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## Research Article

# The domestic pig as human-relevant large animal model to study adaptive antifungal immune responses against airborne *Aspergillus fumigatus*

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Pulmonary mucosal immune response is critical for preventing opportunistic *Aspergillus fumigatus* infections. Although fungus-specific CD4<sup>+</sup> T cells in blood are described to reflect the actual host–pathogen interaction status, little is known about *Aspergillus*-specific pulmonary T-cell responses. Here, we exploit the domestic pig as human-relevant large animal model and introduce antigen-specific T-cell enrichment in pigs to address *Aspergillus*-specific T cells in the lung compared to peripheral blood. In healthy, environmentally *Aspergillus*-exposed pigs, the fungus-specific T cells are detectable in blood in similar frequencies as observed in healthy humans and exhibit a Th1 phenotype. Exposing pigs to 10<sup>6</sup> cfu/m<sup>3</sup> conidia induces a long-lasting accumulation of *Aspergillus*-specific Th1 cells locally in the lung and also systemically. Temporary immunosuppression during *Aspergillus*-exposure showed a drastic reduction in the lung-infiltrating antifungal T-cell responses more than 2 weeks after abrogation of the suppressive treatment. This was reflected in blood, but to a much lesser extent. In conclusion, by using the human-relevant large animal model the pig, this study highlights that the blood clearly reflects the mucosal fungal-specific T-cell reactivity in environmentally exposed as well as experimentally exposed healthy pigs. But, immunosuppression significantly impacts the mucosal site in contrast to the initial systemic immune response.

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**Keywords:** *Aspergillus fumigatus* · Fungal aerosolization · Porcine large animal model · Pulmonary immune response · T cells



Additional supporting information may be found online in the Supporting Information section at the end of the article.

## Introduction

*Aspergillus fumigatus* (*A. fumigatus*) is a ubiquitous airborne fungus. Although humans and animals constantly inhale numerous conidia of *A. fumigatus*, healthy individuals clear the fungus without developing disease [1, 2]. In immunocompromised individuals, *A. fumigatus* has become one of the most prevalent airborne fungal pathogens, causing severe invasive infections as well as hypersensitivity diseases, such as allergic bronchopulmonary aspergillosis (ABPA), in patients suffering from atopic asthma or cystic fibrosis (CF). Invasive fungal infections are difficult to diagnose since specific and highly sensitive noninvasive diagnostic methods for *A. fumigatus* are currently lacking. Infections often result in mortality rates of more than 50% [3], therefore, early initiation of targeted antifungal therapy is key to improve the prognosis [4]. Blood-derived CD4<sup>+</sup> T-cell responses against individual fungal pathogens provide important information on the individual fungus–host interaction status and treatment-related prognostic criteria [5]. In humans, peripheral *A. fumigatus*-specific CD4<sup>+</sup> T cells have been studied using antigen-specific T-cell enrichment (ARTE) [6, 7] and demonstrate that (i) the strongest T-cell immunoreactivity is directed against fungal membrane proteins [8], (ii) the frequencies of *A. fumigatus*-reactive conventional T cells in healthy donors range from 0.06 to 0.37% [9], (iii) the majority of *A. fumigatus*-specific Th cells in healthy humans are in a naive state and only a small subset produces IFN- $\gamma$  [6, 8], (iv) patients with proven invasive infections show markedly increased frequencies of fungus-reactive CD4<sup>+</sup> cells in blood [10], and (v) *A. fumigatus*-specific Th cells contain a small subset of Th17 cells cross-reactive to *Candida albicans* [11]. But, due to patient accessibility and human sampling limitations [12] comparability of T cells in blood and mucosal lung tissues are still unclear. Current animal models, including mice, rats, guinea pigs, and rabbits, have substantially contributed to our understanding of *Aspergillus* pathogenesis and host defence [13], but studying mucosal or time-resolved *A. fumigatus*-specific CD4<sup>+</sup> T-cell responses in most animal models is restricted due to repeated sampling limitations, small organ size, the lack of a pre-existing, naturally primed CD4<sup>+</sup> T-cell pool, model immanent rapid mortality or the unavailability of immunological tools to address rare, antigen-specific CD4<sup>+</sup> T cells. Based on similarities in the anatomy of the respiratory tract and the immune system [14] as well as the natural exposure to fungal aerosols in animal confinement buildings [15], we have chosen to study the domestic pig as human-relevant large animal model to investigate the adaptive antifungal immune response against *A. fumigatus*. The porcine respiratory tract is closely related to humans with regard to the tracheobronchial

tree structure, lung physiology, and size [16], and the numbers of airway submucosal glands [17]. Furthermore, the anatomical similarity of the upper respiratory tracts of pigs and humans [16] leads to a similar lung deposition of inhaled aerosol in comparison to mice [18].

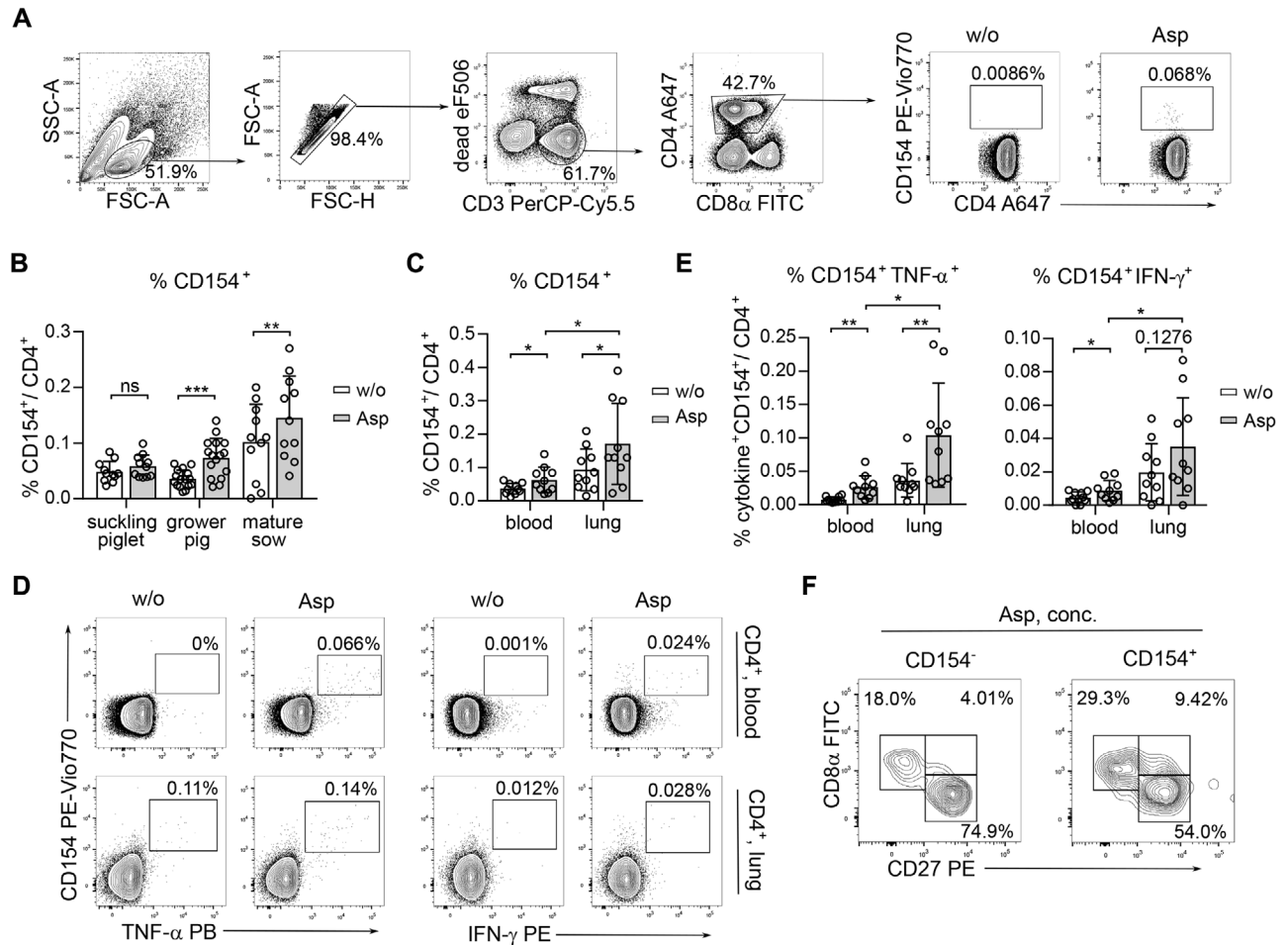
The porcine immune system is characterized reasonable accuracy and offers a wide range of established methods and tools [14, 19]. Blood immune cell composition, innate immune cell function, functional T-cell subsets, and cytokine secretion exhibit prominent similarities to humans [14, 19] which makes the pig a particular relevant model to study infectious diseases [14]. A variety of pig-specific and cross-reactive antibodies exist for multicolour flow cytometry for the identification and characterization of various immune cell subsets [20]. We have recently established the detection of CD154 (CD40L) on porcine T cells for the identification and characterization of porcine pathogen-reactive T cells, which enables to study the porcine adaptive immune response to a given pathogen [21].

Here, we focus on *Aspergillus*-specific T cells present in lung tissues compared to blood in (i) environmentally exposed, healthy pigs, (ii) following experimental, airborne exposure, and (iii) upon predisposing suppressive treatment. Our data show that blood-derived *A. fumigatus*-specific CD4<sup>+</sup> T cells mirror the phenotype of lung-resident fungus-specific T cells. Immunosuppressive treatment caused disturbed clearance of conidia and a drastic reduction in the number of lung-infiltrating *A. fumigatus*-specific CD4<sup>+</sup> T cells more than 2 weeks after experimental exposure.

## Results

### Pigs from conventional husbandry possess circulating and lung-resident *A. fumigatus*-reactive T cells

Indoor and outdoor reared domestic pigs from conventional husbandry are continuously exposed to spores of *A. fumigatus*. To address the naturally acquired, *A. fumigatus*-specific CD4<sup>+</sup> T-cell pool in healthy pigs we analyzed blood samples from different age groups. Following short-term stimulation with *A. fumigatus* lysate (Asp) antigen, intracellular staining of CD154 (CD40L) [21] identified the population of Asp-reactive CD4<sup>+</sup> T cells compared to unstimulated controls (w/o) (Fig. 1A). *Aspergillus fumigatus*-specific T cells among total CD4<sup>+</sup> were detectable in healthy animals in the categories “growers; 1-6 month” and “mature sows; >6 month” in the range of  $0.0736 \pm 0.0344\%$ , and  $0.1457 \pm 0.0749\%$ , respectively, which is comparable to *A. fumigatus*-specific CD4<sup>+</sup>



