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## Toll-like receptor 2 induced cytotoxic T-lymphocyte-associated protein 4 regulates *Aspergillus*-induced regulatory T-cells with pro-inflammatory characteristics

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Patients with cystic fibrosis, chronic obstructive pulmonary disease, severe asthma, pre-existing pulmonary lesions, and severely immunocompromised patients are susceptible to develop infections with the opportunistic pathogenic fungus *Aspergillus fumigatus*, called aspergillosis. Infections in these patients are associated with persistent pro-inflammatory T-helper (T<sub>H</sub>)<sub>2</sub> and T<sub>H</sub>17 responses. Regulatory T-cells, natural suppressor cells of the immune system, control pro-inflammatory T-cell responses, but can also contribute to disease by shifting to a pro-inflammatory T<sub>H</sub>17-like phenotype. Such a shift could play an important role in the detrimental immunopathology that is seen in aspergillosis. Our study demonstrates that *Aspergillus fumigatus* induces regulatory T-cells with a T<sub>H</sub>17-like phenotype. We also demonstrate that these regulatory T-cells with a pro-inflammatory T<sub>H</sub>17-like phenotype can be reprogrammed to their “classical” anti-inflammatory phenotype by activating Toll-like receptor 2 (TLR2), which regulates the induction of cytotoxic T-lymphocyte-associated protein 4 (CTLA4). Similarly, soluble CTLA4 could reverse the pro-inflammatory phenotype of *Aspergillus*-induced regulatory T-cells. In conclusion, our results suggest a role for regulatory T-cells with a pro-inflammatory T<sub>H</sub>17-like phenotype in *Aspergillus*-associated immunopathology, and identifies key players, i.e. TLR2 and CTLA4, involved in this mechanism.

Opportunistic infections caused by *Aspergillus fumigatus* are frequently observed in immunocompromised patients. These infections are considered to be severe complications and result from the absence of a fully functional host defence, thereby increasing susceptibility to this fungus<sup>1</sup>. Other patient groups at risk of developing disease caused by *A. fumigatus* are patients with cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), severe asthma, or individuals with pre-existing pulmonary lesions<sup>2–6</sup>. Clinical manifestations of such disease are called (invasive) aspergillosis, and range from hypersensitivity reactions to *A. fumigatus*, as is seen in allergic bronchopulmonary aspergillosis (ABPA)<sup>5,7</sup>, to insufficient clearance of *A. fumigatus* with long-lasting inflammatory responses and ongoing fungal growth, as is seen in chronic pulmonary aspergillosis (CPA)<sup>2,3</sup>.

Adequate clearance of *A. fumigatus* relies on T-helper cell-mediated pro-inflammatory immune responses, and particularly the T-helper (T<sub>H</sub>)<sub>1</sub> response<sup>8–11</sup>. However, T-helper responses, in particular T<sub>H</sub>2 and T<sub>H</sub>17, are also known to play a detrimental role in the pathogenesis of ABPA and CPA<sup>12–14</sup>. These responses can cause uncontrolled inflammation, resulting in a massive influx of eosinophils and neutrophils<sup>12,15</sup>. Although T<sub>H</sub>17-mediated recruitment of neutrophils plays an important role in the clearance of fungi, this response can

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also play a detrimental role in protective immunity during aspergillosis<sup>10,11</sup>. T<sub>H</sub>17 activation by fungal growth can lead to disruption and necrosis of pulmonary tissue, thereby creating a niche for saprophytic growth of *A. fumigatus*<sup>1</sup>, allowing the fungus to persist within the lungs and continue to induce persistent inflammatory responses<sup>16</sup>.

An important inhibitor of T-helper cell-driven pro-inflammatory responses are the regulatory T (T<sub>reg</sub>) cells. This endogenous immune suppressive cell controls T<sub>H</sub>2 polarization, and neutralizes T<sub>H</sub>17 responses in the lungs<sup>17</sup>. Adoptive transfer of T<sub>reg</sub> cells, or localised over-expression of Forkhead box protein P3 (FoxP3) (the classical transcription factor of T<sub>reg</sub> cells), inhibits T<sub>H</sub>2 and T<sub>H</sub>17 responses in animal models for asthma, resulting in improvement of clinical scores<sup>18,19</sup>. T<sub>reg</sub> cells are known to modulate immune responses through both contact dependant (cytotoxic T-lymphocyte-associated protein 4; CTLA4, Indoleamine-pyrrole 2,3-dioxygenase; IDO, membrane bound Transforming growth factor  $\beta$ ; TGF $\beta$ , and competition for major histocompatibility complex; MHC), and contact independent (Interleukin-10 and TGF $\beta$ ) signalling pathways<sup>20,21</sup>.

Recently, several studies demonstrated that T<sub>reg</sub> cells can acquire pro-inflammatory characteristics by differentiating into Interleukin (IL)-17A producing RAR-related orphan receptor  $\gamma$ t (ROR $\gamma$ t) expressing T-cells<sup>22,23</sup>. This conversion was found to be involved in the pathogenesis of autoimmune arthritis and depends on pro-inflammatory cytokines such as IL-2 and IL-15<sup>23</sup>. T<sub>H</sub>17 differentiation promoting cytokines, like IL-1 $\beta$ , IL-6, and IL-23 further influence this conversion<sup>22</sup>. Interestingly, in CPA patients, IL-1 and IL-15 cytokine levels were found to be upregulated, suggesting a T<sub>reg</sub> – T<sub>H</sub>17 interplay in these patients<sup>14</sup>. Such a shift could play a crucial role in the IL-17 mediated immunopathology that is seen in patients with aspergillosis.

If *A. fumigatus* is capable of inducing T<sub>reg</sub> cells with a pro-inflammatory phenotype, this could have important implications for our understanding of the detrimental immunopathology seen in aspergillosis. In that case, reversal of pro-inflammatory T<sub>reg</sub> cells to their “classical” anti-inflammatory state could be a promising strategy for immunomodulatory therapy. This study shows that human *A. fumigatus*-induced T<sub>reg</sub> cells can exert this pro-inflammatory phenotype. In addition, we investigate the mechanisms involved and the potential of T<sub>reg</sub> cells to be reprogrammed to their “classical” anti-inflammatory phenotype.

