

Robust T cell responses to aspergillosis in chronic granulomatous disease: implications for immunotherapy

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Summary

Chronic granulomatous disease (CGD) patients are highly susceptible to invasive aspergillosis and might benefit from aspergillus-specific T cell immunotherapy, which has shown promise in treating those with known T cell defects such as haematopoietic stem cell transplant (HSCT) recipients. But whether such T cell defects contribute to increased risks for aspergillus infection in CGD is unclear. Hence, we set out to characterize the aspergillus-specific T cell response in CGD. In murine CGD models and in patients with CGD we showed that the CD4⁺ T cell responses to aspergillus were unimpaired: aspergillus-specific T cell frequencies were even elevated in CGD mice ($P < 0.01$) and humans ($P = 0.02$), compared to their healthy counterparts. CD4-depleted murine models suggested that the role of T cells might be redundant because resistance to aspergillus infection was conserved in CD4⁺ T cell-depleted mice, similar to wild-type animals. In contrast, mice depleted of neutrophils alone or neutrophils and CD4⁺ T cells developed clinical and pathological evidence of pulmonary aspergillosis and increased mortality ($P < 0.05$ compared to non-depleted animals). Our findings that T cells in CGD have a robust aspergillus CD4⁺ T cell response suggest that CD4⁺ T cell-based immunotherapy for this disease is unlikely to be beneficial.

Keywords: chronic granulomatous disease (CGD), T cells, therapy/immunotherapy

Introduction

Patients with chronic granulomatous disease (CGD) have defects in the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme [1], a multi-subunit transmembrane enzyme complex that generates reactive oxygen species (ROS) following electron transfer. ROS possess some direct anti-microbial activity [2], but their indirect effects on innate and adaptive cells have increasingly been shown to be important defences against diverse pathogens [3]. Consequently, NADPH oxidase deficiency in CGD increases susceptibility to both bacterial and fungal infections [4].

CGD patients are especially at risk for invasive aspergillosis (IA), an opportunistic fungal infection caused by *Aspergillus fumigatus* and other species of this genus [5]. Invasive fungal diseases cause significant morbidity and mortality in CGD. IA is characterized by invasion of blood

vessels in the lung, resulting in multi-focal infiltrates and dissemination to other organs, particularly the central nervous system [6]. Although pathogenic factors in *A. fumigatus* have been suggested, no single important virulence gene or mechanism has been found that contributes to the development of IA. Therefore, there is increasing interest in evaluating the host's immune response to this pathogen [7].

Studies in mice and humans suggest the importance of the T helper type 1 (Th1) response [8] in conferring protection against invasive aspergillosis. Th1 interferon (IFN)- γ secreting cells are believed to increase innate immune activity against aspergillus by activating macrophages and neutrophils, and enhance anti-microbial activity of respiratory epithelial cells [9]. Mice given neutralizing antibodies against Th2 cytokines, to indirectly enhance Th1 cytokine production, have increased protection against aspergillosis [8]; and Th1 cells harvested from mice immunized

previously with aspergillus antigens were able to protect otherwise susceptible mice from aspergillosis following adoptive transfer [10].

Haematopoietic stem cell (HSCT) recipients are also susceptible to aspergillosis, and epidemiological studies suggest that this is because of the delayed recovery of T cells [11]. Several groups have sought to restore aspergillus-specific T cells as treatment for IA occurring during HSCT [12,13], and a clinical trial has shown promising results [14]. Whether or not similar immunotherapeutic strategies are beneficial for patients with CGD remains unknown. In theory, augmenting T cells in CGD may be effective if patients lack aspergillus-specific Th1 CD4⁺ T cells that produce IFN- γ , a cytokine used commonly in the treatment of CGD [15], at sites of infection. This is, however, relatively speculative as the aspergillus-specific T cell response in the setting of CGD has been largely uncharacterized.

In this study, we evaluated the role of the aspergillus-specific T cell response in CGD. Our murine and human studies suggest that the administration of aspergillus-specific Th1 CD4⁺ T cells may play no role as a primary therapy for neutrophil disorders such as CGD, where the cell-mediated immune response is already augmented.

Materials and methods

Murine studies

Mice. C57Bl/6 (wild-type) and *Cybb*^{-/-} (gp91 phox knock-out) mice (CGD mice) (Jackson Laboratories, Bar Harbor, ME, USA), 8–14 weeks old, were bred and kept under pathogen-free conditions at the animal facilities of Baylor College of Medicine. All experimental manipulations involving animals were performed with the approval of Baylor's Institutional Animal Care and Use Committee. Mice undergoing experimental infection were monitored for signs and symptoms of distress, and euthanized appropriately.

Microorganism, culture conditions and infection. The strain of *A. fumigatus* was obtained from a fatal case of pulmonary aspergillosis at the American Type Culture Collection (ATCC, Manassas, VA, USA; NRC A-4-56/WB5122). The microorganism was grown on Sabouraud dextrose agar (Difco, Detroit, MI, USA) for 6 days at 37°C. Conidia were harvested by washing plates with 10 ml of 1× phosphate-buffered saline (PBS) and scraping the conidia from the mycelium. After washing with saline, conidia were counted and aliquots snap-frozen and stored in liquid nitrogen until future use. Viability was determined by thawing aliquots 1 month or more after freezing and plating on Sabouraud dextrose agar for 24 h and colonies counted. An average of >55% viability was obtained and was used to calculate the number of conidia. On the day of infection, conidia are thawed at room temperature, resuspended at 40×10^6 viable conidia/ml in 1× PBS and administered intranasally to mice

after anaesthetization by isoflurane at the appropriate dose (2×10^6 conidia/50 μ l for antibody depletion experiments, 50 conidia/50 μ l for experiments to determine T cell response) and frequency (every other day, five times for antibody depletion; once for T cell response) following dilution with 1× PBS.

