme-linked immunosorbent assays (mean values ± s.d., $n = 3$) in lung homogenates. (**e**) Relative expression (mRNA-fold increase) of transcription factor genes (by reverse transcriptase-PCR) on purified $CD4^+$ T cells from TLN. Assays were done at three days post-infection. *P*-value was determined by one-way analysis of variance Bonferroni post-test ($n = 3$).

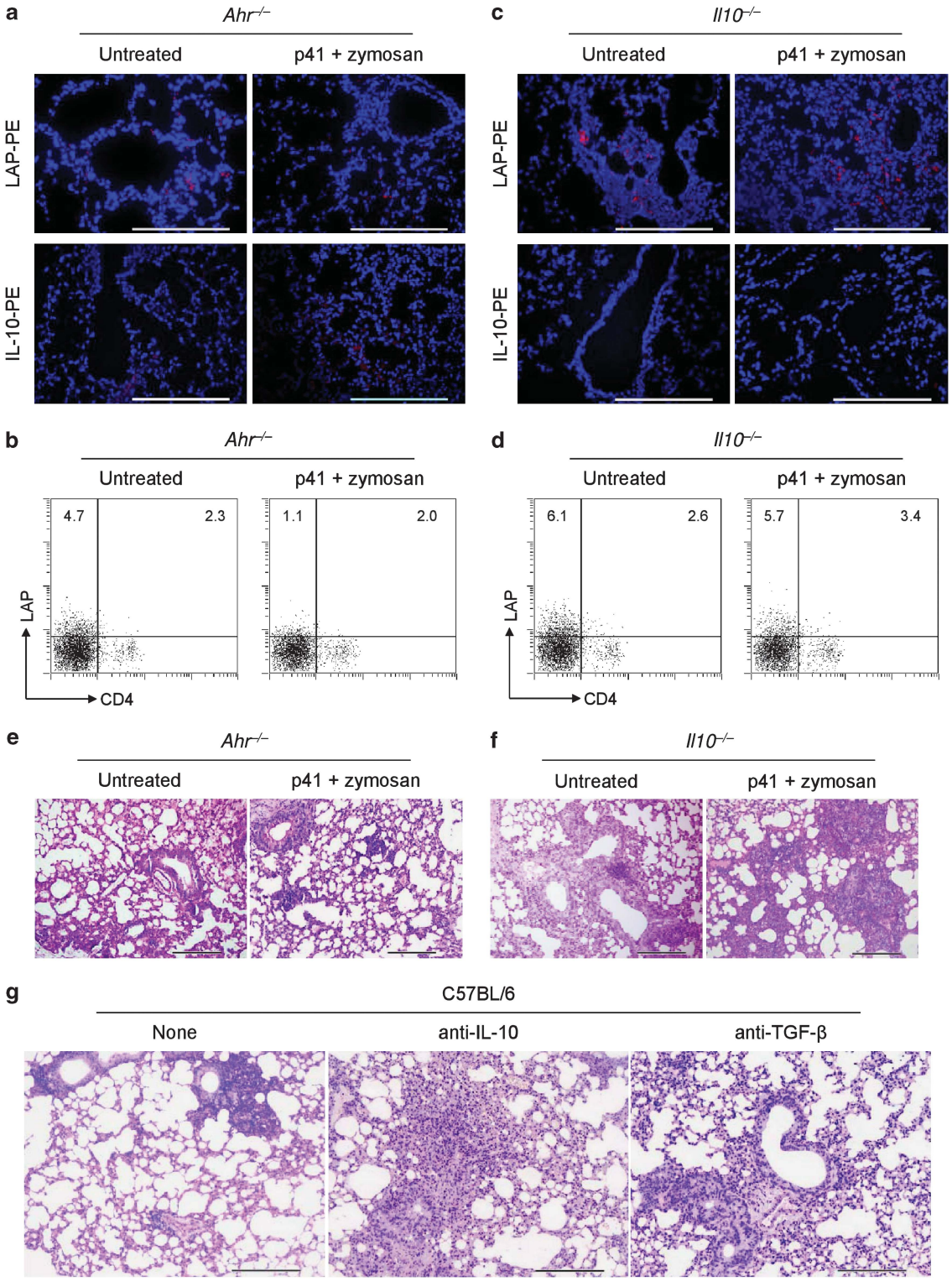


Figure 6 Mouse Tr1 cells are activated through Ahr and require IL-10 and, partly, TGF-β to suppress. *Ahr*$^{-/-}$ or *Il10*$^{-/-}$ mice were treated and infected as decribed previously in Figure 5 and assessed for lung immunofluorescence with anti-LAP-PE or anti-IL-10-PE staining (**a**, **c**); numbers of LAP^{+} CD4^{+} T cells by flow cytometry on anti-CD3 + CD28-stimulated lung cells (**b**, **d**), and lung histology (periodic acid-Schiff staining) (**e**, **f**). Cell nuclei were stained blue with 4′,6-diamidino-2-phenylindole in **a** and **c**. Representative images of two independent experiments were acquired with a ×40 objective. Scale bars, 200 μm. In **b** and **d**, numbers refer to % positive cells. (**g**) C57BL/6 mice were treated with p41 + zymosan, infected and treated with IL-10 or TGF-β-neutralizing antibody at the time of infection. Scale bars, 200 μm. None, rat IgG1 antibody.

cocultures at 1:1 ratio. After 72 h, CFSE signal was analyzed by flow cytometry. Suppression (%) was expressed as the relative inhibition of the percentage of CFSE cells ($100 \times$ (1-% $CFSE^{low}$ $CD4^+CD25^-$ T cells in coculture/% $CFSE^{low}$ $CD4^+CD25^-$ T cells alone)) for CFSE-based measurement of proliferation.

Flow cytometry and intracellular staining of mouse cells

Cells were sequentially reacted with anti-CD4 (Fluorescein, clone: GK1.5), anti-CD25 (Phycoerythrin, clone: 7D4) (Miltenyi Biotech) or anti-LAP (TGF-β1) (Phycoerythrin, clone: TW7-16B4, Biolegend, Campoverde Srl, Milan, Italy). For intracellular cytokine detection purified cells were stimulated with 200 ng ml^{-1} of phorbol 12-myristate 13-acetate (Sigma-Aldrich) and 1 μg ml^{-1}of ionomycin (Sigma-Aldrich) for 2 h at 37 °C. Cells were cultured for four additional hours with brefeldin and then permeabilized with the CytoFix/CytoPerm kit (BD Pharmingen) for intra-cytoplasmic detection of IFN-γ (Alexa Fluor488, clone: XMG1.2, eBioscience) and IL-10 (Fluorescein, clone: JES5-16E3, Biolegend). Cells are analyzed with a FACScan flow cytofluorometer (Becton Dickinson, Mountain View, CA, USA) equipped with CELLQuestTM software.