**Figure 1.** *A. fumigatus*-reactive CD4<sup>+</sup> T cells are present in blood and lungs of healthy pigs from conventional husbandry environmentally exposed to fungal spores. (A–F) Ex vivo detection of *A. fumigatus*-reactive CD4<sup>+</sup> T cells by flow cytometry following short-term (6 h) antigenic stimulation in environmentally exposed pigs. (A) Gating strategy to identify CD154<sup>+</sup> *A. fumigatus*-reactive CD4<sup>+</sup> T cells in PBMC. Percentages of CD154<sup>+</sup> among CD4<sup>+</sup> T cells are indicated within unstimulated (w/o) or stimulated (*A. fumigatus* lysate = Asp, 40 μg/mL) PBMC. (B) Age-dependent frequencies of *A. fumigatus*-reactive CD4<sup>+</sup> T cells in blood of domestic pigs (suckling piglet <1 month, n = 11 (pooled data from three independent experiments); grower pig 1–6 months, n = 16 (pooled from five independent experiments); mature sow > 6 months, n = 11 (pooled from two independent experiments) presented as mean (bars) ± SD, two-tailed paired t-test (\*\*p < 0.01, \*\*\*p < 0.001). (C) Frequencies of *A. fumigatus*-reactive CD154<sup>+</sup> T cells from unstimulated (w/o) or stimulated (Asp, 40 μg/mL) mononuclear cells of PBMC (blood) or corresponding lung tissues (lung) of n = 10 animals presented as mean (bars) ± SD pooled from three independent experiments; paired t test (\*p < 0.05). (D) Cytokine co-producing *A. fumigatus*-reactive CD154<sup>+</sup> T cells for TNF-α<sup>+</sup>CD154<sup>+</sup> (left) and IFN-γ<sup>+</sup>CD154<sup>+</sup> (right) in blood (upper row) and corresponding lung tissues (lung, lower row) (one representative analysis of three independent experiments). Percentages of cytokine co-producing CD154<sup>+</sup> among CD4<sup>+</sup> T cells are indicated within unstimulated (w/o) or stimulated (Asp) PBMC and summarized in (E) as frequencies of TNF-α<sup>+</sup> (left) or IFN-γ<sup>+</sup> (right) co-producing CD154<sup>+</sup> T cells for n = 10 animals, aged 1.5–2 months presented as mean (bars) ± SD, (pooled data from three independent experiments, paired t-test, Wilcoxon matched-pairs signed rank test (\*p < 0.05, \*\*p < 0.01). (F) Analysis of CD8α and CD27 co-expression of *A. fumigatus*-reactive, blood-derived CD154<sup>+</sup>CD4<sup>+</sup> T cells (right panel) compared to CD154<sup>-</sup>CD4<sup>+</sup> T cells (left panel). Concatenated contour plots (from n = 4 healthy animals, aged 3 months) show percentage of the CD8α<sup>+</sup>CD27<sup>-</sup> (effector memory), CD8α<sup>+</sup>CD27<sup>+</sup> (central memory), and CD8α<sup>-</sup>CD27<sup>+</sup> (naive) population. One representative analysis of two independent experiments. Values with p < 0.05 (\*), p < 0.01 (\*\*), p < 0.001 (\*\*\*), and p < 0.0001 (\*\*\*\*) were considered to be significant.

T-cell frequencies in blood of healthy human subjects (Fig. 1B, [8]). However, we did not detect fungus-reactive T cells in blood of suckling piglets aged 3–4 weeks (Fig. 1B). Because *A. fumigatus* conidia are 2–3 μm in size and therefore should reach the lung alveoli of growing pigs [1], we assumed a local fungus-reactive T-cell response in the lungs of pigs being environmentally exposed during husbandry and analyzed mononuclear cells from lung tissues of growers. Compared to corresponding blood samples fre-

quencies of pulmonary *A. fumigatus*-reactive T cells were increased and ranged from 0.023 to 0.39% of mucosal CD4<sup>+</sup> T cells, but we also noted a considerably higher CD154 background expression in unstimulated lung-derived controls (w/o) (Fig. 1C). When subtracting individual background values *A. fumigatus*-specific T-cell frequencies did not significantly differ between lung tissue and blood in healthy, environmentally exposed pigs (Supporting information Fig. S1A). Intracellular staining revealed a similar

predominant expression of TNF- $\alpha$  and IFN- $\gamma$  in both circulating blood- and lung-derived T cells, resembling a characteristic Th1 phenotype (Fig. 1D and E and Supporting information Fig. S1B). In healthy, environmentally exposed pigs *A. fumigatus*-specific T cells did not express significant levels of IL-17A and IL-4 in both blood and lung tissue (Supporting information Fig. S2A–D).

The differentiation/activation state of porcine *A. fumigatus*-reactive CD4<sup>+</sup> T cells was addressed by analysing CD8 $\alpha$  and CD27 memory marker expression on *A. fumigatus*-specific CD154<sup>+</sup> and CD154<sup>-</sup>CD4<sup>+</sup> T cells in blood (Fig. 1F). A discrete proportion of *A. fumigatus*-reactive T cells belonged to either CD8 $\alpha$ <sup>+</sup>CD27<sup>-</sup> or CD8 $\alpha$ <sup>+</sup>CD27<sup>+</sup> subsets (mean 29.3 and 9.42% of total CD154<sup>+</sup>) described as effector memory and central memory compartments [22]. Of note, a major fraction of porcine *A. fumigatus*-reactive T cells (mean 54%) are in a naïve (CD8 $\alpha$ <sup>-</sup>CD27<sup>+</sup>) state, equivalent to findings in healthy humans [9].

In sum, we show that frequency and phenotype of pulmonary *A. fumigatus*-reactive CD4<sup>+</sup> T cells of healthy pigs, constantly exposed to environmental levels of airborne conidia, are mirrored in the peripheral compartment.

### Experimental *A. fumigatus* exposure induces fungus-reactive CD4<sup>+</sup> T-cell responses in pigs

In healthy humans, the frequency of *A. fumigatus*-reactive CD4<sup>+</sup> T cells in blood positively correlates with environmental fungal exposure [23]. In order to employ the pig as human-relevant model to study the mucosal antifungal T-cell response against airborne *A. fumigatus*, we established an experimental aerosolization of *A. fumigatus* conidia in an aerosol chamber (3.2 m<sup>2</sup> base area, 7 m<sup>3</sup> volume) capable of housing piglets (Supporting information Fig. S7). While *A. fumigatus* spore concentrations in the air of pig confinement buildings range from 0 to 100 cfu/m<sup>3</sup> [24], our aerosol chamber allowed high and constant airborne exposure of 10<sup>6</sup> cfu/m<sup>3</sup> conidia for a period of 8 h (Supporting information Fig. S8).

When profiling the blood response after experimental spore exposure (Fig. 2A), we noticed a clear trend in the overall frequency of *A. fumigatus*-specific CD4<sup>+</sup> T cells being induced at day 4 postexposure compared to their naïve, environmentally pre-exposed levels at day 0 (Fig. 2B and C and Supporting information Fig. S4A) and significantly increased frequencies of TNF- $\alpha$ /IFN- $\gamma$  cytokine co-producing, *A. fumigatus*-specific T cells (Fig. 2D, Supporting information Fig. S4D). Similarly, at day 18 postexposure blood levels of cytokine producing *A. fumigatus*-specific T cells significantly increased above baseline values of day 0 (Fig. 2D). CD8 $\alpha$  co-staining revealed increasing CD8 $\alpha$  expression on fungus-reactive CD154<sup>+</sup> blood T cells after experimental exposure, but also a trend in the CD154<sup>-</sup> population (Fig. 2E). We thus cannot directly prove that naïve *A. fumigatus*-specific CD4<sup>+</sup> T cells convert into effector-memory cells.

To compare peripheral and pulmonary *A. fumigatus*-specific T-cell responses and to account for the large organ size of the

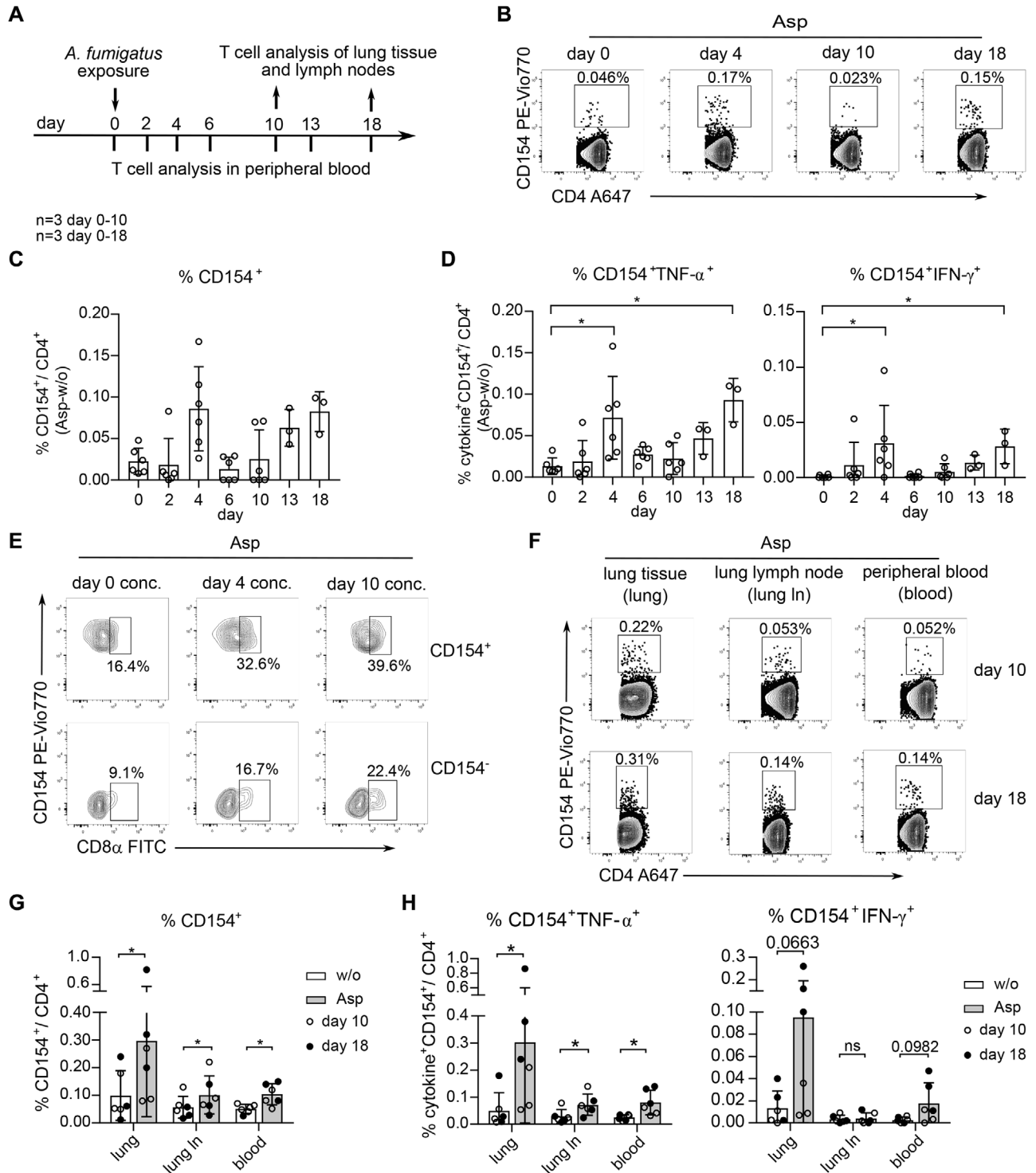
pig's lung and the possibility of local response differences, we pooled tissue samples from seven distinct anatomical lung regions and five draining lung lymph nodes. Combined data from d10 and d18 postexposure revealed a trend to higher frequencies of *A. fumigatus*-specific T cells in lung tissue compared to blood (Fig. 2F and G, Supporting information Fig. S4B and C). This trend, even though not reaching statistical significance, is most likely attributed to higher lung frequencies at day 18 postexposure (Supporting information Fig. S4B and C). However, the overall very low frequency of *A. fumigatus*-recognizing cells within the pool of CD4<sup>+</sup> T cells together with the different, organ-specific background levels detected by antigenic restimulation limit a direct comparison between the organs.