Antibody depletion of animals. Antibody depletion was performed 48 h prior to infection. C57Bl/6 mice were anaesthetized with isoflurane. After an appropriate depth of anaesthesia was achieved, mice were injected intraperitoneally with 50 μ g of anti-CD4 antibodies (GK1.5; eBiosciences, San Diego, CA, USA), 200 μ g anti-neutrophil antibodies (RB6-8C5; eBiosciences), or both. Depletion of CD4 T cells and neutrophils was verified using flow cytometry using antibodies to CD3⁺/CD4⁺ and Ly6⁺ populations 1 day after antibody injection using blood obtained from mice retro-orbitally. Antibody administration was repeated every 5 days until the end of the infection period.

Flow cytometry. Cells obtained from harvested spleens were assessed for surface expression of CD3, CD4, CD8, CD25, CD27, CD69 and CD70 using fluorochrome-conjugated antibodies (BD Biosciences, San Jose, CA, USA). Following homogenization and red blood cell (RBC) lysis, cells were harvested and washed with cold PBS (1× PBS; Sigma, St Louis, MO, USA) supplemented with 1% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA). Antibodies were added to cell pellets and allowed to bind at 4°C for 20 min in the dark. Cells were then washed twice with cold PBS with 1% FBS and acquired on a fluorescence activated cell sorter (FACS) Calibur flow cytometer. Data were analysed using CellQuest software (Becton Dickinson).

CD4⁺ T cell selection. CD4 T cells were obtained from spleens using magnetic selection according to the manufacturer's instructions (Miltenyi Biotec, Auburn, VA, USA). Briefly, cells obtained from spleens following homogenization and RBC lysis were stained with biotinylated anti-CD4 antibodies for 15 min in room temperature. Streptavidin-coated magnetic beads were then added and allowed to incubate for an additional 10 min. Cells were washed with 1× PBS with 0.5% bovine serum albumin (BSA) (Sigma), centrifuged, and passed through magnetic columns. After several washes, cells were removed from the columns and resuspended in the appropriate media.

Murine IFN- γ enzyme-linked immunospot assay (ELISPOT). IFN- γ ELISPOT analysis was used to evaluate the specificity of CD4 T cells derived from previously infected mice in response to bone marrow-derived dendritic cells (DCs) loaded with aspergillus extracts and cytosine-phosphate-guanosine (CpG) adjuvant. Briefly, plates were coated with anti-IFN- γ capture antibodies, and 5×10^5 cells were plated into each well. Control unloaded DCs and DCs

loaded with 100 ng aspergillus antigens (Greer Laboratories, Lenoir, NC, USA) and matured with CpG (Hycult, Plymouth Meeting, PA, USA) were then added onto cells in duplicate or triplicate. Concanavalin A was used as positive control. Secreted IFN bound onto membranes were detected using biotinylated anti-IFN- γ capture antibodies and subsequent addition of avidin-bound enzyme. Colour change upon addition of 3-amino-9-ethylcarbazole (AEC) substrate was evident as spots on the membranes. Membranes were punched out, and spots counted independently by Zellnet Consulting, Inc. (Fort Lee, NJ, USA) to obtain the frequency of aspergillus-specific T cells.

Lung histology. Left lungs were isolated from mice immediately after euthanasia, formalin-fixed, embedded in paraffin, serial-sectioned (3 μ M) and placed onto glass slides. For haematoxylin and eosin (H&E) stains, slides were stained with haematoxylin for 3 min 30 s, rinsed, differentiated with 0.3% acid alcohol for 10 s, rinsed and counterstained with eosin for 30 s. They were subsequently dehydrated with ethanol, treated with xylene and mounted. For silver stains, slides were oxidized with chromic acid for 1 h, rinsed and treated with 1% sodium metabisulphite for 1 min, treated in silver solution at 60°C for 15–20 min, treated with succeeding rinses and incubations with 0.2% gold chloride and 2% sodium thiosulphate for 2 min, and counterstained with working light green for 15 s. They were also dehydrated with ethanol, treated with xylene and mounted.

Human studies

Blood procurement. Peripheral blood mononuclear cells (PBMCs) used to generate DCs and T cell lines were obtained from healthy volunteers and patients diagnosed with CGD after obtaining informed consent from Baylor College of Medicine Institutional Review Board (IRB)-approved protocols.

ELISA. Peripheral blood mononuclear cells from CGD patients and healthy donors were cultured with autologous DCs presenting aspergillus extract (after pulsing for 24 h) for 5 days. Culture supernatants were harvested, centrifuged to remove cell debris and snap-frozen. IFN- γ levels in thawed supernatants were measured by ELISA, as per the manufacturer's instructions (BD Biosciences).

