MAJOR ARTICLE

Multispecific *Aspergillus* T Cells Selected by CD137 or CD154 Induce Protective Immune Responses Against the Most Relevant Mold Infections

Claudia Stuehler,[1] Justyna Nowakowska,[1] Claudia Bernardini,[1,2] Max S. Topp,[4] Manuel Battegay,[2] Jakob Passweg,[3] and Nina Khanna[1,2]

[1]Infection Biology Laboratory, Department of Biomedicine, [2]Division of Infectious Diseases and Hospital Epidemiology, Departments of Biomedicine and Clinical Research, and [3]Division of Hematology, University Hospital of Basel, Switzerland; and [4]Division of Hematology, Department of Internal Medicine II, University of Wuerzburg, Germany

Background. *Aspergillus* and *Mucorales* species cause severe infections in patients after hematopoietic stem cell transplantation (HSCT). Induction of antifungal $CD4^+$ T-helper type 1 (T_h1) immunity is an appealing strategy to combat these infections. Immunotherapeutic approaches are so far limited because of a lack of antigens inducing protective T cells, their elaborate production, and the need of targeting a broad spectrum of pathogenic fungi.

Methods. We examined the response to different *Aspergillus fumigatus* proteins in healthy individuals and patients after HSCT and compared rapid selection protocols for fungus-specific T cells based on CD137 or CD154 expression.

Results. The *A. fumigatus* proteins Crf1, Gel1, and Pmp20 induced strong T_h1 responses in healthy individuals. T cells specific for these antigens expanded in patients with active invasive aspergillosis, indicating their contribution to infection control. T_h1 cells specific for the 3 proteins can be selected with similar specificity within 24 hours, based on CD137 or CD154 expression. These cells recognize naturally processed *A. fumigatus* and the multispecific T-cell lines, directed against all 3 proteins, especially those selected by CD154, additionally cross-react to different *Aspergillus* and *Mucorales* species.

Conclusions. These findings may form the basis for adoptive T-cell transfer for prophylaxis or treatment in patients with these devastating infections.

Keywords. T-cell therapy; fungal infection; multispecific T cells; CD137 and CD154 expression; *Aspergillus*.

Aspergillus and *Mucorales* species cause severe infections in patients with leukemia and those who have undergone allogeneic hematopoietic stem cell transplantation (HSCT) [1, 2].

Antifungal prophylaxis or treatment in high-risk patients with prolonged neutropenia, T-cell suppression, or graft-versus-host disease (GVHD) is often ineffective owing to impaired host immunity and may result in the emergence of uncommon or resistant molds. Treatments are furthermore associated with drug interactions, toxicity, and high costs [2]. Hence, treatment approaches that restore or boost fungus-specific immunity would be desirable.

Adoptive T-cell therapy using T-cell clones generated by stimulation with *Aspergillus fumigatus* conidia in patients with invasive aspergillosis after haploidentical HSCT was promising [3]. However, reproducibility is difficult because of the variation of stimuli and the elaborate production required for compliance with good manufacturing practices.

Therefore, it is crucial to identify immunogenic proteins and peptides in healthy individuals and patients with invasive aspergillosis. This is challenging, however,

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Correspondence: Nina Khanna, MD, Infection Biology Laboratory and Division of Infectious Diseases and Hospital Epidemiology, University Hospital of Basel, Petersgraben 4, 4031 Basel, Switzerland (nina.khanna@usb.ch).

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because the *A. fumigatus* genome encodes for approximately 10 000 genes [4]. Until now, several immunogenic proteins and T-cell epitopes have been characterized in healthy individuals and mice, such as extracellular cell wall glucanases Crf1, Crf2, Scw4 [5–9], 1,3-β-glucanosyl-transferase Gel1 [6, 8, 9], GPI-anchored protein PST1 [9], mycelial catalase 1 [6, 7, 9], serine hydroxylmethyltransferase 2 [9], aspartic protease Pep1 [6, 8], enolase Aspf22 [9, 10], manganese superoxide dismutase Sod2 [9, 10], and cytosolic peroxisomal peroxiredoxin Pmp20 (Asp f3) [9–11]. Only recently, T-cell responses to some of these proteins have been shown to correlate with a beneficial outcome in patients with invasive aspergillosis [8, 12]. Furthermore, it would be desirable that these antigen-specific T cells recognize additional clinically relevant molds.

To facilitate adoptive therapy for invasive aspergillosis, the generation of T-cell products needs to be rapid, to be specific, and to comply with GMP. The CliniMACS IFN-γ Cytokine Capture System that has so far been successfully applied for the generation of virus-specific T cells [13–15] shows limited sensitivity for antigens with low T-cell precursor frequencies [16]. Therefore, other T-cell activation markers that enable selection of antigen-specific T cells with higher sensitivity and irrespective of cytokine production may be more suitable. Selection methods based on activation-dependent expression of CD154 or CD137 have recently been shown to be promising candidates [9, 17, 18]. Moreover, the CliniMACS CD137 isolation reagents were deemed in 2013 to comply with GMP, but their clinical use has not yet been demonstrated.

Here, we characterized the T-cell repertoire specific to different *Aspergillus*-derived peptide pools and epitopes in healthy individuals and patients after HSCT and compared the CD154- and CD137-based isolation methods for specificity and cross-reactive potential.