Importantly, although healthy piglets were exposed to high doses of 10<sup>6</sup> cfu/m<sup>3</sup> of *A. fumigatus* conidia, the animals did not develop clinical signs of invasive aspergillosis. PCR analysis targeting the internal transcribed spacer 1 (ITS1)–5.8S rRNA gene region was applied to detect *A. fumigatus* in BAL samples and positive BAL PCR results (only one of six healthy pigs) were verified using serum samples (Supporting information Fig. S3A). However, BAL and serum PCR together, and microscopic hyphae detection in lung tissue sections revealed no signs of invasive fungal growth (Supporting information Fig. S3B) in line with the common resistance of healthy humans to *A. fumigatus* infection. In addition, immunohistochemistry polyclonal targeting *Aspergillus* on lung tissue sections were negative for growing fungal hyphae (Supporting information Fig. S3B).

Hence, experimental exposure of domestic piglets with high levels of *A. fumigatus* conidia is well tolerated by healthy but environmentally pre-exposed individuals and the defined fungal exposure results in a local antigen-specific T-cell response against *A. fumigatus* in the lung by day 18. In blood, *Aspergillus*-specific T cells show a first peak as early as 4 days postexposure. The overall very low frequency of *A. fumigatus*-recognizing cells, however, limits their detection.

### Magnetic enrichment reveals massive infiltration of *A. fumigates*-reactive T cells into the lung

To overcome this limitation, we adapted the method of ARTE based on magnetic isolation of CD154-expressing CD4<sup>+</sup> T cells [6]. Due to the transient nature of CD154 surface expression and the unavailability of porcine-specific or cross-reactive CD40-blocking agents to prevent its internalization, we applied a combination of CD154 surface trapping [25] and chemical stabilization [26]. Mononuclear cells were stimulated with *A. fumigatus* lysate antigen in presence of CD154-PE antibody to allow complexing of *de novo* synthesized porcine CD154 and fluorescently labelled anti-CD154. Monensin was added after 2 h of stimulation to preserve CD154-antibody complexes on the cell surface [26]. Subsequently, cells were labelled with antifluorochrome magnetic microbeads and incubated for another 2 h in the presence of Monensin and Brefeldin A to enhance intracellular cytokine signals. Magnetic enrichment of CD154-expressing, porcine CD4<sup>+</sup> T cells enabled



**Figure 2.** *Aspergillus fumigatus*-reactive CD4<sup>+</sup> T cells increase in blood and locally in the lungs following experimental *Aspergillus* exposure. (A) Experimental set-up of two independent experiments with different endpoint analysis (days 10 or 18) with n = 3 pigs/experiment and analysed by flow cytometry. (B) Exemplary kinetic analysis (day 0 = environmentally exposed, 4, 10, and 18 postexposure) of *A. fumigatus*-reactive CD4<sup>+</sup> T cells in blood identified by their CD154<sup>+</sup> expression gated on CD4<sup>+</sup>, summarized for n = 3–6 animals and timepoints in (C) presented as mean (bars)  $\pm$  SD combined of two independent experiments with different endpoint analysis (days 10 or 18) of n = 3 pigs/experiment. CD154<sup>+</sup> background of the unstimulated control was subtracted; Kruskal–Wallis test with Dunn’s multiple comparisons test (\*p < 0.05). (D) Frequency of cytokine co-producing *A. fumigatus*-reactive CD4<sup>+</sup> T cells co-expressing TNF- $\alpha$  (left) or IFN- $\gamma$  (right) on day 0, 4, 10, and 18, presented as mean (bars)  $\pm$  SD combined of two independent experiments with different endpoint analysis (days 10 or 18) of n = 3 pigs/experiment. CD154<sup>+</sup> background of the unstimulated control was subtracted; Kruskal–Wallis test with Dunn’s multiple comparisons test (\*p < 0.05). (E) Concatenated contour plots (from n = 6 animals) indicating the percentage of CD154<sup>+</sup>CD8 $\alpha$ <sup>+</sup> and CD154<sup>-</sup>CD8 $\alpha$ <sup>+</sup> among all CD154<sup>+</sup> (upper panel) and CD154<sup>-</sup> (lower panel) T cells before (day 0) and after (days 4 and 11) experimental exposure to *Aspergillus* conidia of two independent experiments of n = 3 pigs/experiment. (F) Analysis of



frequencies of *A. fumigatus*-reactive CD154<sup>+</sup> T cells across lung tissue (lung) and lung draining lymph node (lung ln), and blood gated on CD4<sup>+</sup> and summarized in (G) for unstimulated (w/o) or stimulated (Asp, 40  $\mu$ g/mL) presented as mean (bars)  $\pm$  SD at dissection days 10 (open circles, n = 3) and 18 (black-filled circles, n = 3) combined of two independent experiments with different endpoint analysis (days 10 or 18) of n = 3 pigs/experiment; paired t test (\**p* < 0.05). (H) Frequencies of TNF- $\alpha$ <sup>+</sup> (left) or IFN- $\gamma$ <sup>+</sup> (right) co-producing *A. fumigatus*-reactive CD4<sup>+</sup> T cells in lung tissue, lung lymph node, and blood for unstimulated (w/o) or stimulated (Asp, 40  $\mu$ g/mL) cells presented as mean (bars)  $\pm$  SD at the terminal days 10 (open circles, n = 3) and 18 (black-filled circles, n = 3) of two independent experiments with different endpoint analysis (days 10 or 18) of n = 3 pigs/experiment, paired t test, Wilcoxon matched-pairs signed rank test (\**p* < 0.05, ns = not significant).

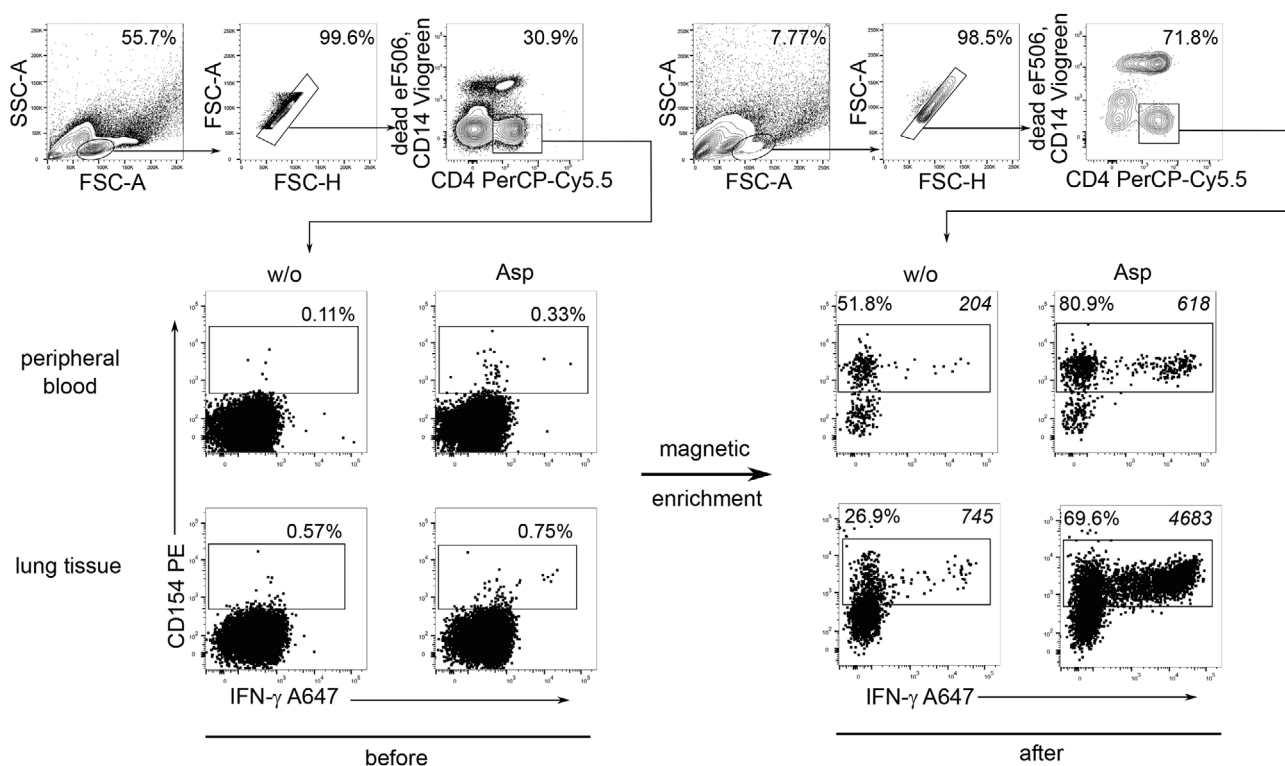
us to investigate *A. fumigatus*-specific T cells and cytokine expression at a reliable resolution as illustrated in Fig. 3.

Importantly, magnetic enrichment showed a massive increase (24-fold) in numbers of lung-resident *A. fumigatus*-specific T cells when comparing experimentally exposed (exp. exp.) versus healthy, age matched, environmentally exposed (env. exp.) pigs (Fig. 4A). Magnetic enrichment further indicated significant higher numbers of *A. fumigatus*-specific T cells in the lung versus draining lymph nodes versus peripheral blood in combined data from days 10 and 18 postexposure (Fig. 4B and D). A separate analysis of both time points reveals stronger organ-specific differences at day 18 compared to day 10 postexposure (Supporting information Fig. S5A to C). Analysing enriched T cells enabled us to address cytokine producing populations within the antigen-

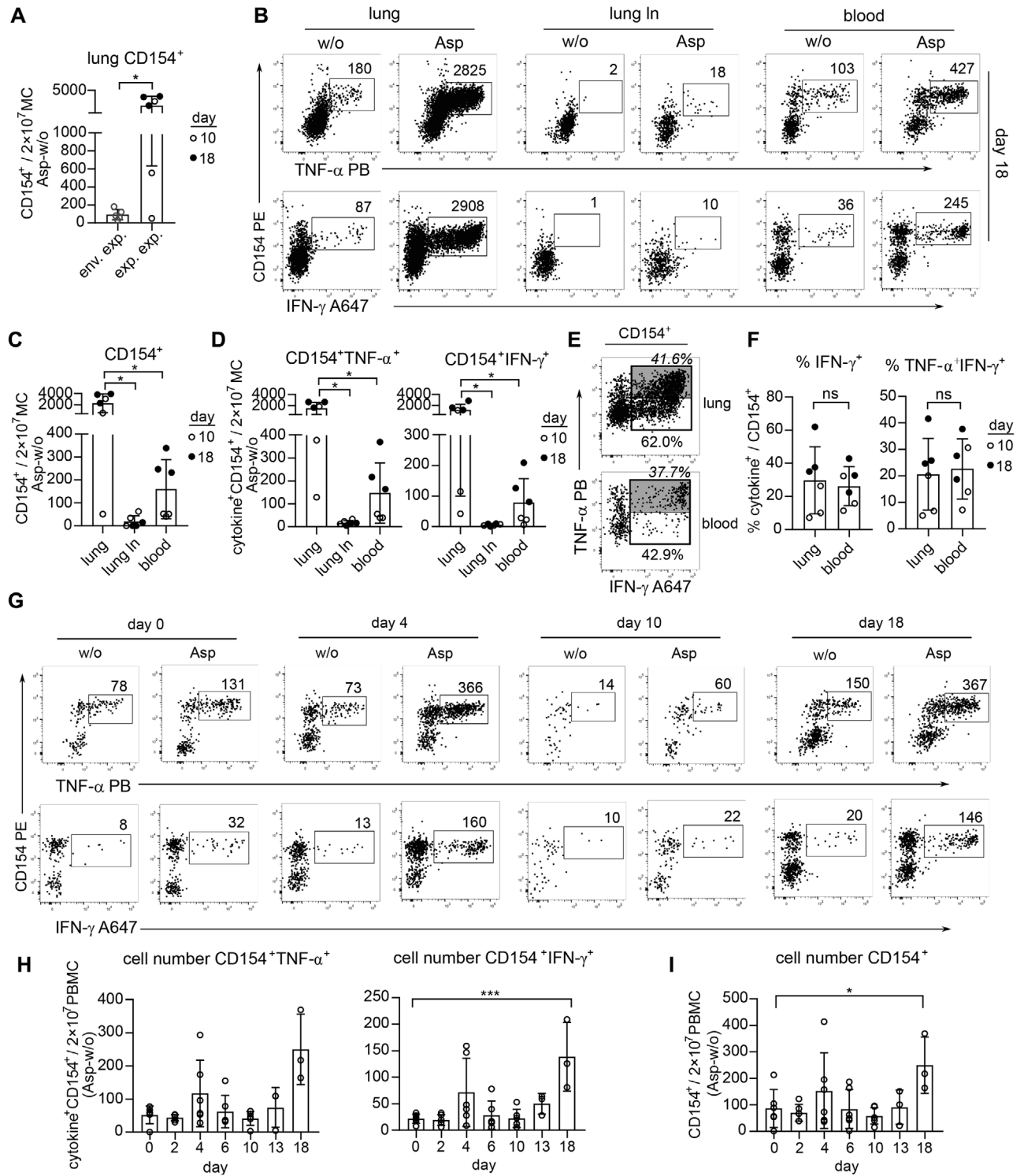
specific T-cell pools. The frequency of IFN- $\gamma$  and IFN- $\gamma$  /TNF- $\alpha$  co-expressing cells was comparable between blood and lung tissue-derived *A. fumigatus*-specific T cells (Fig. 4E and F). Again, these data indicate that the Th1 phenotype of blood-derived fungus-specific T cells mirror the phenotype of lung resident T cells.