Flow cytometry. Peripheral blood mononuclear cells obtained following Ficoll gradient centrifugation and CD14-depletion were assessed for surface expression of CD3, CD4, CD8, CD25, CD27, CD69 and CD70 using fluorochrome-conjugated antibodies against these proteins (BD Biosciences). T cells were harvested and washed with cold PBS ($\times 1$ PBS; Sigma) supplemented with 1% FBS (Invitrogen). They were spun down and antibodies were

added to the pellets and allowed to bind at 4°C for 20 min in the dark. Cells were then washed twice with cold PBS with 1% FBS and acquired on a FACSCalibur flow cytometer. Data were analysed using CellQuest software (Becton Dickinson).

Intracellular cytokine staining. After 9–12 days of DC stimulation, T cells were harvested, resuspended at a concentration of 1×10^6 /ml in T cell media (50% RPMI-1640, 50% Click's, 2 μ M GlutaMAX, 10% human serum) and plated at 200 μ l/well in a 96-well plate. T cells were stimulated with unloaded DCs, DCs loaded with 100 ng of the aspergillus extract or *Staphylococcus enterotoxin* in the presence of brefeldin A (1 μ g/ml; Becton Dickinson). Afterwards, T cells were washed once with PBS containing 2% FBS, pelleted and surface-stained with CD4, CD8 and CD3 (BD Biosciences; 10 μ l/antibody/tube) antibodies. After 20 min incubation at 4°C in the dark, cells were washed twice, pelleted, fixed for 20 min with 4% paraformaldehyde and permeabilized with PBS/2% FBS containing 1% saponin (Calbiochem; EMD Chemicals, Gibbstown, NJ, USA) for 30 min at 4°C in the dark. Cells were washed and incubated with IFN- γ antibodies (Becton Dickinson) for 30 min at 4°C in the dark. After washing, live cells from each population were analysed with a FACSCalibur equipped with Cell Quest software (Becton Dickinson).

Multiplex ELISA. Peripheral blood mononuclear cells from CGD patients and healthy donors were cultured with autologous DCs presenting aspergillus extract (after pulsing for 24 h). Culture supernatants were harvested, centrifuged to remove cell debris and snap-frozen. Cytokine release in supernatants was analysed using a multiplex ELISA (Millipore, Billerica, MA, USA), according to the manufacturer's instructions. Cytokine levels were read and analysed using the xPONENT software in a Luminex® reader.

Statistical analysis

Comparisons of IFN- γ secretion and other cytokines between groups were compared using Student's *t*-test. Flow cytometry results were analysed using Wilcoxon's rank sum test and Student's *t*-test. Survival curves were constructed using the Kaplan–Meier method and compared using the weighted log-rank test. A *P*-value < 0.05 was considered significant.

Results

T cells from Cybb-deficient mice (CGD mice) are phenotypically similar, but secrete increased IFN- γ in response to *A. fumigatus* compared to wild-type controls

We first determined whether aspergillus-specific CD4⁺ T cells in murine models of CGD are dysfunctional compared

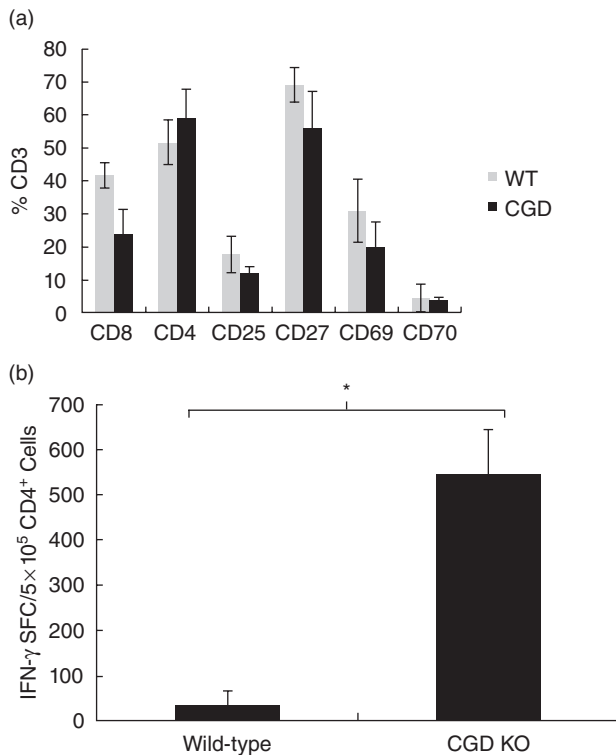


Fig. 1. CD4⁺ T cells derived from chronic granulomatous disease (CGD) mice have a similar phenotype to wild-type but secrete increased interferon (IFN)- γ in response to aspergillus. (a) Lymphocytes harvested from the spleens of CGD and wild-type (healthy) mice were phenotyped using flow cytometry and demonstrated similar percentages of CD4 T cells, higher percentages of CD8 T cells in wild-type mice, as well as similar levels of the activation markers CD25, CD27, CD69 and CD70. (b) CD4 T cells harvested by magnetic affinity cell sorting (MACS) selection from the spleens of CGD and wild-type mice infected with sublethal (50 conidia intranasally) doses of aspergillus were incubated with bone-marrow-derived dendritic cells (DC) loaded with aspergillus extract and matured with cytosine-phosphate-guanosine (CpG) and the number of IFN- γ spot-forming cells per 5×10^5 cells was measured. CGD mice had higher frequencies of IFN- γ -secreting cells compared to wild-type mice ($P < 0.001$).