MATERIALS AND METHODS

Fungal Antigens

Aspergillus fumigatus lysate and Crf1, Gel1, and Pmp20 peptide pools (Miltenyi Biotec) and custom-made peptide libraries of Gel1 and Pmp20 (Proimmune) consisting of 111 and 40 15-mer peptides overlapped by 4 amino acids with a purity of >70% and the Crf1/p41 FHTYTIDWTKDAVTW (JPT Peptide Technologies) were reconstituted at 5 μg/μL in dimethyl sulfoxide (Sigma-Aldrich).

Generation of Heat-Inactivated Fungi

Aspergillus fumigatus strain D141 and clinical fungal isolates were provided by Prof Dr Sven Krappmann (Medical Immunology Campus Erlangen, Germany) and Dr Reno Frei (University Hospital Basel, Switzerland), respectively, and were cultured and heat inactivated as described elsewhere [5].

In Vitro Stimulation, Epitope Mapping, and CD137$^+$ and CD154$^+$ Selection

Blood specimens were obtained from healthy donors and patients after receipt of informed consent and in accordance with a protocol approved by the Ethics Commission of Northwest and Central Switzerland. Isolation of peripheral blood mononuclear cells (PBMCs) and generation of dendritic cells (DCs) and macrophages were performed as published previously [5].

To generate antigen-specific T-cell lines, PBMCs were stimulated for 7 days with *A. fumigatus* lysate (50 μg/mL), Crf1, Pmp20, or Gel1 peptide pools (1 μg/peptide/mL) with 5 U/mL interleukin 2 (Proleukin, Chiron). CD3$^+$/CD4$^+$ and CD3$^+$/CD8$^+$ cells were isolated by fluorescence-activated cell sorting from antigen-specific cell lines after 8 days and restimulated with antigen-pulsed macrophages at a responder to stimulator ratio of 5:1.

For CD137 and CD154 selection, $5–8 \times 10^7$ PBMCs in 4–5 mL of medium were stimulated with *A. fumigatus* lysate, Crf1, Pmp20, Gel1, or all 3 peptide pools. A total of 1 μg/mL anti-CD40 antibody was added for CD154-based selection (Miltenyi Biotech). After 14 hours, cells were enriched by CD137$^+$ or CD154$^+$ selection according to the manufacturers' instructions (Miltenyi Biotech). Up to 4×10^5 CD137$^+$ or CD154$^+$ cells were cocultured with 50:1 γ-irradiated (35 cGy) autologous feeder cells as published previously [16]. The cell lines were expanded as described elsewhere [19].

Functionality Assays and Flow Cytometry

Interferon γ (IFN-γ), interleukin 17 (IL-17), interleukin 10 (IL-10), and interleukin 4 (IL-4) enzyme-linked immunosorbent spot (ELISPOT) analyses were performed according to the manufacturers' instructions, as published previously [16]. The number of spot-forming cells was counted by an ELISPOT reader (Cellular Technologies).

For proliferation, PBMCs were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) according to the manufacturer's instructions and were stimulated with antigens for 8 or 10 days. Intracellular cytokine (ICC) staining was performed as described elsewhere [16]. Cells were stained with Zombie-Aqua-Fixable-Viability Kit, anti-CD4-PacificBlue, anti-CD8-allophycocyanin, anti-CD3-peridinin chlorophyll protein antibodies (all Biolegend), and, for ICC, anti-IFN-γ-phycoerythrin (PE) antibody (Miltenyi Biotech) and acquired on a BD LSRFortessa system (BD Biosciences). Data were analyzed with FlowJo software vX.0.7.

The *A. fumigatus* Crf1/p41-specific major histocompatibility complex class II tetramer DRB1*0401 FHTYTIDWTKDAVTW, labeled with PE (Beckman Coulter), was used according to the manufacturers' instructions.

Granulocyte macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor α (TNF-α), IL-10, and transforming

growth factor β (TGF-β) levels were determined in culture supernatant after 48 hours stimulation (2×10^4 cells/200 μL/well in 96-well plates) by enzyme-linked immunosorbent assay (all R&D Systems) according to the manufacturers' instructions. The TGF-β level was determined with and without previous activation.

To determine the cross-reactivity to different fungi, immature DCs were coincubated with heat-inactivated fungi overnight and used to stimulate T-cell lines in an effector to target ratio of 10:1 by ELISPOT analysis.

Alloreactivity was determined by [^{3}H]-thymidine incorporation. Autologous PBMCs and antigen-specific T-cell lines (2×10^5 cells/well) were stimulated alone or with 2 different third-party DCs at a responder to stimulator ratio of 10:1 for 96 hours. DNA synthesis was measured as published elsewhere [16]. The counts per minute were determined using a liquid scintillation counter.

Statistical Analysis

Data were analyzed by GraphPad Prism 6.0 (GraphPad Software). The statistical tests used for statistical analyses are indicated in the figure legends. *P* values of $< .05$ were considered statistically significant.