## Results

**A. fumigatus induces regulatory T-cells with a pro-inflammatory T<sub>H</sub>17-like phenotype.** By determining the kinetics of IL-17A and IL-10 production over a course of 7 days in PBMCs simulated with *A. fumigatus* conidia, we determined the optimal time point to detect T<sub>H</sub>17-like pro-inflammatory T<sub>reg</sub> cells. Similar to previous studies with *Candida albicans*<sup>24</sup>, *A. fumigatus*-induced IL-17A release was detectable at peak levels in supernatants after 7 days (Fig. 1A). The production of IL-10, an anti-inflammatory cytokine required for T<sub>reg</sub> cell differentiation<sup>25</sup>, which is also produced by T<sub>reg</sub> cells, demonstrated a biphasic response (Fig. 1A). Expression of the transcription factor of T<sub>H</sub>17 cells, ROR $\gamma$ t, started to increase after 5 days exposure to conidia, similar to IL-17A release, whereas FoxP3 expression was already induced after 24 h and continued to increase in expression towards 7 days (Fig. 1B).

To detect *A. fumigatus*-induced T<sub>H</sub>17-like pro-inflammatory T<sub>reg</sub> cells, PBMCs were stained and measured by flowcytometry after 7 days of stimulation with *A. fumigatus* conidia. T-cells were identified through CD4 (Fig. 1C). Within the CD4<sup>+</sup> population, the number of T<sub>reg</sub> cells was quantified as the percentage of CD25<sup>+</sup>FoxP3<sup>+</sup> cells (Fig. 1D). T<sub>H</sub>17 cells were quantified as ROR $\gamma$ t<sup>+</sup>IL-17A<sup>+</sup> cells within the CD4<sup>+</sup> population (Fig. 1E). Finally, the percentage of cells with T<sub>H</sub>17 markers, i.e. ROR $\gamma$ t / IL-17A, was determined within the T<sub>reg</sub> population, i.e. CD25<sup>+</sup> FoxP3<sup>+</sup> (Fig. 1F).

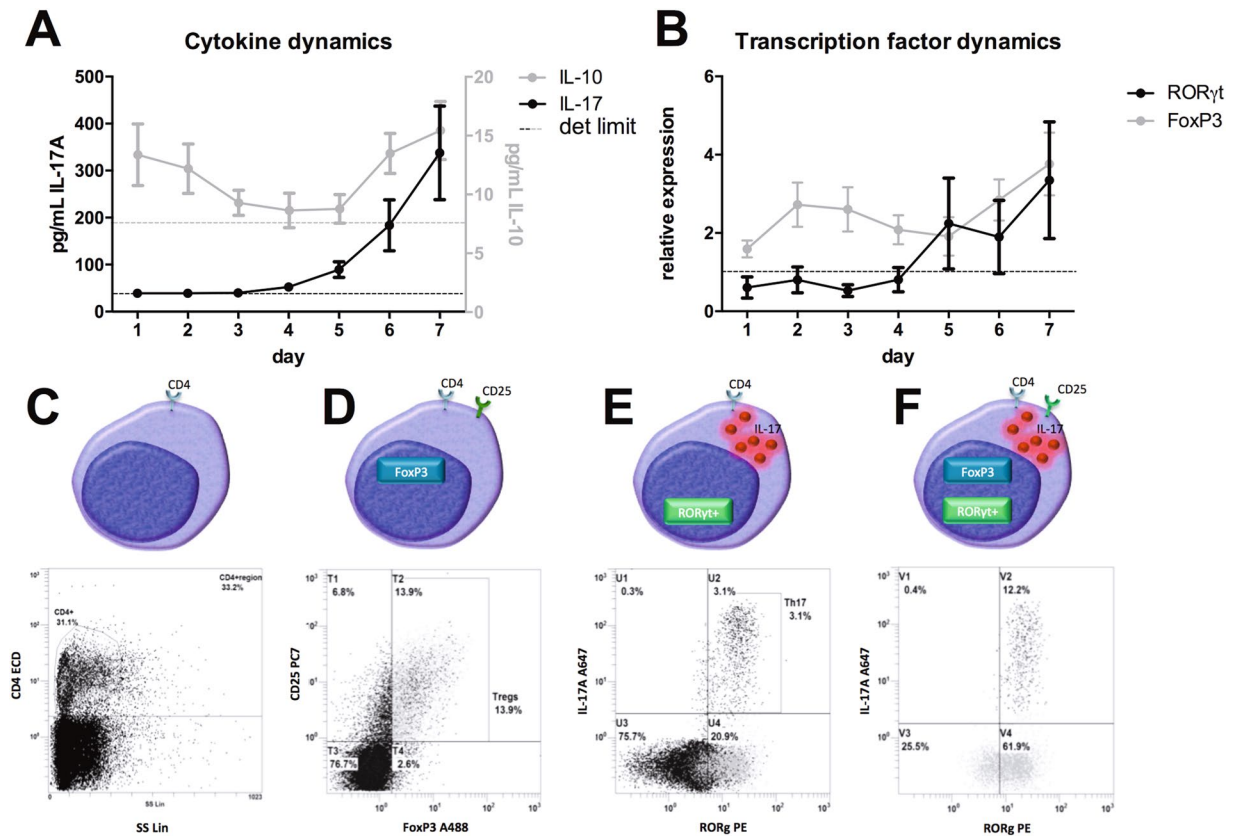
Following stimulation with *A. fumigatus*, a significant induction of CD25<sup>+</sup>FoxP3<sup>+</sup> T<sub>reg</sub> cells ( $p = 0.0017$   $n = 14$ ) (Fig. 2A), ROR $\gamma$ t<sup>+</sup>IL-17A<sup>+</sup> T<sub>H</sub>17 cells ( $p = 0.0017$   $n = 14$ ) (Fig. 2B), and CD25<sup>+</sup>FoxP3<sup>+</sup> ROR $\gamma$ t<sup>+</sup>IL-17A<sup>+</sup> pro-inflammatory T<sub>reg</sub> cells ( $p = 0.0011$   $n = 14$ ) (Fig. 2C) was observed. The observed overlap between regulatory T-cell phenotype (CD25<sup>+</sup>FoxP3<sup>+</sup>), and T<sub>H</sub>17 cell-phenotype (ROR $\gamma$ t<sup>+</sup>, IL-17A<sup>+</sup>, or ROR $\gamma$ t<sup>+</sup>IL-17A<sup>+</sup>), within the CD4<sup>+</sup> cell-population, following stimulation with *A. fumigatus*, is depicted as a Venn diagram in Fig. 2G.