The early peak of *A. fumigatus*-specific CD4<sup>+</sup> T cell numbers detected in blood at day 4 postexposure was confirmed by magnetic enrichment, just as the gradual increase until day 18 (Fig. 4G to I).

In sum, by establishing magnetic, antigen-reactive T-cell enrichment for the pig we could show that upon exposure with conidia *A. fumigatus*-reactive T cells specifically accumulate in lung tissues and that the lung-resident IFN- $\gamma$  /TNF- $\alpha$  T cell



**Figure 3.** Antigen-reactive enrichment of porcine CD4<sup>+</sup> T cells. PBMC or lung-derived mononuclear cells were seeded at a density of  $2 \times 10^7$  cells/mL and stimulated by adding 40  $\mu$ g/mL *A. fumigatus* lysate or left untreated (w/o) in presence of anti-CD154-PE pure antibody for 5 h and Monensin (2  $\mu$ M) was added after 2 h of stimulation. After 5 h, cells were collected, washed, and magnetically labeled using anti-PE microbeads. Labeled cells were incubated for another 2 h in presence of 3  $\mu$ g/mL Brefeldin A and 2  $\mu$ M Monensin to allow for intracellular cytokine detection. Afterwards cells were magnetically enriched and stained on column for flow cytometric analysis. Gating strategy to identify porcine CD4<sup>+</sup> T cells before (left) and after (right) magnetic enrichment (upper panel), *A. fumigatus* stimulated (Asp) and untreated (w/o) PBMC before and after magnetic enrichment (middle panel) and *A. fumigatus* stimulated (Asp) and untreated (w/o) lung-derived mononuclear cells before and after magnetic enrichment (lower panel). Numbers in plots indicate frequencies of CD154<sup>+</sup> among CD4<sup>+</sup> T cells while cell counts are indicated in italics. Exemplary dot plots are representative of two independent animal experiments with up to 24 PBMC and three lung tissue samples/experiment.



**Figure 4.** Lung infiltrating *A. fumigatus*-specific T cells identified by magnetic enrichment. (A) Comparison of *A. fumigatus*-reactive, enriched CD154<sup>+</sup> T cell numbers in lung tissue (lung) of naïve, environmentally exposed pigs (n = 5, one representative experiment of two independent experiments is shown) and on days 10 (n = 3, open circles) and 18 (n = 3, black-filled circles) after exposure to *Aspergillus* conidia presented as mean (bars) ± SD combined of two independent experiments with different duration (10 or 18 days) of n = 3 pigs/experiment; two-tailed paired t test (\*p < 0.05). (B) Exemplary flow cytometry dot plots of enriched *A. fumigatus*-specific CD4<sup>+</sup> T cells expressing TNF-α (upper panel) or IFN-γ (lower panel) compared to unstimulated controls of cells isolated from lung tissue, lung lymph node and blood at day 18 and gated on CD4<sup>+</sup> T cells. Exemplary data of one pig of two independent experiments with n = 3 pigs/experiment are shown. (C) Comparison of fungus-reactive, enriched CD154<sup>+</sup> T cell numbers in lung, lung lymph node, and blood on days 10 (n = 3, open circles) and 18 (n = 3, black-filled circles) after experimental exposure to *Aspergillus* conidia. (D) Comparison of fungus-reactive, enriched CD154<sup>+</sup> T cell numbers co-expressing TNF-α (left) or IFN-γ (right) in lung, lung lymph nodes, and blood on days 10 (n = 3, open circles) and 18 (n = 3, black-filled circles) after experimental exposure to *Aspergillus*



phenotype is reflected by blood-derived T cells, indicating similar mucosal and systemic responses.

### Immunosuppression prior to exposure negatively affects the *Aspergillus*-specific T-cell pool

Healthy human individuals clear constantly inhaled conidia of *A. fumigatus* without developing disease. Immunocompromised patients with pre-existing pulmonary diseases, however, often have to deal with invasive aspergillosis or *Aspergillus*-associated hyperinflammatory diseases including exogenous allergic alveolitis, allergic sinusitis, asthma or ABPA [2]. We therefore studied how immunosuppressive treatment prior to airborne exposure to *Aspergillus* conidia affects the fungus-reactive T-cell response in swine. Piglets were treated with cyclophosphamide and methylprednisolone 6 days in advance, on day 0 and 1 day after experimental exposure (Fig. 5A). Immunosuppressive treatment caused a significant reduction of the total leukocyte number and severe neutropenia on the day of experimental exposure in blood (Fig. 5B). Circulating CD4<sup>+</sup> T cells also showed significantly lower proliferative capacity as detected by Ki-67 expression at day 0 in suppressed compared to nonsuppressed animals (Fig. 5C) indicating an immunocompromised status.

When comparing the pulmonary T-cell response of suppressed (suppr.) versus nonsuppressed (nonsuppr.) versus environmentally exposed (env. exp.) pigs for days 10/11 and 18/19 after experimental exposure, we observed a clear reduction in the number of *A. fumigatus*-specific T cells infiltrating lung tissues in immunocompromised compared to nonsuppressed pigs 18 days after exposure (Fig. 6A). Of note, lung-tissue levels of *A. fumigatus*-specific T cells in experimentally exposed but suppressed animals reflected those of animals that had not been subjected to experimental exposure (environmentally exp.) (Fig. 6A). Similarly, IFN- $\gamma$  /TNF- $\alpha$  cytokine secretion by pulmonary *A. fumigatus*-specific T cells was significantly reduced in suppressed versus nonsuppressed piglets at day 18 (Fig. 6B and C). This indicates a long-lasting restriction in mounting a local T-cell response in only short-term suppressed animals.

Of note, diagnostic *A. fumigatus* PCR analysis of bronchoalveolar lavage samples revealed 3/3 immunosuppressed animals to be positive on day 11, compared to one third of the nonsuppressed group. At the later time points (day 18/19), we detected one-third immunocompromised animals to be positive and none (0/3) of the nonsuppressed group (Supporting information Fig. S3A).

Remarkably, no signs of invasive fungal growth was detected by serum-diagnostic or histology. This indicates the suitability of the porcine model to study predisposing factors for acquiring IA with regard to immunocompetence and adaptive antifungal immunity without, however, inducing rapid morbidity and mortality. Interestingly, when comparing cell numbers of *A. fumigatus*-reactive CD4<sup>+</sup> T cells in lung tissue of suppressed and nonsuppressed animals, we noticed that pigs presenting low numbers of lung-resident fungus-reactive T cells also showed a positive *A. fumigatus* BAL-PCR indicating disturbed fungal clearance (Fig. 6D).

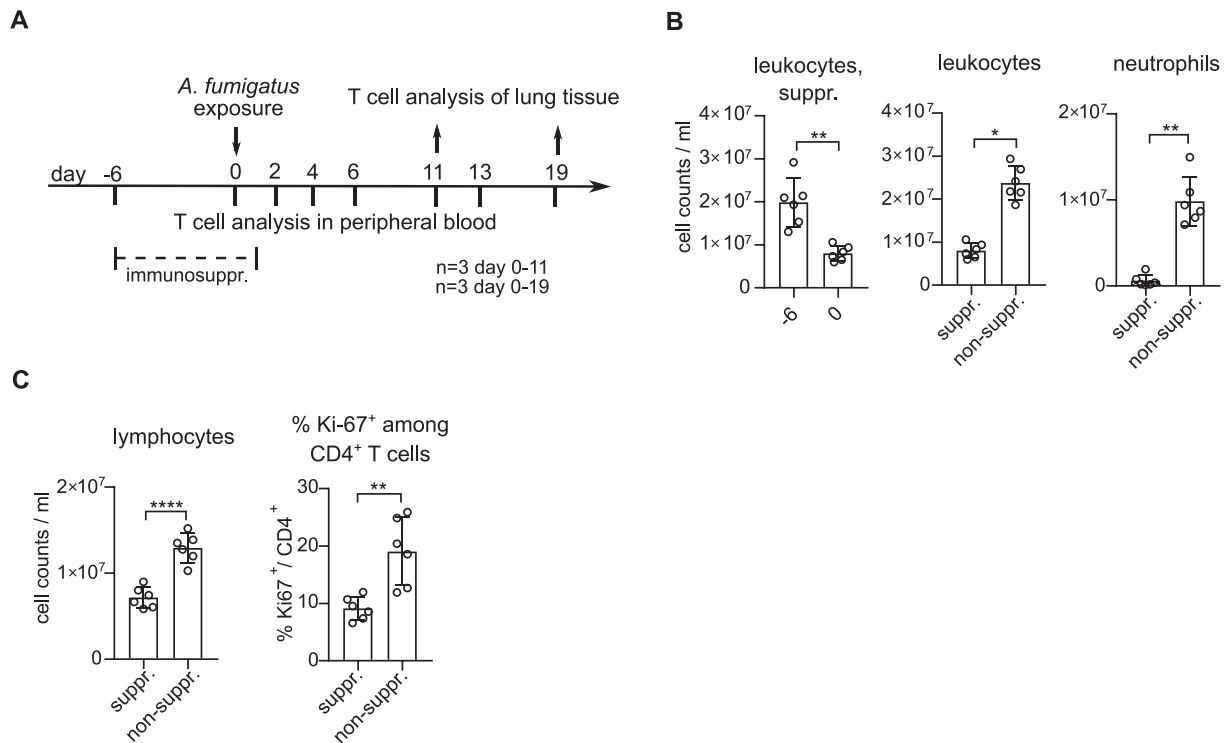
Interestingly, when monitoring the peripheral response in blood, we observed an almost identical early kinetic response in suppressed versus and nonsuppressed pigs with a peak at day 4 postexposure (Supporting information Fig. S6A and B). On day 18/19, however, significantly less *A. fumigatus*-specific T cells were circulating in the blood of immunosuppressed piglets.