RESULTS

A. fumigatus Lysate and Peptide Pools of Crf1, Gel1, and Pmp20 Induce Similar and Predominant T_h1 Responses

To identify immunogenic *A. fumigatus* antigens, we compared *A. fumigatus* lysate, which complies with GMP, to commercially available Crf1, Gel1, and Pmp20 peptide pools in healthy donors ($n \geq 8$). Most of the donors showed IFN-γ production to at least 2 of 3 tested peptide pools after 7 days of in vitro stimulation, indicating that T cells specific for these antigens are present in the healthy population. The response to peptide pools was comparable to stimulation with *A. fumigatus* lysate, except for Gel1, which had a lower stimulatory capacity. There was only low IL-17, IL-4, and IL-10 production detected, except for *A. fumigatus* lysate, which induced a higher IL-10 response (Figure 1*A*).

Since recent publications indicated that, in addition to $CD4^+$ T_h1 cells, $CD8^+$ T cells induce protective antifungal immune responses [20, 21], we next determined the cellular source of IFN-γ. As for Crf1 [5], most individuals showed a dominant $CD4^+$ T-cell response. Only Pmp20 induced, in 2 of 6 donors, a $CD8^+$ T-cell response comparable to or even higher than the $CD4^+$ T-cell response (Figure 1*B*). To verify these data, we determined T-cell proliferation by CFSE staining, which confirmed the predominance of $CD4^+$ T-cell expansion toward all antigens (Figure 1*C*).

In conclusion, in most healthy donors, the peptide pools Crf1, Gel1, and Pmp20 induced a T_h1 response that was comparable in magnitude and cytokine profile to stimulation with *A. fumigatus* lysate.

Patients With Active Invasive Aspergillosis Respond to *A. fumigatus* Antigens Irrespective of Absolute $CD4^+$ T-Cell Count and Show Expansion Over Time

To clarify whether T cells specific for *A. fumigatus* Crf1, Gel1, and Pmp20 play a role during invasive aspergillosis, we analyzed the IFN-γ response to these antigens in 5 patients with active invasive aspergillosis currently under control by antifungal therapy and in 8 patients without any fungal infection or antimold treatment before or after HSCT (Supplementary Table 1). Samples were obtained from patients 5–19 months after HSCT. Importantly, patients with active invasive aspergillosis showed high IFN-γ responses to all antigens, regardless of absolute $CD4^+$ T-cell counts and GVHD (Figure 2*A*), while patients without fungal infection showed comparable immune responses only with higher $CD4^+$ T-cell counts (Figure 2*B*). IL-17 and IL-4 production was low in both groups (data not shown).

Furthermore, using the previously described HLA-DRB1*04 tetramer, 1 patient with well-controlled invasive aspergillosis experienced expansion of Crf1/p41 tetramer-specific cells from an undetectable level to 1.35% of $CD4^+$ T cells over 10 weeks, which corresponded to an improvement of the pulmonary lesion detected by computed tomography (Figure 2*C*). In patients without invasive aspergillosis, as well as in a patient with invasive aspergillosis with poor outcome, Crf1/p41-specific cells were not detectable (data not shown).

Taken together, T cells specific for Crf1, Gel1, and Pmp20 were detectable in peripheral blood specimens from patients with well-controlled invasive aspergillosis, irrespective of $CD4^+$ T cell recovery and GVHD, and Crf1/p41-specific cells expanded over time, concomitantly improving the fungal lesions, suggesting that these cells may contribute to the control of invasive aspergillosis.

Identification of Immunodominant Epitopes of Gel1 and Pmp20

Because of the promising results, we aimed to identify immunodominant peptide epitopes of these proteins. As peptide epitopes for Crf1 have been previously characterized [5, 7, 12], we focused on Gel1 and Pmp20. The mapping procedure is shown in Supplementary Figure 1. Only 5 of 19 and 6 of 17 healthy donors recognized the most common epitopes for Gel1 and Pmp20, respectively, and the overall responses were lower than responses to the complete peptide pools (Table 1). We therefore decided to use the complete peptide pools instead of single epitopes for further experiments.

A. fumigatus–Specific T Cells Can Be Isolated on the Basis of Activation-Dependent Surface Expression of CD137 and CD154

To generate pathogen-specific T cells for adoptive transfer, rapid isolation methods for direct infusion are needed. We compared the previously described CD154-based selection method