In order to assess the cytokine release by these different cell populations, IL-10 production was measured in the culture supernatant after 24 hours and 7 days, and IL-17A production was measured after 7 days. After 7 days of stimulation, production of both IL-10 and IL-17A was significantly increased ( $p = 0.0273$   $n = 15$  and  $p < 0.0001$ ,  $n = 17$ ) (Fig. 2E and F). However, this effect was not observed for IL-10 after 24 hours (Fig. 2D).

**TLR2 regulates regulatory T-cells with a T<sub>H</sub>17-like phenotype.** Toll-like receptor (TLR)2 is involved in the recognition of pathogen-associated molecular patterns (PAMPS) in the *Aspergillus* cell wall (reviewed in ref. 26), and is associated with the induction of T<sub>reg</sub> cells in response to fungi<sup>27,28</sup>. Based on the observation that naïve splenocytes of *Tlr2*<sup>-/-</sup> mice produce higher levels of IL-17A than wild type (WT) mice (Supplementary Figure 1), we hypothesized that TLR2 could have a role in shaping the population of *Aspergillus*-induced regulatory T-cells.

To investigate the role of TLR2 in the induction of T<sub>reg</sub> cells with a T<sub>H</sub>17-like phenotype, human PBMCs were stimulated with *A. fumigatus* conidia for 24 hours and 7 days while TLR2 was blocked with a neutralizing antibody.

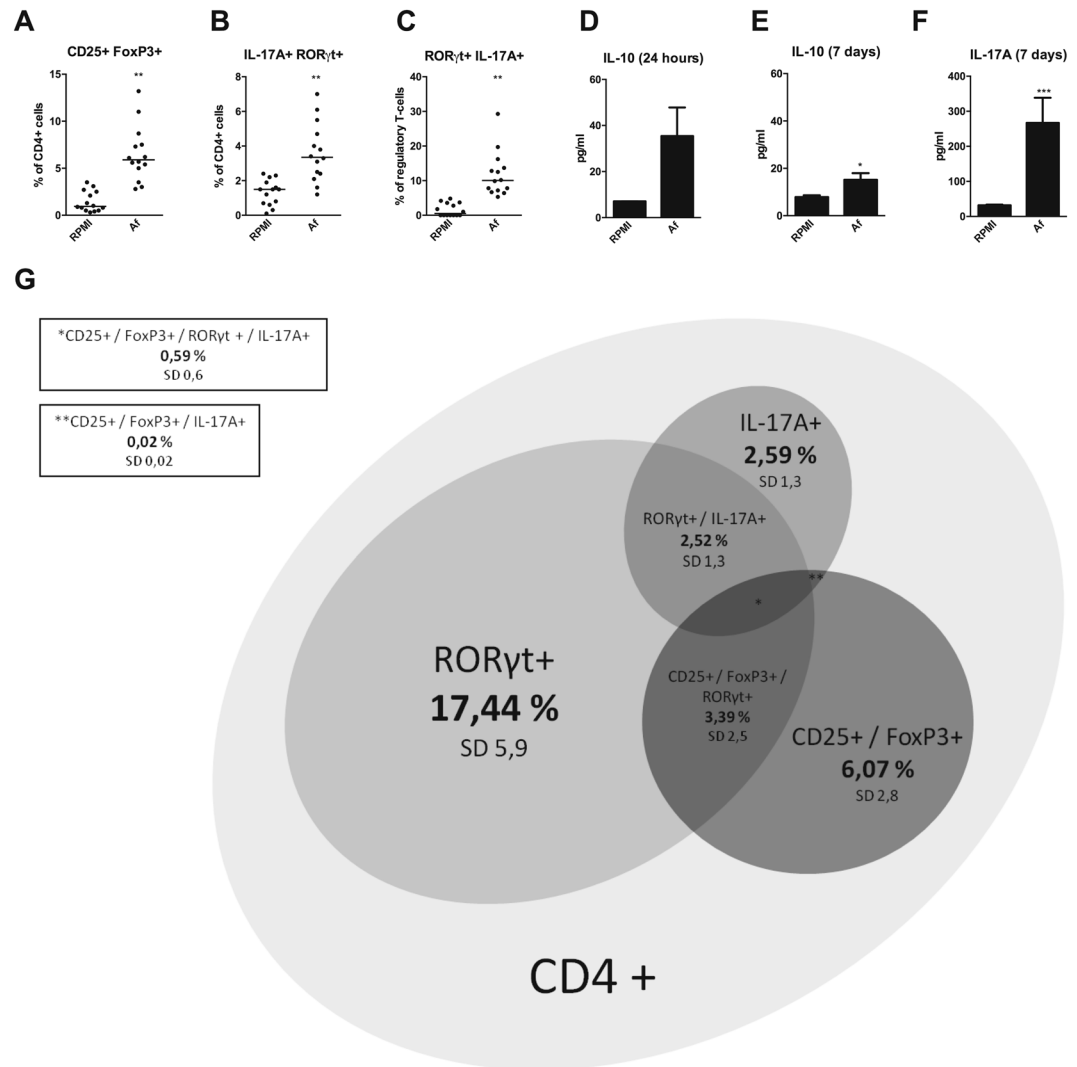
As demonstrated previously<sup>29</sup>, blocking TLR2 before stimulating with *A. fumigatus* conidia resulted in a significant increase in IL-17A production ( $p = 0.0039$   $n = 9$ ). However, no change in IL-10 production after 24 hours, and after 7 days was observed (Fig. 3A). Within the CD4<sup>+</sup> population, the number of CD25<sup>+</sup>FoxP3<sup>+</sup> T<sub>reg</sub> cells significantly decreased with TLR2 blockade ( $p = 0.0117$   $n = 9$ ), while a non-significant trend towards increased expression of T<sub>H</sub>17 cell-characteristics, i.e. ROR $\gamma$ t<sup>+</sup>IL-17A<sup>+</sup> within these cells was observed ( $p = 0.1875$   $n = 6$ ) (Fig. 3B). Expression of T<sub>H</sub>17 cell-characteristics, i.e. ROR $\gamma$ t<sup>-</sup>, ROR $\gamma$ t<sup>+</sup>, and ROR $\gamma$ t<sup>+</sup>IL-17A<sup>+</sup>, within CD25<sup>+</sup>FoxP3<sup>+</sup> T<sub>reg</sub> cells are depicted in Fig. 3C.