to their wild-type counterparts. Mice genetically deficient in the gp91phox subunit (Cybb) of the protein were used as models for CGD, as this mutation corresponds to the most commonly mutated form in humans [16]. We assessed T cell phenotype using flow cytometry and observed no differences in expression between wild-type and CGD mice following infection, with both groups showing similar percentages ($P > 0.05$) of CD4 T cells in their spleens (51.7 ± 6.7 versus 59.2 ± 8.6). There was, however, a higher percentage of CD8 T cells in wild-type animals compared to CGD animals that was statistically significant ($P = 0.03$). T cells from wild-type and CGD mice had comparable expression of the activation markers CD25, CD27, CD69 and CD70 (Fig. 1a).

We next evaluated the ability of T cells derived from CGD mice to respond to stimulation (with IFN- γ secretion) in the presence of *A. fumigatus*. As shown in Fig. 1b, *A. fumigatus*-treated CGD mice ($n = 5$) had higher frequencies of T cells that secrete IFN- γ in response to DCs loaded with *A. fumigatus* extract [mean 545.7 IFN- γ spot-forming cells (SFC)/ 5×10^5 CD4⁺ T cells, median 575 , range 380 – 628] *ex vivo*, than similarly treated wild-type ($n = 5$) animals [mean 37.4 IFN- γ spot-forming cells (SFC)/ 5×10^5 CD4⁺ T cells, median 38 range 2 – 78] ($P < 0.01$).

CD4⁺ T cell deficiency does not increase susceptibility to aspergillus infection in the absence of neutrophil dysfunction or deficiency

To understand the contributions of CD4⁺ T cells to the control of aspergillus infections, we infected CD4-depleted, neutrophil-depleted and CD4/neutrophil-depleted C57/BL6 mice with *A. fumigatus* conidia intranasally, and examined fungal growth, anti-fungal immunity and survival. Mice depleted of neutrophils ($n = 16$) had significantly shorter survival compared to non-depleted animals ($n = 11$) ($P = 0.0494$) (Fig. 2a). These animals displayed physical signs of the disease, a higher fungal burden in their lungs with hyphal mat formation (Fig. 2b) and evidence of invasive disease and granuloma formation in lung parenchyma (Fig. 2c). In contrast, CD4⁺ T cell-depleted mice ($n = 11$) remained as resistant to aspergillus infection as non-immunosuppressed animals. All mice in these groups survived the experimental period (Fig. 2a) without physical signs of disease or evidence of conidia germination into hyphae (Fig. 2b). However, mild inflammation was observed in the lung parenchyma of CD4-depleted mice, with fewer infiltrates than seen in non-depleted animals. Aspergillus-infected mice depleted of both CD4⁺ T cells and neutrophils ($n = 14$) showed no significant differences in fungal burden and overall survival compared with mice depleted of neutrophils alone ($P = 0.1195$) although survival was poorer for the animals depleted of both neutrophils and T cells (Fig. 2a,b).

Hence, the protective benefit for aspergillus-specific CD4⁺ T cells in the absence of intact innate immunity provided by neutrophils appears modest and, despite their increased frequency, CGD mice succumb readily to aspergillus infection [16]. These results suggest that aspergillus-specific T cells, while functional, do not seem to protect fully against aspergillus in the setting of neutrophil deficient states such as CGD.

T cells from CGD patients are phenotypically similar, but elicit elevated IFN- γ in response to *A. fumigatus* compared to healthy donors

To verify our findings in the CGD mice, we next evaluated whether there were phenotypical differences between T cells