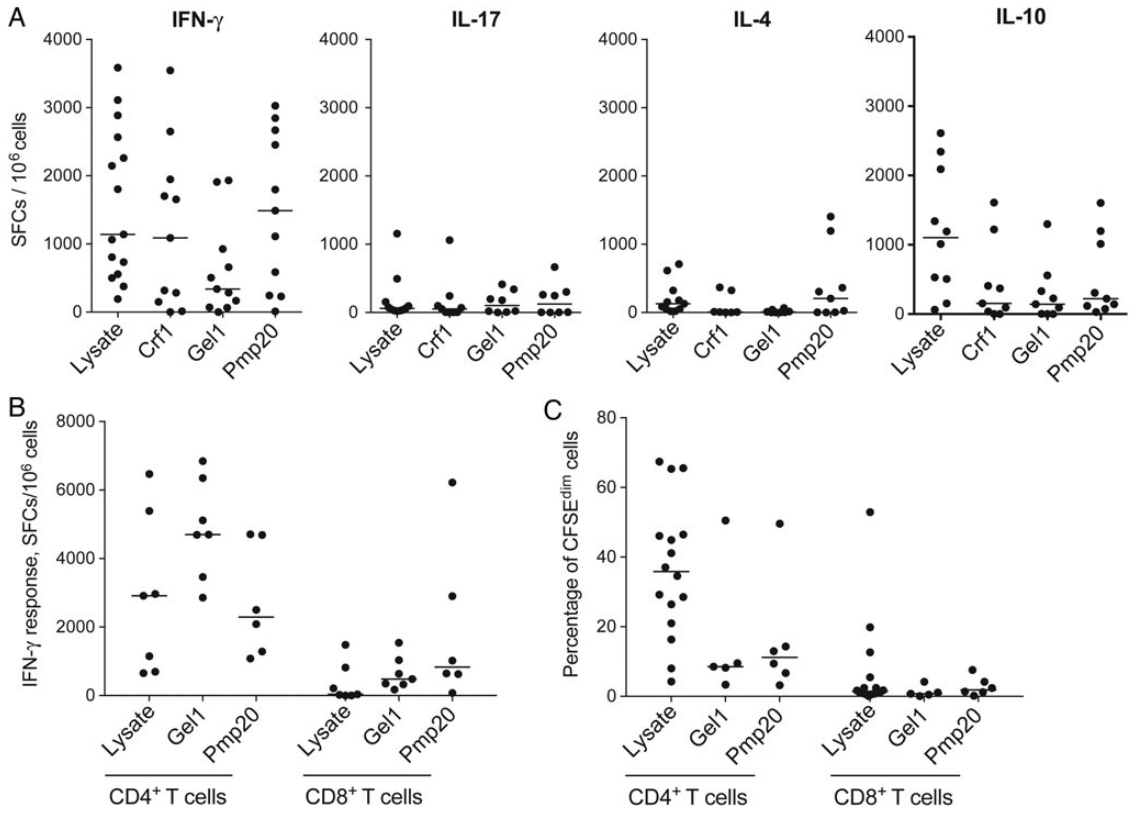

Figure 1. Healthy individuals show similar and predominant T-helper type 1 (T_h1) responses to *Aspergillus fumigatus* lysate and peptide pools. *A*, Peripheral blood mononuclear cells (PBMCs) from healthy donors were prestimulated for 7 days with different *A. fumigatus* antigens, and the interferon γ (IFN-γ), interleukin 17 (IL-17), interleukin 4 (IL-4), and interleukin 10 (IL-10) responses were determined by enzyme-linked immunosorbent spot (ELISPOT) analysis after restimulation. The median IFN-γ responses to *A. fumigatus* lysate, Crf1, Gel1, and Pmp20 were 1140, 1088, 338, and 1488 spot-forming cells (SFCs)/1×10^6 cells, respectively. The median IL-17 and IL-4 responses were ≤126 and ≤205 SFCs/1×10^6 cells, respectively. The median IL-10 response was 1102 SFCs/1×10^6 cells for lysate and ≤221 SFCs/1×10^6 cells for the peptide pools, respectively. Median responses of unstimulated controls for IFN-γ, IL-17, IL-4, and IL-10 were 61 SFCs/1×10^6 cells (range, 0–433 SFCs/1×10^6 cells), 4 SFCs/1×10^6 cells (range, 0–235 SFCs/1×10^6 cells), 0 SFCs/1×10^6 cells (range, 0–293 SFCs/1×10^6 cells), and 42.5 SFCs/1×10^6 cells (range, 0–771 SFCs/1×10^6 cells), respectively ($n \geq 8$). *B*, PBMCs from healthy donors were prestimulated with *A. fumigatus* lysate or Gel1 or Pmp20 peptide pool. After 8 days, $CD4^+$ and $CD8^+$ T-cell subsets were isolated and individually restimulated, and the IFN-γ response was determined by ELISPOT analysis. Median responses of unstimulated controls for $CD4^+$ and $CD8^+$ T cells were 70 SFCs/1×10^6 cells (range, 0–920 SFCs/1×10^6 cells) and 48 SFCs/1×10^6 cells (range, 0–643 SFC/1×10^6 cells), respectively ($n \geq 6$). *C*, Carboxyfluorescein diacetate succinimidyl ester–labeled PBMCs from healthy donors were stimulated with *A. fumigatus* lysate for 8 days or with Gel1 and Pmp20 peptide pool for 10 days. Proliferation of $CD4^+$ and $CD8^+$ T-cell subsets was determined by counterstaining with anti-CD3, anti-CD4, and anti-CD8 antibodies. Median proliferation of unstimulated controls for $CD4^+$ and $CD8^+$ cells was 0.4% (range, 0.1%–1.5%) and 0.6% (range, 0.1%–1.7%), respectively ($n \geq 5$). Data are shown after subtraction of values for the unstimulated control.

to the CD137 selection method, recently judged to be in compliance with GMP.

We performed the selections 14 hours after antigenic stimulation, because surface expression of CD137 and CD154 was highest after 14–24 hours, with decreased viability after 24 hours (data not shown).

T cells specific for *A. fumigatus* lysate, the peptide pools Crf1, Gel1, and Pmp20 or the combination of all 3 peptide pools (C + G + P) were enriched from PBMCs of 4 healthy donors. A median of 1.6×10^5 cells for CD137 and 1.8×10^5 cells for CD154 selection were isolated from 7×10^7 PBMCs, respectively (Supplementary Figure 2*A*). The viability of the selected cells was higher than 95% and similar for both selection methods. There were no differences between *A. fumigatus* lysate and the different peptide pools.