**Figure 1.** Expression markers for regulatory T-cells and T<sub>H</sub>17 cells. **(A)** Dynamics of IL-17 and IL-10 cytokine levels in culture supernatants of PBMCs exposed to heat-inactivated *A. fumigatus* conidia ( $1 \times 10^7$ /mL) for 7 days. **(B)** Dynamics of RORγt and FoxP3 mRNA expression in PBMCs exposed to *A. fumigatus* conidia for 7 days. **(C–F)** Gating strategy to detect regulatory T-cells with T<sub>H</sub>17 characteristics. **(C)** T-helper cells were selected by expression of the marker CD4 (CD4<sup>+</sup>), **(D)** regulatory T-cells were selected within the CD4<sup>+</sup> population using the characteristics (CD25<sup>+</sup> FoxP3<sup>+</sup>), **(E)** T<sub>H</sub>17 cell were selected within the CD4<sup>+</sup> population using the characteristics (RORγt<sup>+</sup> IL-17A<sup>+</sup>), and **(F)** T<sub>H</sub>17 cell characteristics (RORγt<sup>+</sup> IL-17A<sup>+</sup>) were selected within regulatory T-cells (CD4<sup>+</sup> CD25<sup>+</sup> FoxP3<sup>+</sup>) upon stimulation with heat-inactivated *A. fumigatus* conidia ( $1 \times 10^7$ /mL).

**TLR2 co-stimulation reduces expansion of regulatory T-cells with a T<sub>H</sub>17-like phenotype.** To further dissect the role of TLR2 in *Aspergillus*-induced T<sub>reg</sub> cells with pro-inflammatory T<sub>H</sub>17-like characteristics, strong TLR2 ligands, i.e. P3C (Pam3Cys-SKKKK; TLR2 and TLR1 heterodimer agonist) and FSL-1 (Pam2Cys-SKKKK; TLR2 and TLR6 heterodimer agonist), were combined with *A. fumigatus* stimulation assays. Co-stimulation of TLR2 with P3C and FSL-1 resulted in a significant decrease in IL-17A production after 7 days ( $p = 0.0002$   $n = 14$ ) and a significant increase in IL-10 production after 24 hours ( $p = 0.0039$  and  $p = 0.0254$   $n = 10$ ), but not after 7 days (Fig. 3D). No significant change in the expansion of CD25<sup>+</sup> FoxP3<sup>+</sup> T<sub>reg</sub> cells was observed upon co-stimulation of TLR2 with P3C, while the population of CD25<sup>+</sup> FoxP3<sup>+</sup> T<sub>reg</sub> cells expressing the T<sub>H</sub>17 RORγt<sup>+</sup> IL-17A<sup>+</sup> phenotype was significantly smaller ( $p = 0.0313$   $n = 7$ ) (Fig. 3E). Expression of T<sub>H</sub>17 cell-characteristics, i.e. RORγt<sup>-</sup>, RORγt<sup>+</sup>, and RORγt<sup>+</sup> IL-17A<sup>+</sup>, within CD25<sup>+</sup> FoxP3<sup>+</sup> T<sub>reg</sub> cells are depicted in Fig. 3F.

**CTLA4 regulates the induction of *Aspergillus*-induced regulatory T-cells with a T<sub>H</sub>17-like phenotype.** One of the most potent molecules that regulates the induction of pro-inflammatory T-cell subsets is CTLA4. To determine whether there is a role for CTLA4 in controlling the induction of IL-17A-expressing regulatory T-cells by *Aspergillus*, we investigated these cells in a patient with CTLA4 deficiency. The CTLA4 deficient patient showed slightly reduced induction of CD25<sup>+</sup> FoxP3<sup>+</sup> regulatory T-cells and a similar induction of IL-17A<sup>+</sup> T<sub>H</sub>17 cells, compared to the healthy control. T<sub>H</sub>17 cell-characteristics, i.e. IL-17A<sup>+</sup>, within CD25<sup>+</sup> FoxP3<sup>+</sup> T<sub>reg</sub> cells, were substantially more induced by the patient compared to the healthy control (Supplementary Figure 2). To assess whether the capacity of TLR2 to reduce the expansion of regulatory T-cells with a T<sub>H</sub>17 phenotype was due to modulation of CTLA4, the capability to induce CTLA4 through TLR2 signalling was investigated. Activation of TLR2 by the agonist P3C resulted in a significant upregulation of CTLA4 expressing CD4<sup>+</sup> T-cells ( $p = 0.0049$   $n = 12$ ). Similarly, co-stimulation of TLR2 with *A. fumigatus* significantly upregulated the induction of CTLA4 expressing CD4<sup>+</sup> T-cells, compared to stimulation with *A. fumigatus* ( $p = 0.0005$   $n = 12$ ) (Fig. 4A). Soluble CTLA4-Ig (Abatacept) is known to inhibit the T-cell co-stimulatory molecule B7-1 (CD80), similar