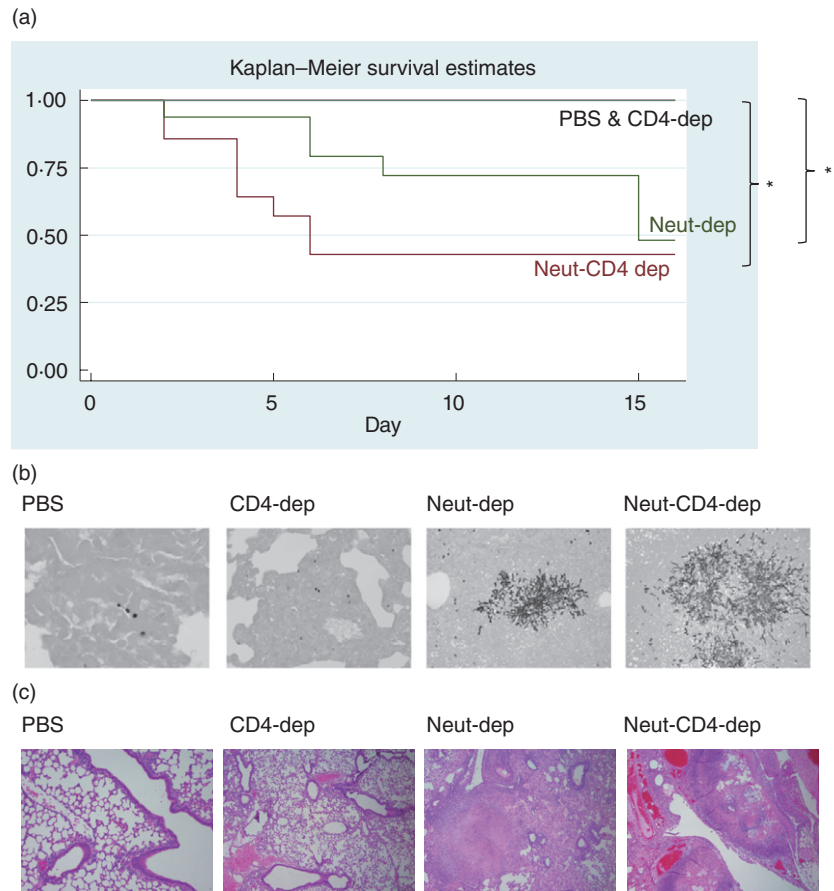


Fig. 2. Animals depleted of CD4 T cells alone do not have increased susceptibility to invasive aspergillosis. (a) The effects of *in-vivo* cell depletion using CD4-depleting antibodies (CD4-dep) or Ly6 (neutrophil)-depleting antibodies (neut-dep) or dual depletion (neut-CD4-dep) on survival compared to undepleted phosphate-buffered saline (PBS)-treated control animals (PBS) are shown. (b) Representative slides from methenamine silver-stained slices of lung tissue show hyphal mat formation only in animals that have been depleted of neutrophils (neut-dep and neut-CD4-dep). (c) Representative haematoxylin and eosin (H&E) stains of lung tissue are shown. The inflammatory response is diminished in CD4 T cell-depleted animals. Interestingly, granuloma formation is seen only in mice depleted of neutrophils alone.

obtained from healthy donors ($n=9$) and CGD patients ($n=9$). CGD patients had T cell populations which did not differ significantly from those expressed by healthy donors ($60.2 \pm 9.7\%$ versus $54.9 \pm 10.6\%$ CD4 T cells and $44.9 \pm 15.3\%$ versus $42.6 \pm 9.6\%$ CD8 T cells). Similar to their murine counterparts, both groups showed comparable expression of the activation markers CD25, CD27, CD69 and CD70 (Fig. 3a).

We next determined whether T cells derived from CGD patients also secreted elevated levels of IFN- γ in response to aspergillus antigens, similar to our murine models. In ELISA assays, T cells derived from CGD patients secreted significantly higher amounts of IFN- γ (915 pg/ml, median 653, range 19–1847) in response to aspergillus antigens than healthy donors (164 pg/ml, median 25, range 19–1400), $n=10$ /group ($P=0.02$) (Fig. 3b). Using intracellular flow cytometry, we show that the IFN- γ was derived from CD4 $^{+}$ T cells (Fig. 3c). Thus, consistent with the murine studies, secretion of IFN- γ , predominantly from the CD4 $^{+}$ fraction, was not only intact but elevated in patients with CGD compared to their immunocompetent counterparts.

T cells from CGD patients also secrete elevated Th1 inflammatory cytokines in response to aspergillus compared to healthy donors

To evaluate the breadth of the Th1 inflammatory cytokine response, using a luminex assay we observed that in addition to increased IFN- γ secretion, T cells from CGD patients ($n=7$) trended towards higher secretion of interleukin (IL)-1 β ($P=0.17$), and significantly higher secretion of IL-6 and tumour necrosis factor (TNF)- α compared to healthy donors ($n=6$), following contact with antigen-presenting cells loaded with aspergillus proteins ($P=0.014$ and $P=0.007$, respectively) (Fig. 4).

Discussion

The overarching goal for this study was to evaluate: (i) whether or not there was an intrinsic defect within the CGD T cells; and (ii) whether or not T cell adoptive immunotherapy would be a reasonable approach for the management of these patients. Our findings show that the IFN- γ CD4 T cell response is elevated in murine models of CGD