The percentage of cells expressing the activation marker after isolation was significantly higher for CD137, compared with CD154 selection (median, 48.8% and 2.9%, respectively; $P \leq .01$ for all lines; Figure 3*A* and 3*B*). However, part of the CD137 population had probably been enriched unspecifically, because CD137 expression was detected in unstimulated controls on up to 2.5% of lymphocytes (data not shown). As expected, CD154-based selection favored $CD4^+$ T cells (median, 79.8%), whereas after CD137 selection only a median of

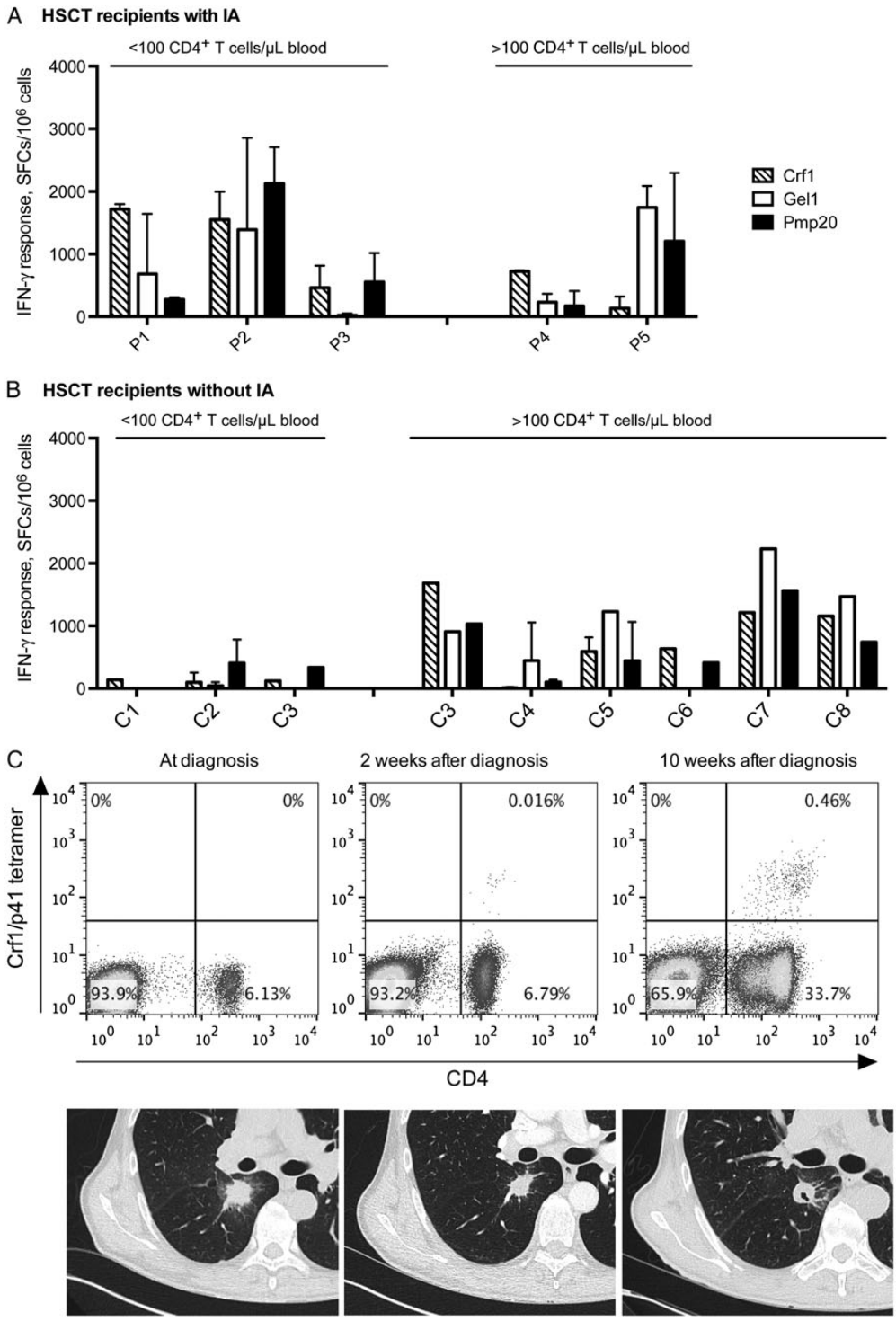

Figure 2. Hematopoietic stem cell transplant (HSCT) recipients with active invasive aspergillosis (IA) respond to *Aspergillus fumigatus* antigens irrespective of absolute $CD4^+$ T-cell counts and Crf1/p41-specific cells show expansion over time. Peripheral blood mononuclear cells (PBMCs) from 5 HSCT recipients with active IA (*A*) and 8 patients without fungal infection (*B*) 5–19 months after HSCT were prestimulated for 7 days with different *A. fumigatus* antigens, and the interferon γ (IFN-γ) response was determined by enzyme-linked immunosorbent spot analysis after restimulation. Median responses of unstimulated controls in patients with active IA and without fungal infection were 63 spot-forming cells (SFCs)/1×10^6 cells (range, 0–448 SFC/1×10^6 cells) and 128 SFC/1×10^6 cells (range, 12–756 SFCs/1×10^6 cells, respectively. Data are shown after subtraction of the unstimulated control ($n \geq 2$ for patients with IA). *C*, PBMCs obtained from a HLA-DRB1*04–positive HSCT recipient at different time points after diagnosis of IA were prestimulated with *A. fumigatus* Crf1/p41 peptide for 7 days, and the frequency of Crf1/p41-specific T cells was determined by tetramer staining. Computed tomography of the chest at the respective time points is shown in the panels below. Patients P1-P5, C1-C3, and C7 developed acute GVHD grade ≥ 2 and were treated with corticosteroids.