Collectively, these data show that a temporary-restricted immunosuppressive treatment prior to experimental exposure to *A. fumigatus* conidia leads to a significant and long-lasting reduction of the amount and cytokine expression of fungus-reactive CD4<sup>+</sup> T cells in the lung. While the early antigen-specific T-cell response against *A. fumigatus* in blood is very similar between suppressed and nonsuppressed animals, the long-term depression observed in lung tissue is also reflected in the blood later on, even though to a much lesser extent. Moreover, low numbers of mucosal *A. fumigatus*-reactive CD4<sup>+</sup> T cells correlated with disturbed fungal clearance in the lungs.

### Experimental *A. fumigatus* exposure induces IL-17A production in fungus-reactive, lung CD4<sup>+</sup> T cells

While we were not able to detect significant *A. fumigatus*-specific IL-17A-production in CD4<sup>+</sup> T cells from healthy, environmentally exposed piglets, we wondered whether differentiation of IL-17-secreting effector/memory cells might be depicted directly in the lung after experimental exposure with high doses of *A. fumigatus* conidia. We therefore analyzed blood- and lung-derived CD4<sup>+</sup> T cells isolated from piglets at day 4 after experimental exposure with 10<sup>6</sup> cfu/m<sup>3</sup> conidia for a period of 8 h (Fig. 7A). In line with our previous results (Supporting information Fig. S2), no detection of IL-17A producing *A. fumigatus*-specific T cells was seen before exposure (day 0) in the blood of healthy pigs (Fig. 7B and C). Upon experimental exposure no IL-17A-producing T cells were detected in blood and draining lymph nodes, but

conidia. (C) and (D) presented as mean (bars)  $\pm$  SD combined of two independent experiments with different endpoint analysis (days 10 or 18) with  $n = 3$  pigs/experiment; one-way ANOVA with Tukey's multiple comparisons test (\* $p < 0.05$ ). (E) Exemplary flow cytometry dot plots showing frequency of total IFN- $\gamma$ -producing cells and IFN- $\gamma$ /TNF- $\alpha$  co-producing cells (grey background, frequency in italic) gated on CD154<sup>+</sup> T cells in lung compared to blood at day 18, summarized for  $n = 6$  pigs (day 10,  $n = 3$ , open circles and day 18,  $n = 3$ , black-filled circles) in (F); presented as mean (bars)  $\pm$  SD combined of two independent experiments with different endpoint analysis (days 10 or 18) with  $n = 3$  pigs/experiment; two-tailed paired t-test (ns = not significant). (G) Exemplary flow cytometry dot plots of enriched *A. fumigatus*-specific T cells expressing TNF- $\alpha$  (upper panel) or IFN- $\gamma$  (lower panel) and unstimulated controls at days 0, 4, 11, and 18 from one representative pig and gated on CD4<sup>+</sup> T cells. Numbers indicate cell counts/ $2 \times 10^7$  PBMC. Data are summarized in (H) for cytokine co-expressing CD154<sup>+</sup> cells and in (I) for CD154<sup>+</sup> cells presented as mean  $\pm$  SD combined of  $n = 3$ -6 pigs of two independent experiments with different endpoint analysis (days 10 or 18) with  $n = 3$  pigs/experiment; one-way ANOVA with Tukey's multiple comparisons test (\* $p < 0.05$ , \*\*\* $p < 0.001$ ). All data were analyzed by flow cytometry.



**Figure 5.** Immunosuppressive treatment of pigs causes leukopenia and severe neutropenia. (A) Experimental scheme of two independent experiments with different endpoint analysis (days 10 or 18) with  $n = 3$  pigs/experiment. Pigs were treated with immunosuppressive medication 6 days in advance and until 1 day after experimental exposure to *Aspergillus* conidia. Porcine fungus-reactive T cells in blood were analyzed before (day 0) and on day 2, 4, 6, 11, 13, and 19 after experimental exposure to *A. fumigatus* followed by T-cell analysis in lung tissue and lung lymph nodes after necropsy on day 11 ( $n = 3$  pigs) and day 19 ( $n = 3$  pigs). (B) Comparison of total leukocytes numbers on days 6 and 0 of immunosuppressed pigs (suppr.,  $n = 6$ ) (left) and leukocytes and neutrophils on day 0 of immunosuppressed (suppr.,  $n = 6$ ) and nonimmunosuppressed (nonsuppr.,  $n = 6$ ) pigs (middle and right) presented as mean (bars)  $\pm$  SD of two independent experiments of both immunosuppressed and nonimmunosuppressed animals with  $n = 3$  pigs/experiment; unpaired t test with Welch's correction, Mann-Whitney test ( $*p < 0.05$ ,  $**p < 0.01$ ). (C) Comparison of lymphocyte numbers (left) and frequencies of Ki-67<sup>+</sup> among CD4<sup>+</sup> T cells on day 0 of immunosuppressed (suppr.,  $n = 6$ ) and nonimmunosuppressed (nonsuppr.,  $n = 6$ ) pigs (right) presented as mean (bars)  $\pm$  SD of two independent experiments of both immunosuppressed and nonimmunosuppressed animals with  $n = 3$  pigs/experiment; unpaired t test with Welch's correction ( $**p < 0.01$ ,  $****p < 0.0001$ ). All data were analyzed by flow cytometry.

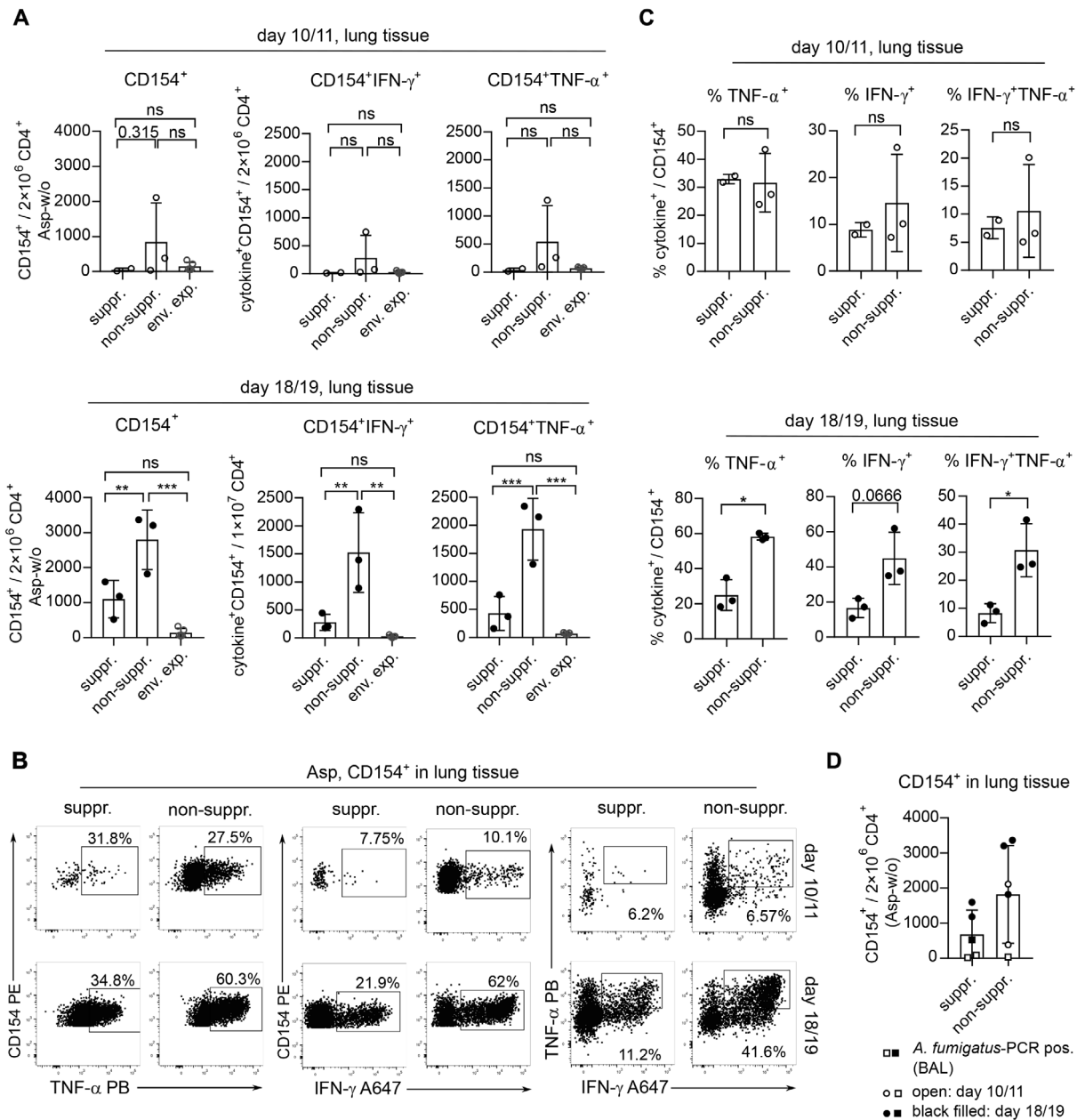
a trend in increased frequencies of IL-17A-producing *A. fumigatus*-specific T cells isolated from lung tissues was observed (Fig. 7B to D). Of note, at day 4 postexposure, the overall numbers of *A. fumigatus*-specific T cells enriched from lung tissues did not differ between experimentally (exp. exp.) and environmentally (env. exp.) exposed piglets (Fig. 7E). For more detailed analysis, we used a protocol previously published by us [21] to expand the population of *Aspergillus*-specific T cells isolated from the lung-tissue of experimentally versus environmentally exposed pigs (Fig. 7F). This method enabled us to address the IL-17A production in a restimulation assay with *A. fumigatus*-antigen loaded, autologous dendritic cells (Fig. 7G and H). Our data show that expanded lung lymphocytes from experimentally exposed piglets significantly upregulated CD154 expression upon restimulation with *A. fumigatus*-antigens and in comparison to expanded lung lymphocytes from environmentally exposed pigs (Fig. 7H). Moreover, IL-17A production was significantly increased in *A. fumigatus*-specific T cells isolated and expanded from experimentally versus environmentally exposed pigs (Fig. 7I and J), indicating a role for Th17 cells in pathogen clearance at the site of infection.

Together, these data highlight the significance of using domestic pigs to directly address lung-specific, adaptive immunity against airborne *A. fumigatus* in health and disease.