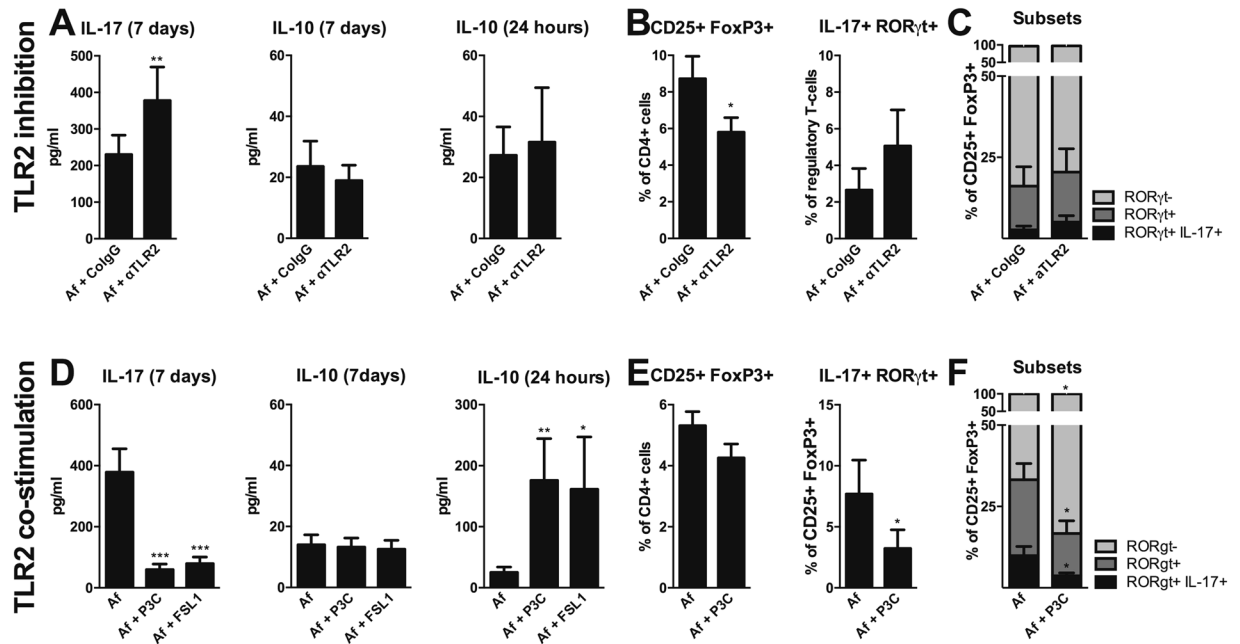


**Figure 2.** *Aspergillus* induces regulatory T-cells with a T<sub>H</sub>17-like phenotype. Scatter plots with median showing (A) Regulatory T-cell (CD25<sup>+</sup> FoxP3<sup>+</sup>) induction after 7 days in human PBMCs stimulated with either RPMI, or heat-inactivated *A. fumigatus* conidia (1 × 10<sup>7</sup>/mL). (B) IL-17A and RORγt expression within regulatory T-cells after 7 days in human PBMCs stimulated with either RPMI, or heat-inactivated *A. fumigatus* conidia (1 × 10<sup>7</sup>/mL). (C) T<sub>H</sub>17 cell (IL-17A<sup>+</sup> RORγt<sup>+</sup>) induction after 7 days in human PBMCs stimulated with either RPMI, or heat-inactivated *A. fumigatus* conidia (1 × 10<sup>7</sup>/mL) (n = 17 donors). (D) IL-10 production after 24 hours in human PBMCs (n = 5 donors) stimulated with either RPMI, or heat-inactivated *A. fumigatus* conidia (1 × 10<sup>7</sup>/mL). (E) IL-10 and (F) IL-17A production after 7 days in human PBMCs (n = 17 donors) stimulated with either RPMI, or heat-inactivated *A. fumigatus* conidia (1 × 10<sup>7</sup>/mL). (G) Proportional Venn diagram showing overlap in CD4 cell-phenotype after PBMCs (n = 17 donors) were stimulated with heat-inactivated *A. fumigatus* conidia (1 × 10<sup>7</sup>/mL) for 7 days. Results are depicted as percentage of CD4<sup>+</sup> cell population with concomitant standard deviation (SD). Data are represented as scatter dot plots with median. Abbreviations: Af = *Aspergillus fumigatus*. \*p-value ≤ 0.05, \*\*p-value ≤ 0.01, \*\*\*p-value ≤ 0.001.

to endogenous CTLA4<sup>30</sup>. Using soluble CTLA4, we validated whether increased CTLA4 levels could reduce *Aspergillus*-induced CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory T-cells with a T<sub>H</sub>17 phenotype. Addition of CTLA4-Ig to human PBMCs resulted in a decreased IL-17A production after 7 days (p = 0.0234 n = 7) (Fig. 4B). Also, induction of the number of CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory T-cells expressing a T<sub>H</sub>17 phenotype, i.e. IL-17A<sup>+</sup>, CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory T-cells and IL-17A<sup>+</sup> T<sub>H</sub>17 cells significantly decreased upon addition of CTLA4-Ig (p = 0.0343, p = 0.0343 and p = 0.0469 n = 7) (Fig. 4D).

## Discussion

In this study we investigated whether *A. fumigatus*-induced T<sub>reg</sub> cells exert a pro-inflammatory phenotype, and if this pro-inflammatory phenotype can be reprogrammed to the “classical” anti-inflammatory T<sub>reg</sub> phenotype. Human PBMCs exposed to *A. fumigatus* conidia showed induction of T<sub>reg</sub> cells with pro-inflammatory T<sub>H</sub>17-like characteristics. Induction of classical (CD25<sup>+</sup>FoxP3<sup>+</sup>) T<sub>reg</sub> cells and (IL-17A<sup>+</sup>RORγt<sup>+</sup>) T<sub>H</sub>17 cells was also