Table 1. Epitope Mapping of *Aspergillus fumigatus* Gel1 and Pmp20 Peptide Pools

Positive Donors, No.	Gel1 Epitopes (n = 19)	Pmp20 Epitopes (n = 17)
6	. . .	p21 (VDVVAVLAYNDAYVM)
5	p34 (DVYLQYIFATVDAFA)	p30 (ARFSKSIGWADEEGR) p31 (KSIGWADEEGRTKRY)
4	p38 (YKNTLAFFSGNEVIN)	p7 (KGEITACGIPINYNA) p12 (DKKVILFALPGAFTP) p14 (LPGAFTPVCSARHVP) p22 (AVLAYNDAYVMSAWG) p29 (SDPDARFSKSIGWAD) p34 (KRYALVIDHGKITYA) p40 (NHLEFSSAETVLKHL)
3	p39 (LAFFSGNEVINDGPS) p59 (CDPSSFKTSGWDQKV) p89 (WDVDNDALPAIPEPA)	p9 (IPINYNASKEWADKK) p24 (YVMSAWGKANQVTGD) p35 (LVIDHGKITYAALEP) p36 (HGKITYAALEPAKNH) p37 (TYAALEPAKNHLEFS)

Data are for 19 (Gel1) or 17 (Pmp20) healthy donors. Peripheral blood mononuclear cells were stimulated for 7 days with the complete Gel1 or Pmp20 peptide pools, and the response to different subpools were determined by interferon γ enzyme-linked immunosorbent spot analysis. Only subpools that induced a response of >100 spot-forming cells/1×10^6 cells were subsequently used in a second round of stimulation to determine the response to single peptides. Only responses of >100 spot-forming cells/1×10^6 cells were defined as relevant.

30.7% were $CD4^+$ T cells, 13.7% were $CD8^+$ T cells, and 56.5% were $CD4^-CD8^-$ T cells, including γδ T cells and natural killer cells (Figure 3*C* and data not shown).

In conclusion, CD137- and CD154-based isolation allowed selection of *A. fumigatus* lysate and peptide-specific T cells with similar cell numbers and viability. The percentage of cells expressing CD137 was significantly higher than of CD154, probably because of unspecific enrichment.

Aspergillus-Specific T-Cell Lines Show a Predominant T_h1 Cytokine Profile

The cell populations isolated by CD137 and CD154 expression could be expanded by a median of 22-fold and 27-fold, respectively (Supplementary Figure 2*B*), and the percentage of the different subpopulations remained unchanged for CD154-selected lines but showed an increase of $CD4^+$ T cells after CD137 selection (Figures 3*C* and 4*A*).

The expanded T-cell lines of all donors showed similar specificity, as evaluated by IFN-γ ICC staining, independent of antigen and selection method, despite the higher purity after CD137 selection confirming unspecific expression (Figure 4*B*). In all lines, the response was predominately mediated by $CD4^+$ T cells, except in 2 donors, who showed high $CD8^+$ T-cell responses in CD137-selected Pmp20 and Crf1 + Gel1 + Pmp20-specific lines.

To further characterize the cytokine profile we analyzed IL-17, IL-4, GM-CSF, TNF-α, IL-10, and TGF-β secretion. The cytokine responses were similar for all CD137- and CD154-selected lines, and in addition to IFN-γ, all antigens induced high production of IL-17 and GM-CSF and low production of TNF-α, IL-10, and TGF-β (Figure 4*C* and 4*D* and data not shown). The only difference was the higher IL-4 response in the lines specific for *A. fumigatus* lysate, compared with the other cell lines.

T-Cell Lines Generated With Crf1 + Gel1 + Pmp20 Remain Multispecific

As the combination of all 3 peptide pools could potentially cover a broader repertoire of *Aspergillus*-specific T cells than the single pools, we investigated whether T-cell lines generated with Crf1, Gel1, and Pmp20 were specific to all antigens. Indeed, all multispecific T-cell lines showed specificity for all 3 peptide pools (Figure 4*E*), indicating limited antigenic competition and the feasibility of this combinatory approach.

Generated T-Cell Lines Respond to Naturally Processed Fungi and Cross-react to *Aspergillus* and *Mucorales* Species and *Candida albicans*

We further investigated the ability of the T-cell lines to respond to naturally processed *A. fumigatus*. Overall, multispecific lines showed higher IFN-γ and IL-17 responses to *A. fumigatus* hyphae than single-specific lines, and the CD154-selected lines responded better than the CD137 lines (Figure 5*A* and 5*C*).