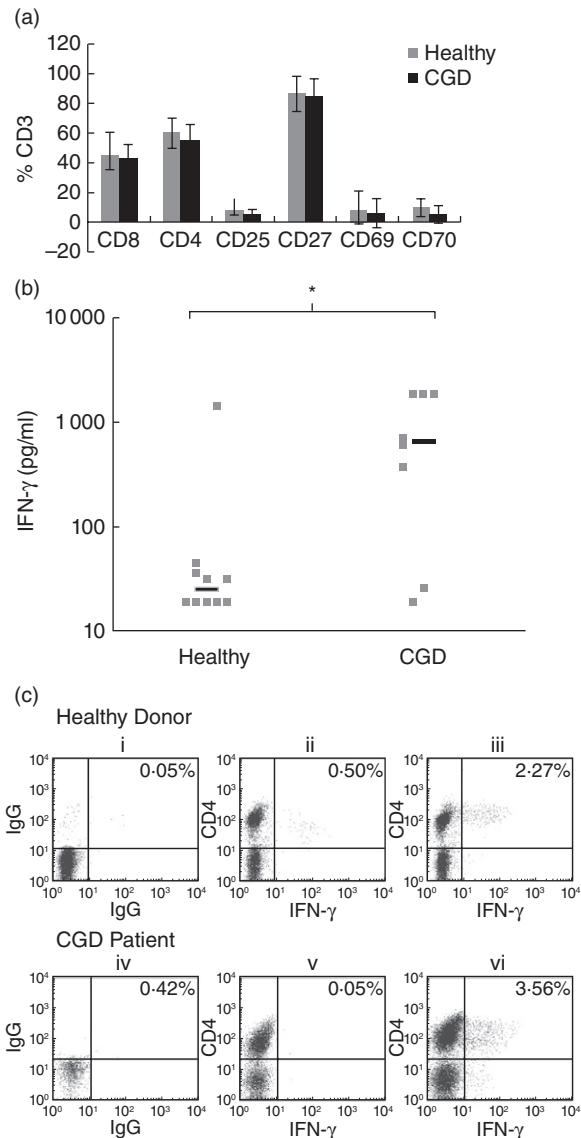


Fig. 3. CD4⁺ T cells derived from patients with chronic granulomatous disease (CGD) secrete increased interferon (IFN)- γ in response to aspergillus compared to healthy donors. (a) Peripheral blood mononuclear cells (PBMC) from CGD patients ($n = 9$) and healthy donors ($n = 9$) were analysed by flow cytometry. Percentages of CD4⁺ and CD8⁺ cells are similar between CGD patients and healthy donors, along with expression of the activation markers CD25, CD27, CD69 and CD70. (b) Peripheral blood T cells from 10 CGD patients and 10 healthy donors were incubated for 5 days with monocyte-derived dendritic cells (DCs) pulsed with aspergillus extract for 24 h, and the supernatants harvested and analysed for IFN- γ secretion using enzyme-linked immunosorbent assay (ELISA) assay. IFN- γ concentrations (pg/ml) are shown for healthy donors and CGD patients. Black bars show median, grey squares denote average of two readings per donor. (c) Examples of intracellular stains from a healthy donor (i–iii) and a CGD patient (iv–vi) are shown. (i, iv) Isotype controls. (ii, v) Percentage of IFN- γ -secreting CD4⁺ T cells responding to dendritic cells (DCs) with no aspergillus antigens, while (iii, vi) show percentage of IFN- γ -secreting CD4⁺ T cells responding to DCs pulsed with aspergillus extract.

and in CGD patients when compared to their wild-type and healthy counterparts, respectively. We also show for the first time that CD4⁺ T cell-depleted mice control aspergillus infection similar to undepleted animals. Further, aspergillus-infected mice depleted of CD4⁺ T cells and neutrophils do not have a significantly different phenotype to mice depleted of neutrophils alone. Our findings do not demonstrate that CD4 T cells are completely dispensable in neutrophil-deficient states such as CGD, but they suggest that infusion of IFN- γ -secreting CD4⁺ T cells may not be beneficial as a sole therapeutic option for these patients. We initially focused on CD4⁺ T cells because previously published studies have already identified a role for these cells to orchestrate the immune response against invasive aspergillosis [9,10,17,18]. Further, aspergillus-specific CD4 T cells have been infused as an immunotherapeutic for the treatment of aspergillosis after allogeneic HSCT [14]. While we showed a significantly higher percentage of CD8 T cells in wild-type animals compared to CGD animals, we did not observe an increase in CD8⁺ T cells in patients with CGD. Hence, this increase in CD8⁺ T cell percentages in the mice may not reflect pathology that is relevant in the CGD and IA setting. Nevertheless, the role of CD8⁺ T cells in the anti-fungal immune response warrants investigation in future studies.

There is scant evidence to suggest that intrinsic T cell defects exist in CGD. Previous studies have suggested a possible role for ROS resulting from NADPH oxidase activity in CGD T cells. This is based primarily on an observation that the NADPH oxidase isoform defective in CGD is also found to be expressed in T cells [19]. Deficient ROS levels in T cells are believed to disrupt the tryptophan pathway [20],

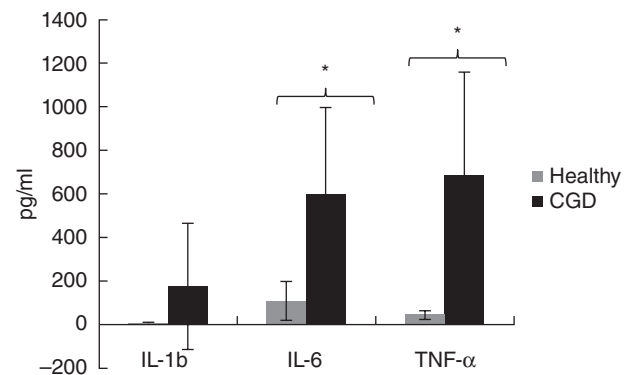


Fig. 4. CD4⁺ T cells derived from patients with chronic granulomatous disease (CGD) secrete increased T helper type 1 (Th1) and decreased Th2 cytokines in response to aspergillus compared to healthy donors. Following stimulation with monocyte-derived dendritic cells loaded with aspergillus antigen, cytokine secretion of T cells obtained from healthy donors and CGD patients was determined using a multiplex enzyme-linked immunosorbent assay (ELISA). Increased levels of cytokines involved in the acute inflammatory response [interleukin (IL)-1 β , IL-6 and tumour necrosis factor (TNF)- α] are seen ($P = 0.17$; $P = 0.014$, $P = 0.007$, respectively).

and thus affect CD4 T cell cytokine secretion. The expression of NADPH oxidase in CGD T cells is, however, markedly lower than that found in myeloid cells [21], and the significance of such low levels of ROS following TCR stimulation is still unclear. Further, other studies were unable to observe deficiencies in tryptophan metabolism in CGD T cells [22]. Moreover, our results demonstrate that proinflammatory cytokine production is unimpaired in CGD T cells and that production of other cytokines are similar to healthy donor T cells (data not shown). These are more indicative of a compensatory, heightened response from T cells rather than an intrinsic T cell defect existing in CGD.