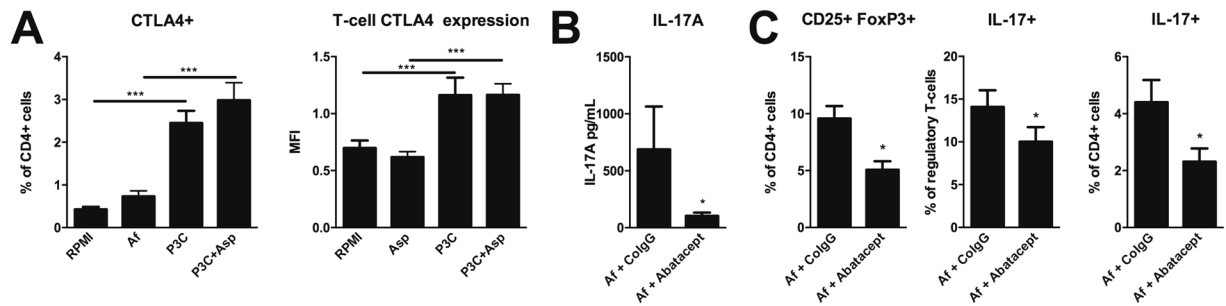


**Figure 3.** TLR2 regulates *Aspergillus*-induced regulatory T-cells with a  $T_H17$ -like phenotype. **(A)** IL-17A production after 7 days, IL-10 production after 24 hours and 7 days, regulatory T-cell ( $CD25^+ FoxP3^+$ ) induction after 7 days, and IL-17A and  $ROR\gamma_t$  expression within regulatory T-cells after 7 days in human PBMCs ( $n = 6$  donors) stimulated with heat-inactivated *A. fumigatus* conidia ( $1 \times 10^7$ /mL) after 1 hour pre-incubation with  $10 \mu\text{g}/\text{mL}$   $\alpha\text{TLR2}$ , or CoIgG. **(B,C)** Different subsets within regulatory T-cell ( $CD25^+ FoxP3^+$ ) induction, defined as  $ROR\gamma_t^-$ ,  $ROR\gamma_t^+$ , or  $ROR\gamma_t^+ IL-17A^+$  regulatory T-cells ( $CD25^+ FoxP3^+$ ), after 7 days in human PBMCs ( $n = 6$  donors) stimulated with heat-inactivated *A. fumigatus* conidia ( $1 \times 10^7$ /mL) after 1 hour pre-incubation with  $10 \mu\text{g}/\text{mL}$   $\alpha\text{TLR2}$ , or CoIgG. Data are represented as mean  $\pm$  SEM. **(D)** IL-17A production after 7 days, IL-10 production after 24 hours and 7 days ( $n = 14$  donors), regulatory T-cell ( $CD25^+ FoxP3^+$ ) induction after 7 days, and IL-17A and  $ROR\gamma_t$  expression within regulatory T-cells after 7 days in human PBMCs ( $n = 9$  donors) stimulated with heat-inactivated *A. fumigatus* conidia ( $1 \times 10^7$ /mL) in the presence or absence of  $10 \mu\text{g}/\text{mL}$  P3C or FSL1. **(E,F)** Different subsets within regulatory T-cell ( $CD25^+ FoxP3^+$ ) induction, defined as  $ROR\gamma_t^-$ ,  $ROR\gamma_t^+$ , or  $ROR\gamma_t^+ IL-17A^+$  regulatory T-cells ( $CD25^+ FoxP3^+$ ), after 7 days in human PBMCs ( $n = 6$  donors) stimulated with heat-inactivated *A. fumigatus* conidia ( $1 \times 10^7$ /mL) in the presence or absence of  $10 \mu\text{g}/\text{mL}$  P3C. Data are represented as mean  $\pm$  SEM. Abbreviations: *Af* = *Aspergillus fumigatus*; *CoIgG* = Control immunoglobulin (G);  $\alpha\text{TLR2}$  = anti-TLR2 antibody; *P3C* = Pam3Cys-SK4444; *FSL-1* = Pam2Cys-SK4444. \* $p$ -value  $\leq 0.05$ , \*\* $p$ -value  $\leq 0.01$ , \*\*\* $p$ -value  $\leq 0.001$ .

significantly increased and related with increased production of IL-10 and IL-17A. These results indicate that *A. fumigatus* can indeed induce  $T_{reg}$  cells with a pro-inflammatory  $T_H17$ -like phenotype, which potentially could contribute to detrimental IL-17-mediated immunopathology.

Although the IL-17A axis plays an important role in the protective immunity against fungal pathogens such as *A. fumigatus*<sup>31</sup>, it has been demonstrated that in some cases IL-17A mediated immune responses overwhelm this protective effect, promoting infection and impairing antifungal immunity<sup>13,32</sup>. Diseases such as CPA and ABPA are characterized by a persistent hyper inflammatory state with massive influx of neutrophils and eosinophils<sup>12,15</sup>, which may be attributed to exaggerated  $T_H17$  responses.  $T_{reg}$  cells are potent suppressors of  $T_H17$  cells<sup>17</sup>, yet this suppressive effect is annulled when regulatory T-cells also acquire  $T_H17$  characteristics. In our study, we observed that the reduction of  $T_{reg}$  cells with a  $T_H17$  phenotype, either through TLR2 stimulation or Abatacept, correlated with a reduction of the  $T_H17$  cytokine IL-17. Since it is technically difficult to determine the origin of IL-17 in culture supernatants we cannot ascertain that this reduction of IL-17A is strictly due to a reduction of IL-17<sup>+</sup>  $T_{reg}$  cells.

TLR2, together with TLR4 and TLR9, is an important Toll-like receptor in the host defence against *Aspergillus* (reviewed in refs 26, 33). The observation that splenocytes of *Tlr2*<sup>-/-</sup> mice have an increased *A. fumigatus*-induced production of IL-17A, directed us towards a possible suppressive role for TLR2 in the induction of IL-17 responses. In addition, many studies have demonstrated a critical role for TLR2 in the induction of regulatory T-cells<sup>27,28,34</sup>. We found that blocking TLR2, while stimulating with *A. fumigatus* conidia, exaggerated the induction of  $T_{reg}$  cells with pro-inflammatory  $T_H17$ -like characteristics. Reversely, we observed that TLR2 co-stimulation not only increased the induction of regulatory T-cells, but also dampened the induction of regulatory T-cells with a  $T_H17$  phenotype. We suggest that altered TLR2 signalling may be involved in the aberrant induction of  $T_{reg}$  cells with pro-inflammatory  $T_H17$ -like characteristics. Whether alterations in TLR2 signalling, pathogen- or host-related, are actually involved in IL-17 mediated immunopathology needs further investigation.



**Figure 4.** CTLA4 regulates *Aspergillus*-induced regulatory T-cells with a  $T_H17$ -like phenotype. (A) Percentage of CTLA4 expressing CD4<sup>+</sup> T-cells and Mean fluorescence intensity (MFI) of CTLA4 staining following stimulation of human PBMCs ( $n = 13$  donors) with heat-inactivated *A. fumigatus* conidia ( $1 \times 10^7$ /mL), 10  $\mu$ g/mL P3C or a combination of both. (B) IL-17A concentration in culture supernatants of human PBMCs ( $n = 6$  donors) stimulated with heat-inactivated *A. fumigatus* conidia ( $1 \times 10^7$ /mL) after 1 hour pre-incubation with 24  $\mu$ g/mL Abatacept, or CoIgG. (C) Regulatory T-cell (CD25<sup>+</sup>FoxP3<sup>+</sup>) induction after 7 days in human PBMCs ( $n = 7$  donors) stimulated with heat-inactivated *A. fumigatus* conidia ( $1 \times 10^7$ /mL) after 1 hour pre-incubation with 24  $\mu$ g/mL Abatacept, or CoIgG.  $T_H17$  cell (IL-17A<sup>+</sup>) induction after 7 days in human PBMCs stimulated with heat-inactivated *A. fumigatus* conidia ( $1 \times 10^7$ /mL) after 1 hour pre-incubation with 24  $\mu$ g/mL Abatacept, or CoIgG. IL-17A<sup>+</sup> regulatory T-cell induction after 7 days in human PBMCs stimulated with heat-inactivated *A. fumigatus* conidia ( $1 \times 10^7$ /mL) after 1 hour pre-incubation with 24  $\mu$ g/mL Abatacept, or CoIgG. Data are represented as mean  $\pm$  SEM. Abbreviations: P3C = Pam3Cys-SKKKK; Af = *Aspergillus fumigatus*; CoIgG = Control immunoglobulin G. \*p-value  $\leq 0.05$ , \*\*p-value  $\leq 0.01$ , \*\*\*p-value  $\leq 0.001$ .