## Discussion

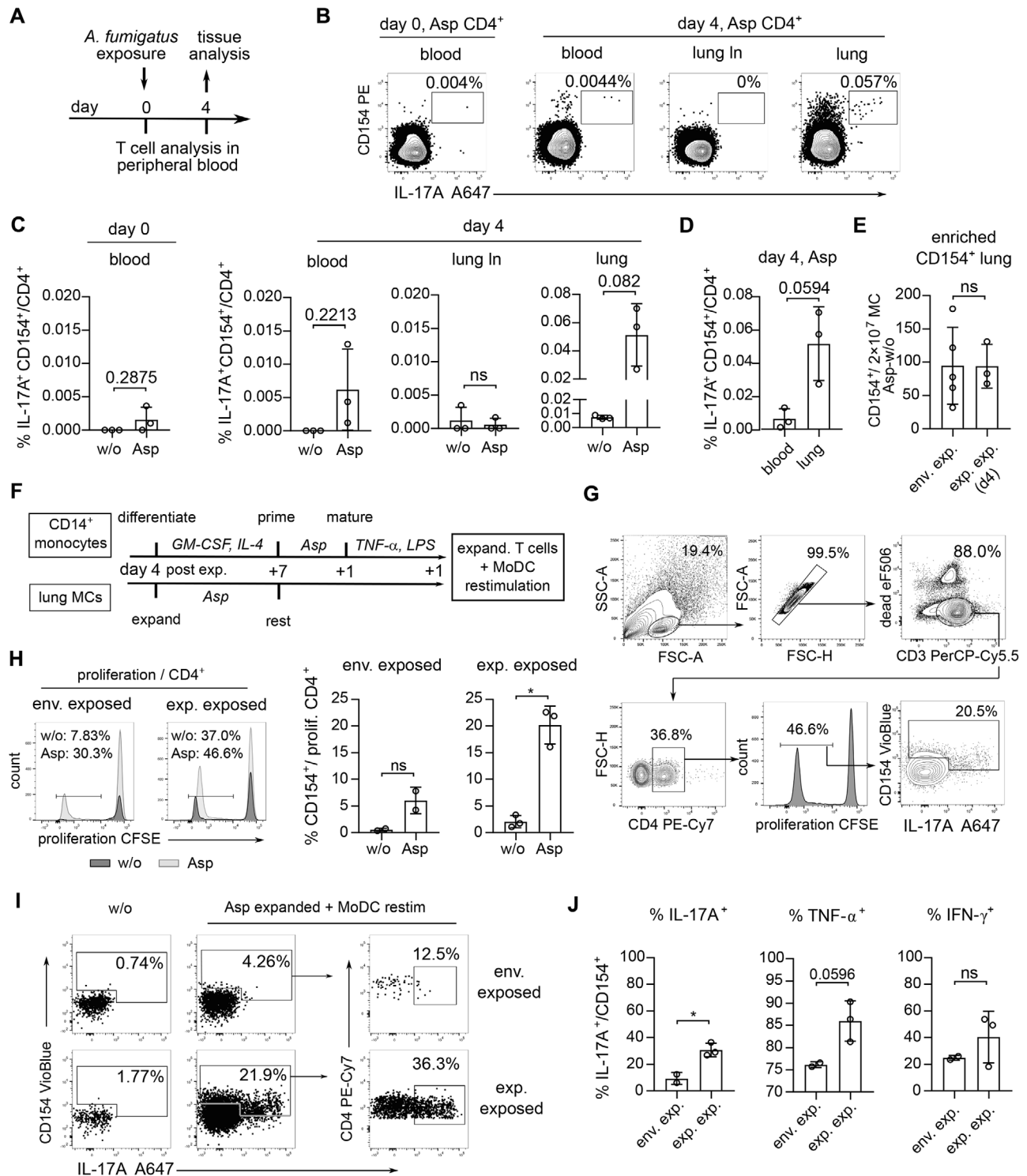
Many of the differences in *A. fumigatus* infection dynamics and infection clearance reported for rodent models versus humans [27] can be explained by differences in size and morphometry of the lungs besides the increasing evidence of functional differences between the human and murine immune systems [28]. The use of domestic pigs as translational models for respiratory diseases is therefore becoming more important in bridging between small laboratory rodent models and human medicine [16], as they, compared to mice, more closely reflect human lung capacity, alveoli size, bronchial anatomy, and respiratory rates [14, 29–31].

The adaptive antifungal immune response in humans, particularly CD4<sup>+</sup> T cells recognizing *Aspergillus* antigens, have gained increasing attention for both, as noninvasive diagnostic targets to



**Figure 6.** Immunocompromised animals demonstrate a reduced fungus-reactive T-cell response locally in the lungs. (A–D) Ex vivo detection of *A. fumigatus*-reactive CD4<sup>+</sup> T cells by antigen-reactive enrichment. (A) Cell numbers of CD154<sup>+</sup>, TNF-α<sup>+</sup>CD154<sup>+</sup> and IFN-γ<sup>+</sup>CD154<sup>+</sup> corrected for individual CD4<sup>+</sup> frequencies and presented as 2 × 10<sup>6</sup> CD4<sup>+</sup> T cells of mononuclear lung cells (MCs) that were stimulated with *A. fumigatus* lysate after experimental exposure to *A. fumigatus* in suppressed (suppr., day 11, upper panel, n = 2, (enrichment analysis of lung tissue from one animal is missing due to a technical error) and 19, lower panel, n = 3) versus nonsuppressed (nonsuppr., day 10; n = 3 and day 18; n = 3) pigs and compared to environmentally exposed (env. exp.) pigs (n = 5). CD154<sup>+</sup> background signals enriched from nonstimulated control (w/o) were subtracted and data presented as mean (bars) ± SD combined of two independent experiments of both immunosuppressed (endpoint analysis on days 11 or 19) and nonimmunosuppressed (endpoint analysis on days 10 or 18) animals with n = 3 pigs /experiment; data of day 10/11 are indicated in open circles and of day 18/19 in black-filled circles; one-way ANOVA with Tukey's multiple comparisons (\*\*p < 0.01, \*\*\*p < 0.001, ns = not significant). (B) Exemplary flow cytometry dot plots of lung-derived cytokine co-expressing *A. fumigatus*-reactive Th cells gated on CD154<sup>+</sup> on day 10/11 (upper panel) and day 18/19 (lower panel) after experimental exposure. Percentages of TNF-α producing cells (left), IFN-γ producing cells (middle) and TNF-α and IFN-γ double-producing cells (right) of suppressed and nonsuppressed pigs are indicated and summarized in (C) for n = 5 (suppr.) and n = 6 (nonsuppr.) pigs presented as mean (bars) ± SD combined of two independent experiments of both immunosuppressed (endpoint analysis on days 11 or 19) and nonimmunosuppressed animals (endpoint analysis on days 10 or 18) with n = 3 pigs/experiment; data of day 10/11 are indicated in open circles and of day 18/19 in black-filled circles. Data are presented separately for days 10/11 in the upper row and for days 18/19 in the lower one; unpaired t test with Welch's correction (\*p < 0.05, ns = not significant). (D) Cell number of *A. fumigatus*-reactive CD4<sup>+</sup> T cells in lung tissue of suppressed (n = 5) versus nonsuppressed (n = 6) pigs. Pigs with a positive *Aspergillus* BAL-PCR are indicated in squares, pigs with negative BAL-PCR are indicated in circles. Data of day 10/11 are presented in open circles or squares and data from day 18/19 in black-filled circles or squares. Data are pooled of two independent experiments of both immunosuppressed (endpoint analysis on days 11 or 19) and nonimmunosuppressed (endpoint analysis on days 10 or 18) animals with n = 3 pigs/experiment. All data were analyzed by flow cytometry.





**Figure 7.** Early after experimental exposure with *A. fumigatus* conidia IL-17A-producing CD4 T cells are induced in the lung. (A) Experimental set-up. Porcine fungus-reactive T cells in blood were analyzed before (day 0) and on day 4 after experimental exposure to *A. fumigatus* followed by T-cell analysis in lung tissue and lung lymph nodes after necropsy on day 4 ( $n = 3$  pigs). (B–D) Ex vivo detection of *A. fumigatus*-reactive CD4<sup>+</sup> T cells by flow cytometry following short-term (6 h) antigenic stimulation. (B) Exemplary flow cytometry plots (day 0 and 4) of IL-17A co-producing *A. fumigatus*-reactive CD4<sup>+</sup> T cells in blood, lung lymph nodes (lung ln) and lung tissue (lung) identified by their CD154<sup>+</sup> expression among all CD4<sup>+</sup>, summarized in (C) for  $n = 3$  animals for each time point for unstimulated (w/o) or stimulated (Asp = *A. fumigatus* lysate, 40  $\mu$ g/mL) cells of one experiment of  $n = 3$  pigs presented as mean (bars)  $\pm$  SD; paired t test. (D) Comparison of IL-17A co-producing *Aspergillus*-reactive CD4<sup>+</sup> T cells in blood and lung tissue (lung) on day 4 after experimental exposure of  $n = 3$  pigs from one experiment presented as mean (bars)  $\pm$  SD; paired t test. (E) Numbers of enriched *Aspergillus*-reactive CD154<sup>+</sup> T cells in lung tissue of environmentally exposed (env. exp.,  $n = 5$  pigs) compared to experimentally exposed (exp. exp.,  $n = 3$ ) pigs 4 days after exposure, presented as mean (bars)  $\pm$  SD of one experiment; unpaired t test with Welch's

monitor invasive fungal growth and identifying patients at risk, and for potentially contributing to the protection against fungal infections [32, 33]. Evidence for the latter, however, is sparse [5,34]. In healthy human individuals constantly inhaling environmental conidia, *A. fumigatus*-specific Th cells are present at low frequencies [6, 9] and are likely kept under control by a robust *A. fumigatus*-specific Treg response [9, 35]. The fact that also domestic pigs from conventional husbandry are continuously confronted with fungal conidia—in sharp contrast to SPF-raised laboratory mice—makes them a promising model to study the naturally acquired, adaptive, antifungal immunity. This study therefore aimed at evaluating the domestic pig as a model to study antifungal T-cell responses against airborne *A. fumigatus* directly in the lung. We could show that in healthy, environmentally exposed grower pigs there is a pool of *A. fumigatus*-specific circulating CD4<sup>+</sup> Th cells present at similar frequencies and with a similar phenotype as compared to healthy human individuals [6, 8, 9, 36]. While human studies have shown that conventional T-cell frequencies reactive against *A. fumigatus* in cord blood were almost identical to those in adult blood [37], we were not able to detect fungus-reactive T cells in suckling piglets aged 3–4 weeks only, indicative for an exposure-driven increase in the number of circulating *A. fumigatus*-specific CD4<sup>+</sup> T cells during husbandry [15]. In line with data from healthy human subjects, porcine *A. fumigatus*-reactive CD4<sup>+</sup> T cells also displayed in large parts a naïve phenotype as shown by lacking CD8 $\alpha$  and expressing CD27 [22] and also co-expressing TNF- $\alpha$  and IFN- $\gamma$ , suggesting a Th1 phenotype [6, 8, 9, 36]. IL-17A producing *A. fumigatus*-reactive CD4<sup>+</sup> T cells, however, were barely detectable in both blood and lung-tissue samples of healthy, environmentally exposed pigs.

Immunocompetent individuals eliminate conidia efficiently by innate defense mechanisms without developing disease. Fungal particles that overcome anatomical barriers, the ciliary action of the mucous epithelium, and the lung surfactant are mainly internalized directly and thereby eliminated by lung-resident alveolar macrophages [38, 39]. While conidial uptake by CCR2<sup>+</sup>Ly6C<sup>+</sup> inflammatory monocytes (CCR2<sup>+</sup>Mo) leads to their differentiation into Mo-DCs and direct spore killing, CCR2<sup>+</sup>Mo and their derivatives also transport conidia or antigens to lung-draining lymph nodes where they induce Th1 differentiation [40–42]. To establish

the pig inhalation model, we set up an aerosolization chamber with a volume of 7 m<sup>3</sup> exposing piglets to 10<sup>6</sup> cfu/m<sup>3</sup> *A. fumigatus* conidia for a period of 8 h to deliver fungal conidia via the respiratory tract. Considering that *A. fumigatus* conidia levels of 100 cfu/m<sup>3</sup> have been detected in animal breeding facilities, this dose represents a 10<sup>4</sup>-fold increased exposure. In healthy humans *A. fumigatus*-reactive T cells positively correlate with environmental mold exposure levels [23]. Until recently, lacking tools to directly address pathogen-specific CD4<sup>+</sup> T cells was a major limitation when working with pigs. Based on the ARTE [6, 7] and our previous work on CD154 as a marker for porcine pathogen-specific T cells [21], we now developed an assay to enrich porcine *A. fumigatus*-specific CD154<sup>+</sup> T cells in the absence of a CD40-blocking agent which is not yet available for pigs. This method allows to reliably investigate even small changes in the levels of circulating and tissue-derived fungus-specific CD4<sup>+</sup> T cells.