The results from this study also support the findings from Smeekens *et al.*, who observed increased secretion of IFN- γ as well as the proinflammatory cytokines IL-6 and TNF- α , following stimulation of PBMCs from CGD patients with aspergillus antigens [22], and those of Jackson *et al.*, who report increased proinflammatory cytokine secretion following non-specific stimulation in CGD murine models [19]. Hence, these findings, combined with our results, provide compelling reasons to suggest that: (i) T cells are not impaired in CGD and (ii) T cell therapy alone will prove insufficient to prevent IA in CGD.

Because the Th1 response to aspergillus appears to be intact in CGD, the utility of T cell therapies as a primary immunotherapeutic strategy directed against *A. fumigatus* might not be prudent for three reasons, as follows.

First, CGD is already associated with increased endogenous IFN- γ . Despite this increased IFN- γ secretion in response to aspergillus in T cells derived from CGD animals, these mice still succumbed readily to aspergillus infection compared to wild-type mice [16]. Interferon is believed to stimulate innate immune cells such as neutrophils [23], but these results suggest that while the aspergillus-specific T cells are able to recognize and respond to aspergillus antigen, high levels of endogenous IFN secreted by the T cell populations cannot protect adequately against aspergillus in the setting of neutrophil dysfunction.

In CGD patients, elevated IFN- γ responses from T cells is in contrast to stem cell transplant recipients who may benefit from the administration of aspergillus T cells, as patients with haematological malignancies have reduced levels of IFN- γ secretion in response to antigen compared with healthy donors [24]. Furthermore, patients after HSCT have increased susceptibility to aspergillosis post-engraftment at a time when T cell numbers have not recovered [11]. After neutrophil engraftment, while the neutrophil numbers may be in the normal range, they may not be fully functional. Deficits in neutrophil response to microorganisms have been observed in the post-engraftment period after allogeneic HSCT [25]. Hence, these deficits in neutrophil function may contribute to the development of IA post-engraftment [11]. T cells secrete cytokines that stimulate neutrophils, which may enable the

expansion of functional neutrophils. Therefore, the infusion of T cells in the HSCT setting may still be warranted. This is in contrast to the situation in CGD, where all neutrophils are dysfunctional and remain so even with a robust T cell response. Nevertheless, with the advent of newer immune monitoring assays, studying the tempo of functional neutrophil recovery after both HSCT and adoptive T cell transfer may be important to comprehensively evaluate patients at risk for fungal disease.

Secondly, despite compelling epidemiological evidence in the transplant setting [11], the exact role of CD4 T cells in aspergillus immunity is still unclear. While it was long believed that a balance towards increased Th1 and decreased Th2 confer protection against aspergillus [8,9], other studies report that decreased Th1 in immune-competent animals normally occurs following exposure to beta glucans from aspergillus, and serves to diversify the T cell response [26]. Similarly, while some studies argue for the importance of increased regulatory T cell (T_{reg}) activity and harmful Th17 responses in CGD [20,27], other groups report the opposite – implicating heightened Th17 responses as important mediators of anti-fungal immunity [28].

Finally, the clinical benefits of IFN- γ as a strategy to protect CGD patients against aspergillosis may need to be re-evaluated, especially in light of newer anti-fungal therapeutics. Control of aspergillosis in CGD patients treated solely with new anti-fungal drugs without IFN- γ has been reported [29]. Additionally, in Europe, where IFN- γ is administered to only 30% of the total CGD population, survival is comparable to the United States, where 70% of the CGD population receives IFN- γ [30,31].

In conclusion, increased aspergillus-specific T cell responses are observed in CGD, and do not appear to afford complete protection in the absence of functional neutrophils. When neutrophils are present and functional, T cells do not seem to play a major role. These results have novel and important clinical consequences, because they imply that T cells may not form the 'frontline' of aspergillus defence. Therefore, adoptive T cell therapy may not provide a comprehensive therapy for the treatment of fungal infections in patients with CGD. Future studies investigating strategies to enhance both innate and adaptive immune response are thus warranted.

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Disclosure

The authors have no financial conflicts of interest to disclose.