An attractive approach would be to investigate whether common genetic variations that slightly alter TLR2 function are associated with detrimental IL-17-mediated inflammation in aspergillosis.

TLR2 activating therapy is currently under consideration as a potential immunotherapy to increase the number of regulatory T-cells, reducing  $T_H2$  mediated hypersensitivity<sup>35</sup>, and increasing anti-tumour capacity<sup>36</sup>. However, whether such therapies are attractive to employ for treatment of aspergillosis remains to be determined. Experimental evidence highlights that TLR2 plays an important role in the antifungal host response against *Aspergillus*. TLR2 deficient mice for example show an impaired recruitment of neutrophils to the site of infection<sup>37</sup>, and a higher lethality and fungal burden<sup>33</sup>. Interestingly, these mice also show an increased  $T_H2$  responses compared to WT mice upon stimulation with *A. fumigatus*<sup>37</sup>. This  $T_H2$  response, associated with ABPA, suppresses the protective  $T_H1$  response<sup>38</sup>. In line with our data it is tempting to speculate that the TLR2 deficient mice are more susceptible to aspergillosis due to modulation of  $T_{reg}$  cells, as TLR2 mediated induction of  $T_{reg}$  cells is crucial for maintaining pro-inflammatory responses and promoting fungal clearance in aspergillosis<sup>33,37</sup>. Several studies have clearly demonstrated a protective role of  $T_{reg}$  cells in fungal infection<sup>39,40</sup>, and that TLR2 plays a crucial role in maintaining this population<sup>28,34</sup>. It can however not be excluded that TLR2 has multiple lines of action in host defence against aspergillosis, such as induction of pro-inflammatory responses crucial for recruitment and activation of innate immune cells that are responsible for clearing the fungi from the lungs.

$T_{reg}$  cells partly exert their anti-inflammatory function through contact dependent mechanisms such as CTLA4<sup>20</sup>. PBMCs of one patient who was deficient for CTLA4 were stimulated with *A. fumigatus*. Interestingly, a higher induction of  $T_{reg}$  cells with a pro-inflammatory  $T_H17$ -like phenotype was observed, compared to the healthy control. In the field of rheumatology, therapeutics such as Abatacept (CTLA4-Ig) are often administered to reduce pro-inflammatory responses in which the  $T_H17 - T_{reg}$  balance shifts towards the  $T_H17$  cell-response<sup>41</sup>. Therefore, such therapeutics might help reverse  $T_{reg}$  cells with pro-inflammatory  $T_H17$ -like characteristics back to their "classical" anti-inflammatory phenotype. We examined the role of Abatacept in preventing the induction of  $T_{reg}$  cells with  $T_H17$ -like characteristics, and a significantly decreased induction of  $T_{reg}$  cells with  $T_H17$ -like characteristics was seen. This indicates that Abatacept has the potential to hamper the induction of  $T_{reg}$  cells with pro-inflammatory  $T_H17$ -like characteristics. These results also indicate that CTLA4 might play a role in hampering the induction of  $T_{reg}$  cells with pro-inflammatory  $T_H17$ -like characteristics, and warrants further investigation to explore whether Abatacept could be used as a targeted therapy in patients with aspergillosis that are suffering from infection-induced immunopathology.

We observed that TLR2 stimulation increases the number of CTLA4 expressing CD4 T-cells and the level of expression on these cells. Separately we demonstrated that either TLR2 stimulation or CTLA4 could reduce the induction of  $T_{reg}$  cells with pro-inflammatory  $T_H17$ -like characteristics. The fact that TLR2 signalling boosts CTLA4 expression could suggest that TLR2 modulates the  $T_H17$  phenotype of *Aspergillus*-induced  $T_{reg}$  cells through CTLA4. It should be noted that *Aspergillus* itself did not significantly induce CTLA4 expression. It is therefore tempting to suggest that *Aspergillus* induces such a pro-inflammatory  $T_{reg}$  phenotype due to the fact that it fails to induce CTLA4 expression, but does induce the inflammatory mediators required for induction of pro-inflammatory T-cell responses.

Collectively, our study demonstrates that *A. fumigatus* is capable of inducing regulatory T-cells with a pro-inflammatory  $T_H17$ -like phenotype. In addition, we found that TLR2 and CTLA4 play a role in regulating the induction of these cells. These findings could pave the way for novel therapeutic approaches that target  $T_{reg}$  cells with pro-inflammatory  $T_H17$ -like characteristics in order to return them to their natural immune regulatory state.

## Methods

**Healthy volunteers and patients.** Blood was collected from healthy volunteers and patients by venous blood puncture, after informed consent was obtained. One patient that was deficient for CTLA4 was included, and for those experiment the healthy control was age ( $\pm 5$  years) and sex-matched. All experiments were performed and conducted in accordance to Good Clinical practice, the Declaration of Helsinki, and the approval of the Arnhem-Nijmegen Ethical Committee (nr.2010/104).

**Aspergillus fumigatus.** *A. fumigatus* V05-27, a previously characterized clinical isolate<sup>42</sup>, was used for all stimulations. Resting conidia were heat-inactivated, for 1 h at 65 °C and were checked for viability on Sabouraud agar. Conidia were stored at  $-80$  °C until use. A concentration of  $1 \times 10^7$ /mL was used in the experiments, unless otherwise indicated.

**Patter recognition receptor blockers and ligands.** P3C (10  $\mu$ g/mL) (Pam3Cys-SKSKK; TLR2/TLR1 heterodimer ligand, EMC microcollections, Tübingen, Germany), FSL-1 (1  $\mu$ g/mL) (Pam2Cys-SKSKK; TLR2/TLR6 heterodimer ligand, EMC microcollections, Tübingen, Germany), and anti-TLR2 blocking antibody/control IgG (10  $\mu$ g/mL) (eBioscience, Halle-Zoersel, Belgium) were used for pre-incubation and co-stimulation with heat-inactivated *A. fumigatus* conidia. Abatacept (CTLA4-Ig, 24  $\mu$ g/mL) (inhibits the T-cell co-stimulatory molecule B7-1 (CD80), Orenica, Mulgrave, Australia) was used for pre-incubation with heat-inactivated *A. fumigatus* conidia.