In the circulation of healthy piglets, we detected an increase of *A. fumigatus*-specific CD4<sup>+</sup> T cells by day 4 after experimental exposure followed by a drop at days 6 and 10 that presumably reflects additional migration of activated T cells into the airways. Interestingly, we monitored a second increase in circulating *A. fumigatus*-specific CD4<sup>+</sup> T cells of healthy animals much later at days 13 and 18 postexposure. Whether this early wave of *A. fumigatus*-specific T cells at day 4 is a consequence of local reactivation of lung-tissue resident memory T cells or reactivation of circulating memory T cells, is not yet clear. However, at day 4 postexposure *A. fumigatus*-specific T-cell levels isolated directly from lung tissues do not differ from environmentally exposed piglets, arguing against the expansion of tissue-resident memory T cells. In *Aspergillus* infection CCR2<sup>+</sup>Ly6C<sup>hi</sup>, Mo-DCs can prime T cells in the lung, but there is also evidence for antigen-transfer from Mo-DCs to lymph-node resident DCs [43]. Whether different routes and locations of antigen delivery and subsequent T-cell priming account for the second increase in circulating *A. fumigatus*-specific T cells remains to be addressed. If, on the long run, fungus-specific T cells are considered as diagnostic sensors [5], it is important to ask whether blood-derived T cell characteristics mirror the local immune response in the primarily affected organ. Our data from the pig model show, that indeed the phenotype of lung-resident fungus-specific T cells is mimicked in blood-derived T cells,

correction (ns = not significant). (F) Experimental set-up of antigen-specific T-cell expansion. Mononuclear cells from lung tissue were isolated 4 days after experimental exposure and expanded for 7 days by adding *A. fumigatus* lysate (Asp, 40  $\mu$ g/mL). CD14<sup>+</sup> monocytes were differentiated for 7 days, primed with *A. fumigatus* antigen (Asp, 40  $\mu$ g/mL), and matured. Expanded T cells were restimulated with *Aspergillus*-primed monocyte derived dendritic cells (MoDCs) for 6 h and later analyzed by flow cytometry. (G) Gating strategy of expanded and proliferated *A. fumigatus*-reactive CD4<sup>+</sup> T cells. (H) Exemplary histograms of the proportion of proliferated CD4<sup>+</sup> of environmentally exposed (env. exp.) and experimentally exposed (exp. exp.) pigs of *Aspergillus*-specific, expanded T cells (Asp) or without antigen expanded T cell controls (w/o). Frequencies of CFSE<sup>low</sup> cells among CD4<sup>+</sup> T cells are indicated as percentages. Exemplary data of one animal of either environmentally (n = 2) or experimentally (n = 3) exposed pigs of one experiment are shown (left) and depicted (right) are CD154 expressing CD4<sup>+</sup> T cells among the fraction of CFSE<sup>+</sup> (proliferated) T cells and presented as mean (bars)  $\pm$  SD; paired t test (\*p < 0.05, ns = not significant). (I) Exemplary flow cytometry plots of unstimulated and unexpanded CD154<sup>+</sup> T cells (w/o, left) and expanded *A. fumigatus*-reactive CD4<sup>+</sup> T cells after restimulation with *Aspergillus*-primed MoDCs (middle and right) of an environmentally exposed pig (env. exp., upper row) and an experimentally exposed pig (lower row). Frequencies of CD154<sup>+</sup> T cells among CFSE<sup>+</sup> proliferated CD4<sup>+</sup> T cells (left, middle) or frequencies of IL-17A<sup>+</sup> T cells among CD154<sup>+</sup> T cells (right) are indicated. Exemplary data of one animal of either environmentally (n = 2) or experimentally exposed (n = 3) pigs of one experiment are shown. (J) Frequencies of IL-17A<sup>+</sup> (left), TNF- $\alpha$ <sup>+</sup> (middle) or IFN- $\gamma$ <sup>+</sup> cells among expanded *A. fumigatus*-reactive CD154<sup>+</sup> T cells of environmentally exposed (env. exp., n = 2) versus experimentally exposed (exp. exp., n = 3) pigs presented as mean (bars)  $\pm$  SD of one experiment; unpaired t test with Welch's correction (\*p < 0.05, ns = not significant).

however, the overall number of *A. fumigatus*-specific T cells isolated from lung tissues was drastically increased after experimental exposure indicating strong migration into the airways of exposed pigs. Surprisingly, the accumulation of *A. fumigatus*-specific T cells in the lungs after exposure to high amounts of conidia is not occurring immediately, but rather builds up for more than 2 weeks postexposure. Of note, healthy animals did not develop clinical or macroscopical signs for invasive fungal growth in the lung.

While healthy human individuals quickly clear the fungus without developing disease, immunocompromised patients are at high risk to develop invasive aspergillosis (IA) or *A. fumigatus*-associated hyperinflammatory diseases [2]. We therefore studied the impact of immunosuppression on the adaptive antifungal T-cell immunity, by applying a restricted immunosuppressive treatment to a group of pigs around the day of fungal exposure. Since we did not intend to establish a severe model of invasive aspergillosis, we restricted the immunosuppression in both duration and dosage of cyclophosphamide and methylprednisolone. When monitoring blood responses over time, we noticed a very similar early kinetic of *A. fumigatus*-specific T cells rising at day 4 postexposure followed by a drop later on. This could indicate that reactivation of pre-existing memory T cells took place and proves that exposure-driven T-cell responses are detectable in immunocompromised individuals. However, immunosuppressed animals did not mount the second increase in blood *A. fumigatus*-specific T cells levels, that potentially arises from primed effector T cells.

For immunocompromised IA patients, the relative frequency of *A. fumigatus*-specific T cells within the CD4<sup>+</sup> blood T cells but not their absolute numbers provided excellent potential for diagnosing IA [10]. After abrogation of immunosuppression 2 days postexposure, blood leucocytes rapidly increased to normal levels. The short-term suppression we applied drastically reduced the number of lung-infiltrating *A. fumigatus*-specific and cytokine-producing T cells in exposed animals. Whether this is a consequence of reduced phagocytosis [44] and antigen-presentation [45] or reduced migratory capacity of Mo-DC [46] remains to be addressed in future studies. Importantly, none of the piglets developed invasive *aspergillosis* although all immunosuppressed animals were detected positive for the *Aspergillus* internal transcribed spacer 1 (ITS1)–5.8S rRNA gene region by PCR of BAL. In the absence of histological, seral or macroscopical proof of invasive fungal growth, we interpret these findings as a disturbed fungal clearance of *A. fumigatus* conidia. This in turn might lead to reduced fungal-antigen presentation and consequently less *A. fumigatus*-specific Th-cell instruction. In the absence of an immediate invasive fungal growth which would lead to high mortality rates, the pig model will allow to study to what extent Th cells contribute to the protection of invasive fungal infections, predisposing factors, and even tipping events.

In healthy animals CD154<sup>+</sup> lung T-cell numbers and also their cytokine production increased from d10 to d18. This might correspond to a study from Rivera et al [47] showing that the full differentiation program of primed *A. fumigatus*-specific T cells into competent Th1 cells is happening upon arrival in the airways [47] and

therefore occurs incrementally [34]. In immunocompromised animals we did not detect such phenotype progression, indicating that *A. fumigatus*-specific T cells trafficking to the lungs do not receive additional lung specific differentiation signals to fully instruct the Th1 program. This is particularly interesting with regard to our brief immunosuppressive treatment applied and should be considered for patients with short phases of immunosuppression not yet considered at high risk of disturbed antifungal immunity. Interestingly we could show, that high doses of inhaled conidia led to a particularly strong increase of Th17 responses already at day 4 postexposure. This suggests that *A. fumigatus* infection but not environmental exposure preferentially enhances Th17 responses, as recently reported for humans [11], and which may contribute to exaggerated inflammation and lung damage. As the role of T cells for human antifungal immunity is largely unknown, our studies on the domestic pig as a large, human-relevant animal model for *A. fumigatus*-specific T-cell immunity may provide new opportunities to decipher their instruction, trafficking, tissue-specific phenotypes, and their potential as diagnostic sensors.

## Materials and methods

### Ethics statement

The porcine study was performed in accordance to the principles outlined in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and the German Animal Welfare Law. Ethical approval was obtained from the State Office of Health and Social Affairs Berlin, Germany (Landesamt für Gesundheit und Soziales Berlin, Germany) for sampling blood and lung tissue from healthy pigs (regulation number T0002/17) and for experimental exposure (approval number G0381/17) of healthy and immunocompromised pigs.

### Animals, sampling, and necropsy

For detection of *A. fumigatus*-specific CD4<sup>+</sup> T cells in conventionally raised, blood was sampled in sterile lithium heparin containing tubes (S-Monovette<sup>®</sup> LH, Sarstedt, Nümbrecht, Germany) from healthy crossbred landrace and large white pigs of different ages and sexes either by taking blood from the external jugular vein or by heart puncture after sedation with ketamine hydrochloride and azaparon (20 mg/kg BW, Ursotamin, Serumwerk Bernburg AG and 2 mg/kg BW, Stresnil, Janssen-Cilag GmbH, Germany). Lung tissue was sampled after euthanization by intracardial injection of tetracaine hydrochloride, mebezoniom iodide, and embutramid (10 mg/kg T61, Intervet Deutschland GmbH). For analyzing the fungus-specific immune response to a defined exposure of *A. fumigatus* conidia, crossbred landrace and large white castrated, male pigs aged 5 weeks were purchased from a conventional breeder. Pigs were maintained for 14 days to allow for acclimatization



before being exposed once to a defined concentration ( $10^6$  colony forming units/ $m^3$ ) of airborne *A. fumigatus* conidia for 8 h in an aerosol chamber. Following exposure, pigs were cleared of remaining aerosol precipitate on their skin and transferred back to their stables.

Pigs were immunosuppressed by receiving a combination of cyclophosphamide and methylprednisolone every day (10 mg/kg p.o., Endoxan, Baxter Oncology GmbH and 4 mg/kg, Urbason, Sanofi-Aventis Deutschland GmbH) applied *per os* starting 6 days before *A. fumigatus* aerosolization for 8 days in total and received Amoxicillin i.m. (15 mg/kg i.m., Duphamox L.A., Zoetis Deutschland GmbH) every second day.

During experimental exposure with *A. fumigatus* conidia, the aerosol chamber was equipped with rubber mats and a water reservoir. After an observation period of either 10/11 or 18/19 days with regular blood sampling via the jugular vein (on days 2, 4, 6, 11, 13), pigs were sedated with ketamine hydrochloride and azaparone (20 mg/kg i.m.; Ursotamin; Serumwerk Bernburg AG and 2 mg/kg i.m.; Stresnil; Janssen-Cilag GmbH) and blood was taken by heart puncture followed by euthanization using T61 for final tissue sampling. Tissues were sampled from the two parts of the cranial left lung lobe, the caudal left lung lobe, the cranial right lung lobe, the middle right lung lobe, the accessory right lung lobe, and the caudal right lung lobe. Moreover, we collected cranial, right, middle, and left tracheobronchial lymph nodes.