Enzyme-linked immunosorbent assay and real-time PCR analysis of mouse cells

The level of cytokines in lung homogenates and culture supernatants was determined by Kit enzyme-linked immunosorbent assay (R&D Systems, Milan, Italy). Real-time Reverse Transcriptase-PCR was performed using the iCycler-iQdetection system (Bio-Rad, Segrate (MI), Italy) and SYBR Green chemistry (Finnzymes Oy, Espoo, Finland). Cells were lysed and total RNA was extracted using RNeasy Mini Kit (QIAGEN, Milan, Italy) and was reverse transcribed with Sensiscript Reverse Transcriptase (QIAGEN) according to the manufacturer's directions. PCR primers for transcription factors were used as described.[30] The following *c-maf* primers were used: sense 5′-GTAGACCAC CTCAAGCAGGA-3′; antisense 3′-GAAAAATTCGGGAGAGGAAG-5′. Amplification efficiencies were validated and normalized against *Gapdh*. The thermal profile for SYBR Green real-time PCR was at 95 °C for 3 min, followed by 40 cycles of denaturation for 30 s at 95 °C and an annealing/extension step of 30 s at 60 °C. Each data point was examined for integrity by analysis of the amplification plot. The mRNA-normalized data were expressed as relative cytokine mRNA expression in treated cells compared with that of unstimulated cells.

Statistical analysis

Paired student's *t*-tests were used for statistical analysis of data obtained from human studies. For the analysis of murine studies, Student's *t*-test or analysis of variance with Bonferroni's adjustment were used to determine statistical significance ($P<0.05$). The data reported are either from one representative experiment out of 3–5 independent experiments (histology and FACS analysis) or pooled from 3–5 experiments. The *in vivo* groups consisted of 6–8 mice per group. Data were analyzed by GraphPad Prism 4.03 program (GraphPad Software, San Diego, CA, USA).

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