References

- Segal AW. The electron transport chain of the microbicidal oxidase of phagocytic cells and its involvement in the molecular pathology of chronic granulomatous disease. *J Clin Invest* 1989; **83**:1785–93.
- Forman HJ, Thomas MJ. Oxidant production and bactericidal activity of phagocytes. *Annu Rev Physiol* 1986; **48**:669–80.
- Lambeth JD. NOX enzymes and the biology of reactive oxygen. *Nat Rev Immunol* 2004; **4**:181–9.
- Segal BH, Romani L, Puccetti P. Chronic granulomatous disease. *Cell Mol Life Sci* 2009; **66**:553–8.
- Segal BH, Romani LR. Invasive aspergillosis in chronic granulomatous disease. *Med Mycol* 2009; **47** (Suppl. 1):S282–90.
- Segal BH. Aspergillosis. *N Engl J Med* 2009; **360**:1870–84.
- Latge JP. *Aspergillus fumigatus* and aspergillosis. *Clin Microbiol Rev* 1999; **12**:310–50.
- Cenci E, Perito S, Enssle KH *et al.* Th1 and Th2 cytokines in mice with invasive aspergillosis. *Infect Immun* 1997; **65**:564–70.
- Romani L. Immunity to fungal infections. *Nat Rev Immunol* 2004; **4**:1–23.
- Cenci E, Mencacci A, Bacci A, Bistoni F, Kurup VP, Romani L. T cell vaccination in mice with invasive pulmonary aspergillosis. *J Immunol* 2000; **165**:381–8.
- Marr KA, Carter RA, Boeckh M, Martin P, Corey L. Invasive aspergillosis in allogeneic stem cell transplant recipients: changes in epidemiology and risk factors. *Blood* 2002; **100**:4358–66.
- Tramsen L, Koehl U, Tonn T *et al.* Clinical-scale generation of human anti-*Aspergillus* T cells for adoptive immunotherapy. *Bone Marrow Transplant* 2009; **43**:13–9.
- Ramadan G, Davies B, Kurup VP, Keever-Taylor CA. Generation of cytotoxic T cell responses directed to human leucocyte antigen Class I restricted epitopes from the *Aspergillus* fl6 allergen. *Clin Exp Immunol* 2005; **140**:81–91.
- Perruccio K, Tosti A, Burchielli E *et al.* Transferring functional immune responses to pathogens after haploidentical hematopoietic transplantation. *Blood* 2005; **106**:4397–406.
- Aoki N, Xing Z. Use of cytokines in infection. *Exp Opin Emerg Drugs* 2004; **9**:223–36.
- Pollock JD, Williams DA, Gifford MA *et al.* Mouse model of X-linked chronic granulomatous disease, an inherited defect in phagocyte superoxide production. *Nat Genet* 1995; **9**:202–9.
- Rivera A, Ro G, Van Epps HL *et al.* Innate immune activation and CD4+ T cell priming during respiratory fungal infection. *Immunology* 2006; **25**:665–75.
- Beck O, Koehl U, Tramsen L *et al.* Enumeration of functionally active anti-*Aspergillus* T-cells in human peripheral blood. *J Immunol Methods* 2008; **335**:41–5.
- Jackson SH, Devadas S, Kwon J, Pinto LA, Williams MS. T cells express a phagocyte-type NADPH oxidase that is activated after T cell receptor stimulation. *Nat Immunol* 2004; **5**:818–27.
- Romani L, Fallarino F, De Luca A *et al.* Defective tryptophan catabolism underlies inflammation in mouse chronic granulomatous disease. *Nature* 2008; **451**:211–5.
- Leto TL, Geiszt M. Role of Nox family NADPH oxidases in host defense. *Antioxid Redox Signal* 2006; **8**:1549–61.
- Smeekens SP, Henriët SS, Gresnigt MS *et al.* Low interleukin-17A production in response to fungal pathogens in patients with chronic granulomatous disease. *J Interferon Cytokine Res* 2012; **32**:159–68.
- The International Chronic Granulomatous Disease Cooperative Study Group. A controlled trial of interferon gamma to prevent infection in chronic granulomatous disease. The International Chronic Granulomatous Disease Cooperative Study Group. *N Engl J Med* 1991; **324**:509–16.
- Hebart H, Bollinger C, Fisch P *et al.* Analysis of T-cell responses to *Aspergillus fumigatus* antigens in healthy individuals and patients with hematologic malignancies. *Blood* 2002; **100**:4521–8.
- Scholl S, Hanke M, Hoffken K, Sayer HG. Distinct reconstitution of neutrophil functions after allogeneic peripheral blood stem cell transplantation. *J Cancer Res Clin Oncol* 2007; **133**:411–5.
- Rivera A, Hohl TM, Collins N *et al.* Dectin-1 diversifies *Aspergillus fumigatus*-specific T cell responses by inhibiting T helper type 1 CD4 T cell differentiation. *J Exp Med* 2011; **208**:369–81.
- Zelante T, De Luca A, Bonifazi P *et al.* IL-23 and the Th17 pathway promote inflammation and impair antifungal immune resistance. *Eur J Immunol* 2007; **37**:2695–706.
- Werner JL, Metz AE, Horn D *et al.* Requisite role for the dectin-1 beta-glucan receptor in pulmonary defense against *Aspergillus fumigatus*. *J Immunol* 2009; **182**:4938–46.
- Cale CM, Jones AM, Goldblatt D. Follow up of patients with chronic granulomatous disease diagnosed since 1990. *Clin Exp Immunol* 2000; **120**:351–5.
- van den Berg JM, van Koppen E, Ahlin A *et al.* Chronic granulomatous disease: the European experience. *PLoS ONE* 2009; **4**:e5234.
- Winkelstein JA, Marino MC, Johnston RB Jr. *et al.* Chronic granulomatous disease. Report on a national registry of 368 patients. *Medicine (Balt)* 2000; **79**:155–69.