As HSCT recipients are highly susceptible to other mold and yeast infections, we challenged the expanded cell lines with clinical isolates of different *Aspergillus* species (*A. flavus*, *A. terreus*, and *A. niger*), *Scedosporium apiospermum, Scedosporium prolificans, Fusarium solani, Rhizopus microsporus, Lichtheimia corymbifera*, and *Candida albicans*. As shown in Figure 5*A* and 5*C*, the CD154-generated multispecific lines showed high IFN-γ and IL-17 responses to all *Aspergillus* species, *Mucorales* species, and *C. albicans*, but responses to *Fusarium* and *Scedosporium* species were not as strong. The CD137-selected cells showed overall lower responses to *A. fumigatus* and other molds. By contrast, all cell lines generated with lysate cross-reacted to all fungi (Figure 5*B* and 5*D*).

In summary, multispecific T-cell lines, particularly those selected by CD154, respond to naturally processed *Aspergillus* species, *Mucorales* species, and *C. albicans* rendering them highly applicable for clinical use.

Alloreactivity of Ex Vivo–Generated T-Cell Lines to Third-Party DCs Is Significantly Reduced

Because adoptive transfer of donor-derived T cells into HSCT recipients may induce GVHD, we assessed their alloreactivity. All lines, regardless of antigen or isolation method, showed significantly reduced alloreactivity to γ-irradiated, partially mismatched third-party DCs, compared with autologous PBMCs (Figure 6).