**Isolation and stimulation of peripheral blood mononuclear cells (PBMCs).** Blood was diluted in phosphate buffered saline (PBS) (1:1) and fractions were separated by Ficoll (Ficoll-Paque Plus; GE healthcare, Zeist, The Netherlands) density gradient centrifugation. Cells were washed twice with PBS and resuspended in Roswell Park Memorial Institute medium (RPMI) 1640 Dutch modification culture medium (Life Technologies/Invitrogen, Breda, The Netherlands) supplemented with 50  $\mu$ g/mL gentamicin, 2 mM Glutamax, and 1 mM pyruvate (Life Technologies). Cells were counted using a particle counter (Beckmann Coulter, Woerden, The Netherlands) after which, the concentration was adjusted to  $5 \times 10^6$ /mL. PBMCs were plated in 96-well round-bottom plates (Corning) at a concentration of  $5 \times 10^5$ /mL in a total volume of 200  $\mu$ L. The samples were stimulated with *A. fumigatus* heat inactivated conidia ( $1 \times 10^7$ /mL), with or without TLR2 blockers/ligands or Abatacept, or remained unstimulated for either 24 hours or 7 days at 37 °C with 5% CO<sub>2</sub>. After stimulation, supernatants were collected and stored at  $-20$  °C until cytokine assays were performed. Cell pellets were used for flowcytometry.

**qPCR.** RNA was isolated using TRIzol reagent (Invitrogen) according to the protocol supplied by the manufacturer, and RNA was converted into cDNA using iScript cDNA synthesis kit (Biorad, Hercules Ca). Quantitative real-time PCR (qPCR) was performed using power SYBR Green PCR master mix (Applied Biosystems, Carlsbad, CA) and the following primers: GAPDH FWD-5'-AGGGGAGATTCAGTGTGGTG-3' REV-5'-CGACCACTTTGTCAAGCTCA-3', ROR $\gamma$ t FWD-5'-TGAGAAGGACAGGGAGCCAA-3' REV-5'-CCACAGATTTTGCAAGGGATCA-3' and FoxP3 FWD-5'-CTGCCCTAGTCATGGTGG-3' REV-5'-CTGGAGGAGTGCCTGTAAGTG-3'. PCR was performed using an Applied Biosystem StepOne PCR system using PCR conditions 2 min 50 °C, 10 min 95 °C, followed by 40 cycles at 95 °C for 15 sec, and 60 °C for 1 min. RNA expression was corrected for differences in loading concentration using the signal of housekeeping protein GAPDH.

**Cytokine measurements.** IL-17A (R&D Systems, Mineapolis, MN) and IL-10 (Sanquin, Amsterdam, the Netherlands) were measured using commercially available ELISAs according to the protocols that were supplied by the manufacturers. Mouse IL-17A was assessed in splenocyte stimulations using the Luminex multiplex platform (Millipore, Billerica, MA).

**Flowcytometry.** For assessment of regulatory T-cells with T<sub>H</sub>17 characteristics, PBMCs were re-stimulated for 6 hours with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL) (Sigma-Aldrich, Zwijndrecht, the Netherlands), Ionomycin (1  $\mu$ g/mL) (Sigma-Aldrich), and GolgiPluginhibitor (1  $\mu$ L/mL) (BD Biosciences, Breda, The Netherlands) after 7 day stimulation with *A. fumigatus*. Cells were stained extracellularly using Phycoerythrin(PE)-conjugated anti-CD4 (clone RPA-T4; ITK Diagnostics BV, Uithoorn, the Netherlands) or Phycoerythrin- Texas Red-X(ECD)-conjugated anti-CD4 (clone SFC112T4D11; Beckman Coulter) and Phycoerythrin-Cyanine7-conjugated anti-CD25 (clone BC96; eBioscience) antibody. Subsequently, the cells were fixed and permeabilized with Cytofix/Cytoperm solution (eBioscience) according to the protocol that was supplied by the manufacturer. Following permeabilization, the cells were stained intracellularly with Alexa Fluor 647-conjugated anti-IL-17A (clone N49-653; BD Biosciences), PE-conjugated ROR $\gamma$ t (clone AFKJS-9; eBioscience, only used in non-patient experiments) and Alexa Fluor 488-conjugated FoxP3 (clone PCH101; eBioscience) according to the protocols supplied by the manufacturers.

For assessment of CTLA4 expression, PBMCs were stained extracellularly using Fluorescein Isothiocyanate (FITC)-conjugated anti-CD4 (clone RPA-T4; ITK Diagnostics BV) and PE-conjugated anti-CD152 (CTLA4) (clone 14D3; eBiosciences).

The cells were measured on a FC500 flow cytometer (Beckman Coulter) and the data were analysed using CXP analysis software v2.2 (Beckman Coulter).

**Ex vivo Stimulation of WT and Tlr2<sup>-/-</sup> murine splenocytes.** Animal studies were carried out in accordance with guidelines and regulations approved by St. Jude Children's Research Hospital Committee on Use and Care of Animals (protocol no 482-100265-1-/13). Wild-type (WT) and Tlr2 knockout (Tlr2<sup>-/-</sup>) C57Bl/6

mice were bred and maintained in the St. Jude Children's Research Hospital, Memphis, TN, USA. Spleens were homogenized in 0,4 µM cell strainer (BD) and the cell number was adjusted to  $1 \times 10^7$ /mL. The cell suspensions (500 µL/well) were placed in a 24 well plate (Corning) and incubated with culture medium or *A. fumigatus* conidia for 1 or 5 days at 37 °C and 5% CO<sub>2</sub>.

**Statistical analysis.** Data of PBMC stimulation with either RPMI or *A. fumigatus* were analysed using the Wilcoxon signed rank test. P-values < 0.05 were considered statistically significant. Data are shown as scattered dot plots, columns with mean ± standard error of mean (SEM), or as a Venn diagram. All data were analysed using GraphPad Prism v5.0. The proportional Venn diagram was drawn using the eulerAPE application v2.0.3<sup>43</sup>.

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## Author Contributions

Conceived and designed the experiments: M.S.G., F.L.v.d.V. Performed the experiments: R.P.H.R., E.G.G.S., F.E.A., C.W.M.J., L.A.B.J. Analysed the data: R.P.H.R., E.G.G.S., M.S.G., C.W.M.J. Wrote the manuscript: R.P.H.R., M.S.G. Provided valuable reagents: T.D.K. Amended the manuscript: L.A.B.J., F.L.v.d.V., M.S.G., T.D.K.

## Additional Information

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**Competing Interests:** The authors declare that they have no competing interests.

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