### Experimental *A. fumigatus* aerosolization set-up

The exposure of pigs to airborne conidia of *A. fumigatus* (strain ATTC46645) was conducted in a stainless steel aerosol chamber with a base area of  $3.2 m^2$  and a volume of  $7 m^3$  (relative humidity of 70%, temperature of  $26^\circ C$ , air flow  $100 m^3/h$ , Supporting information Fig. S6) as previously described [48]. *Aspergillus fumigatus* conidia suspension was generated by plating conidia on *Aspergillus* minimal medium containing 50 mM glucose and cultivated for 3 days at  $37^\circ C$  until sporulation. Conidia were harvested in sterile water and filtered through a  $40 \mu m$  cell strainer (BD Biosciences, USA). For aerosolization, the suspension was transferred into a 50 mL syringe and stored at  $4^\circ C$ . Conidia suspension was transported via a perfusion pump (LAMBDA Instruments GmbH) and aerosolized with an ultrasonic nebulizer (Broadband Ultrasonic Generator, Sono-Tek Corporation, NY, USA) installed on the ceiling of the chamber. The *Aspergillus* conidia aerosol was then dispersed in the chamber by an axial ventilator. In initial blank aerosolization studies to verify airborne concentrations of *A. fumigatus* conidia, we set up technical parameters to reach an airborne concentration of  $10^6$  cfu/ $m^3$  conidia constantly over 8 h. Air samples were taken during the animals' exposure at two different levels (0.8 and 0.3 m) above ground by using AGI-30 impinger (Neubert-Glas GbR, Geschwenda, Germany) filled with 30 mL of distilled, filtered, and autoclaved water on the two time points 0.5 and 6 h after starting the aerosolization. A 100  $\mu L$  volume of the remaining water in the impinger were plated threefold on malt extract agar (Carl Roth GmbH + Co. KG) and incubated for 42 h at

$37^\circ C$ . The concentration of airborne conidia was calculated by the mean number of colony forming units, the amount of remaining water in the impinger, the airflow, and the duration of air sampling formulated in the following equation: aerosol concentration (cfu/ $m^3$ ) = [mean of colony forming units  $\times$  dilution factor  $\times$  10 (cfu/mL)]  $\times$  amount of remaining water in the impinger (mL)  $\times$  1000/airflow (12.5 L/min)  $\times$  sampling duration (30 min). Supporting information Fig. S7 shows calculated airborne *A. fumigatus* conidia concentration in the aerosol chamber.

### Leukocyte isolation

PBMC were freshly isolated on the day of blood sampling by density gradient centrifugation of diluted (1:2 in 0.9% NaCl) porcine blood using Pancoll solution (density 1.077 g/mL, PAN-Biotec GmbH). For gradient isolation of mononuclear cells of the lung,  $2 \times 2$  cm sized lung tissues were sampled from the seven locations and placed in complete RPMI-1640 medium (PAN-Biotec GmbH) supplemented with 100 U/mL Penicillin and 100  $\mu g/mL$  Streptomycin (P/S, PAN-Biotec GmbH) and cut into small pieces. After washing in 30 mL of cRPMI-1640, digestion was performed by resuspending the mashed up material in RPMI-1640 containing 1% P/S, 0.125 U/mL Collagenase D (Sigma-Aldrich), 0.180 mg/mL DNaseI (Sigma-Aldrich), 0.125 mg/mL Liberase DH (Sigma-Aldrich), and 0.125 mg/mL Liberase TM (Sigma-Aldrich). The material was incubated at  $37^\circ C$  in a shaking water bath (250 rpm) for 2 h in total with an intermediate filtering step ( $70\text{-}\mu m$ ) after 1 h. After final filtering and washing with ice-cold HBSS (PAN-Biotec GmbH), erythrocyte lysis was performed for 5 min at room temperature (RT). Cells were washed with complete RPMI-1640 and the cell pellet was resuspended in 40% Percoll solution (GE Healthcare) and layered onto 70% Percoll. After density gradient centrifugation at 1500 rpm for 20 min at RT, mononuclear cells were obtained at the interface and washed before culture. Leukocytes from lung lymph nodes were isolated by passing lymph node tissue through a  $70\text{-}\mu m$  cell strainer followed by erythrocyte lysis for 5 min and additional washing with complete RPMI-1640 (P/S, PAN-Biotec GmbH).

### Restimulation with fungal lysates

Lyophilized extracts of *A. fumigatus* (ATTC 46645) were purchased from either Miltenyi Biotec GmbH or generated from frozen mycelium as described in [8]. For detection of *A. fumigatus*-reactive  $CD4^+$  T cells, isolated PBMCs, mononuclear cells from the lung or leukocytes from lung lymph nodes were resuspended in complete IMDM (PAN-Biotec GmbH) supplemented with 10% FCS (PAN-Biotec GmbH), 100 U/mL Penicillin and 100  $\mu g/mL$  Streptomycin (PAN-Biotec GmbH), and rested overnight in cIMDM at  $37^\circ C$  and 5%  $CO_2$ . The following day, cells were stimulated with *A. fumigatus* lysate (Asp, 40  $\mu g/mL$ ) for 6 h at  $37^\circ C$  and 5%  $CO_2$ . Brefeldin A (eBioscience, Inc.) was added after 2 h of restimulation at a final concentration of 3  $\mu g/mL$ .

## Antigen-reactive enrichment of porcine CD4<sup>+</sup> T cells

A total of 1 to  $2 \times 10^7$  mononuclear cells of blood or lung were resuspended in 1 mL cIMDM (PAN-Biotec GmbH), supplemented with 10% FCS (PAN-Biotec GmbH), 100 U/mL Penicillin and 100 µg/mL Streptomycin (PAN-Biotec GmbH), seeded into 12-well culture plates and rested overnight at 37°C and 5% CO<sub>2</sub>. On the following day, the cells were stimulated for 5 h with *A. fumigatus* lysate (Asp, 40 µg/mL) in presence of anti-CD154-PE (1:50 dilution, clone 5C8, Miltenyi Biotec GmbH) at 37°C, 5% CO<sub>2</sub>. Unstimulated cells (w/o) served as control. Monensin (final concentration 2 µM, eBioscience, Inc.) was added after 2 h of stimulation. After 5 h, cells were collected and washed, followed by labeling with anti-PE MicroBeads (Miltenyi Biotec) for 15 min at 4°C. After washing, cells were resuspended in 1 mL cIMDM containing Brefeldin A (3 µg/mL, eBioscience, Inc.) and Monensin (2 µM, eBioscience, Inc.), placed back into the 12-well culture plate and incubated for another 2 h to allow for intracellular cytokine staining. Cells were collected and magnetically enriched by two sequential MS columns (Miltenyi Biotec). Surface staining was performed on the first column, followed by fixation and intracellular staining using Inside stain kit (Miltenyi Biotec) on the second column, as described by Bacher et al [6, 7].

## Flow cytometry

Cells were stained in different combinations with fluorochrome-conjugated antibodies for flow cytometry analyses (BD FACS Canto II, BD FACSAriaIII, BD FACS Diva software, FlowJo v10 software by Tree Star) according to agreed standards [49]. The following porcine-specific antibodies were used: anti-CD3<sub>ε</sub>-PerCP-Cy5.5 (clone BB23-8E6-8C8, IgG2a, BD Biosciences), anti-CD4<sub>α</sub>-AlexaFluor<sup>®</sup> 647 or -PE-Cy7 or -PerCP-Cy5.5 (clone 4-12-4, IgG2b, BD Biosciences), anti-CD8<sub>α</sub>-FITC (clone 76-2-11, IgG2a, BD Biosciences), IFN- $\gamma$ -PE or -AlexaFluor<sup>®</sup> 647 (clone P2G10, IgG1, BD Biosciences) and anti-CD27 (clone B30C7, IgG1, Bio-Rad Laboratories, Inc.). In addition, several cross-reactive antibodies were used: antihuman CD14-Viogreen (clone Tük4, IgG2a, Miltenyi Biotec), antihuman CD154-PE-Vio770 or -PE (clone 5C8, IgG2a, Miltenyi Biotec), antihuman TNF- $\alpha$ -Pacific Blue (clone Mab11, IgG1, BioLegend), antihuman IL-17A-AlexaFluor<sup>®</sup> 647 (clone SCPL1362, IgG1, BD Biosciences), and antihuman IL-4 PE-Cy7 (clone MP4-25D2, IgG1, BioLegend). Fixable viability dyes in eFluor<sup>®</sup> 506 were used to exclude dead cells, and CFSE for monitoring proliferating cells (both purchased from Thermo Fisher Scientific). For intranuclear staining, cells were fixed and permeabilized with Transcription Factor Staining Buffer Set (eBiosciences) or the Inside Stain Kit (Miltenyi Biotec).

## Histological examination

The two parts of the cranial left lung lobe, the caudal left lung lobe, the cranial right lung lobe, the middle right lung lobe, the

accessory right lung lobe and the caudal right lung lobe of the porcine was sampled for histology and fixed in a formalin solution (Roti-Histofix 10%, Carl Roth GmbH + Co. KG) for 6 h at RT and then stored at 4°C. The tissue was embedded in paraffin and sections were stained via Periodic acid-Schiff (PAS) and with an Anti-*Aspergillus* antibody ab20419 (Abcam Plc.) for immunohistochemistry.

## DNA extraction from BAL

Samples were vortexed and 0.5 mL of BAL fluid was centrifuged at 10 000 g for 5 min. The supernatant was transferred to a new tube. The remaining pellet was bead-beaten for 90 s using ceramic beads. Using a commercial extraction kit both fractions were combined and extracted as described before [50].

## DNA extraction from serum samples

DNA was extracted from 1 mL of serum using the QIAamp UltraSens virus kit (Qiagen, Hilden, Germany) with the following modification: (a) no carrier RNA was used, (b) lysate centrifugation was adjusted to 3000 g, and (c) the elution volume was adjusted to 35 µL. In each DNA extraction run, one negative control (human serum) and one *Bacillus*-positive serum (spiked with 10 000 plasmid copies *Bacillus subtilis*-DNA) were included as quality controls [51].

## DNA amplification and detection of *Aspergillus fumigatus*

An *Aspergillus*-specific real-time PCR assay [52] was used to detect fungal DNA. Briefly, 20-µL reaction mixtures contained 0.3 µM primers, 0.15 µM hydrolysis probe, 10 µL TaqMan gene expression master mix (Applied Biosystems), and 5 µL template DNA. Amplification was carried out in a StepOnePlus machine (Applied Biosystems) with the following steps: 50°C for 2 min, 95°C for 10 min, and 60 cycles of 95°C for 5 s, 54°C for 15 s (detection step), and 72°C for 1 s. Negative and positive PCR controls were included in each run. Samples were analyzed in duplicate. In each DNA extraction run, one negative control (DNA-free water spiked with 5000 plasmid copies of *B. subtilis* DNA) was included as a quality control. Beside the detection of fungal DNA, *B. subtilis* DNA was detected. This internal control was tested independently of the fungal target (monoplex), but within the same PCR run [51]. Beside the use as extraction control, *B. subtilis* DNA was also used to monitor PCR inhibition. For this, 1000 plasmid copies of *B. subtilis* DNA were spiked in every PCR reaction. PCR inhibition was claimed if the sample Cq value differs more than 3.3 from the controls [50].

## Total leukocyte and differential cell count

Whole blood was diluted 1:20 with a 3% acetic acid solution in a Thoma blood diluting pipette (Glaswarenfabrik Karl Hecht GmbH & Co KG). Leukocytes were manual counted (C-Chip, DHC-NO1, NanoEnTek, Inc.). Blood smears were air-dried, fixed, and stained using HAEMA Quick-Stain kit from Labor + Technik Eberhard Lehmann GmbH according to the manufacturer's instruction. Differential cell counting was performed on 200 cells per slide.

## Statistical analysis

Graphics and statistics were created with GraphPad PRISM software 7.03 (GraphPad Software, USA). Normality was tested with the Shapiro–Wilk test. Values with  $p < 0.05$  (\*),  $p < 0.01$  (\*\*),  $p < 0.001$  (\*\*\*), and  $p < 0.0001$  (\*\*\*\*) were considered to be significant.

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**Abbreviations:** ABPA: allergic bronchopulmonary aspergillosis · ARTE: antigen-specific T-cell enrichment · CF: cystic fibrosis · IA: invasive aspergillosis · RT: room temperature

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