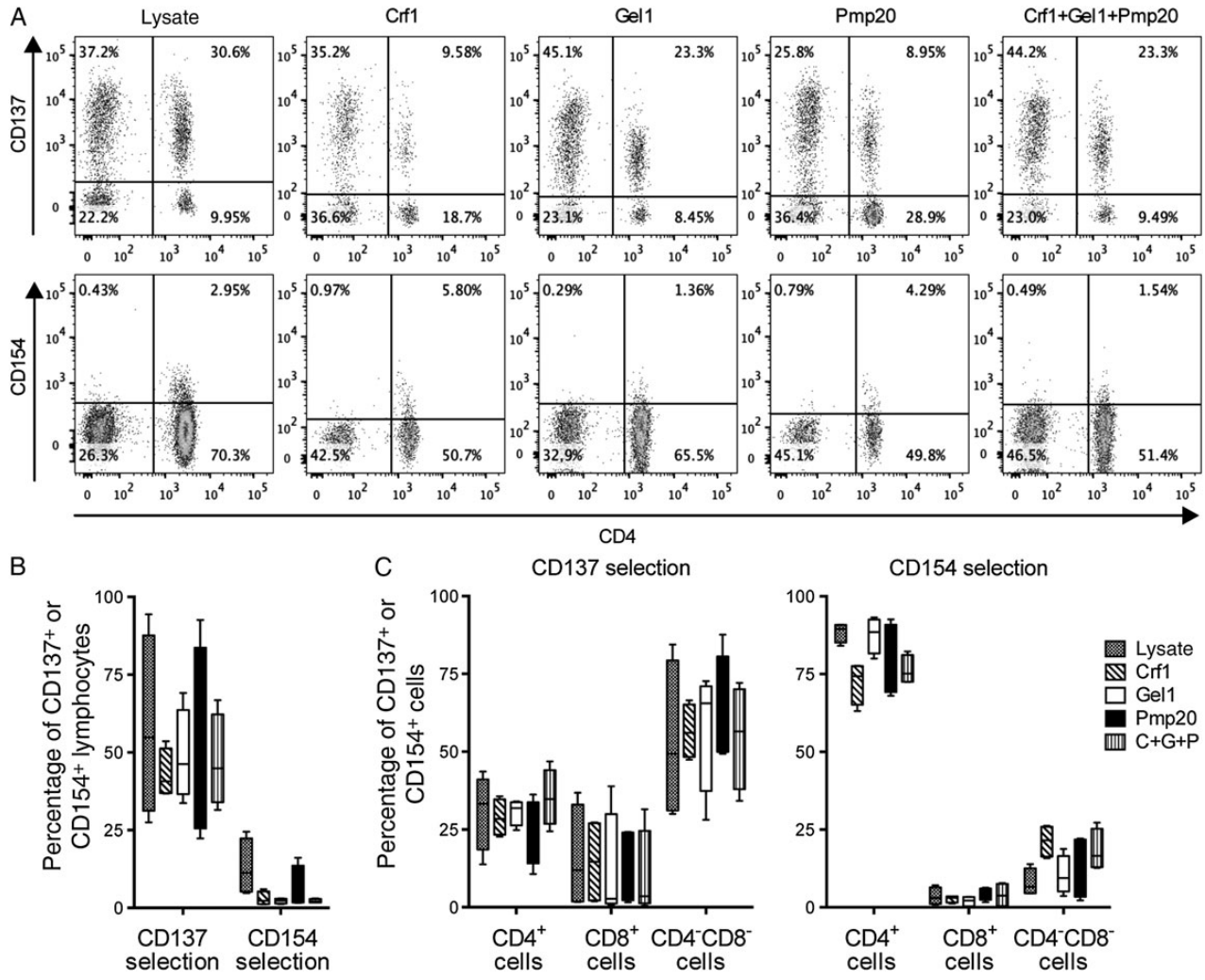

Figure 3. *Aspergillus fumigatus*–specific T cells can be isolated on the basis of activation-dependent expression of CD137 and CD154. *A*, Peripheral blood mononuclear cells (PBMCs) from healthy donors were stimulated for 14 hours with different *A. fumigatus* antigens, and specific cells were isolated by CD137 or CD154 positive selection. Shown are the positive fractions gated on lymphocytes from 1 representative donor (n = 4). *B*, The purity of CD137 and CD154-positive fractions of 5 different antigens (n = 4). The purity of CD137-selected lines is significantly higher than that of CD154-selected lines for all antigens ($P \leq .01$, by 2-way analysis of variance with the Bonferroni multiple comparisons test). *C*, Phenotypic characterization of the isolated $CD137^+$ and $CD154^+$ cells in the positive fractions. The abbreviation "C + G + P" indicates that all 3 antigens—Crf1, Gel1, and Pmp20—were used for stimulation.

DISCUSSION

In this study we show that *A. fumigatus* proteins Crf1, Gel1, and Pmp20 are immunogenic and likely protective by inducing T_h1-cell responses in healthy individuals and patients with active invasive aspergillosis after HSCT. Based on activation-dependent expression of CD137 or CD154, these cells can be selected and show similar specificity. Moreover, the multispecific CD154-selected lines cross-react to the most relevant human-pathogenic molds. Therefore, our results greatly foster adoptive T-cell transfer for these problematic infections.

Clinical application of adoptive T-cell transfer strongly relies on identification of potent and safe fungal antigens. This is challenging, however, owing to the high number of fungal proteins. We focused on Crf1, Gel1, and Pmp20 because previous studies indicated high immunogenicity in healthy individuals or mice [5, 6, 10, 12, 22, 23], commercial availability, and potential cross-reactivity to other human-pathogenic fungi [5, 6]. We detected IFN-γ responses primarily mediated by $CD4^+$ T_h1 cells to at least 2 of 3 peptide pools in all healthy donors. A recent study reported lower responses to Gel1 and Pmp20, compared with Crf1, which may be due to the different experimental setups. But these findings also support interindividual variability of the immune responses [9].

Nevertheless, patients with active invasive aspergillosis responded similarly to these antigens, regardless of $CD4^+$ T-cell

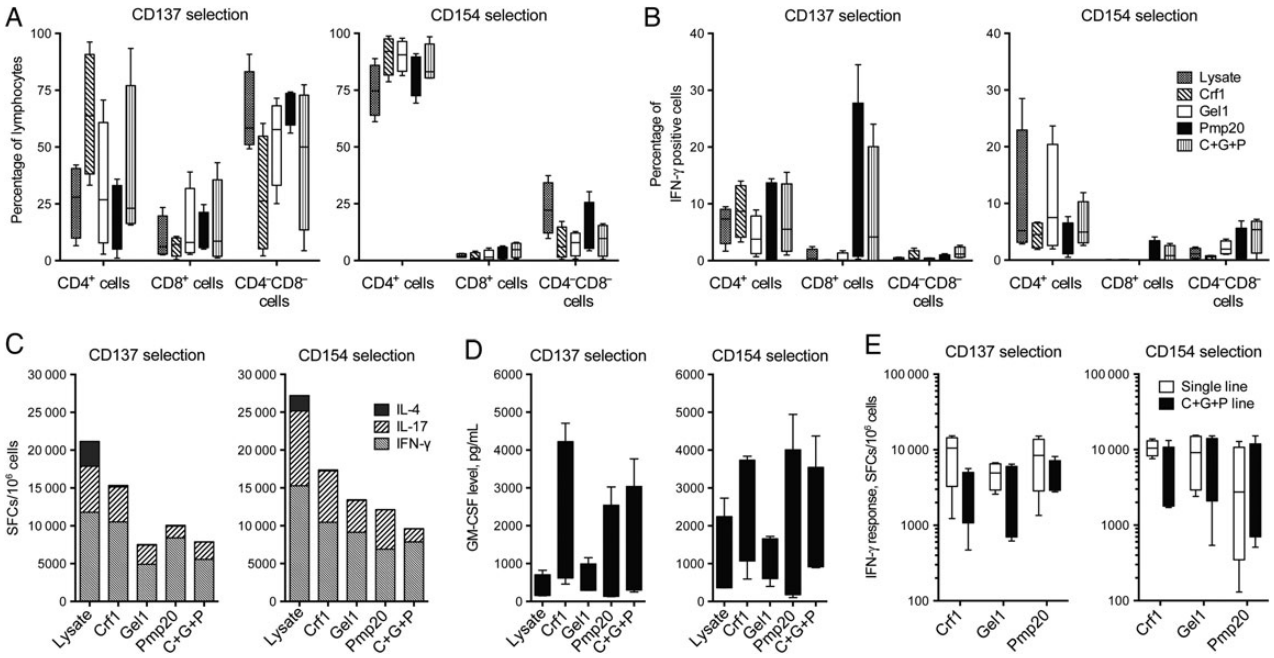

Figure 4. T-cell lines generated by CD137 or CD154 selection show similar cytokine responses after expansion, and Crf1, Gel1, and Pmp20-specific T cells can be simultaneously expanded. *A*, Phenotypic characterization of CD137 or CD154-selected cell lines after 14 days of expansion (n = 4). *B*, The percentage of interferon γ (IFN-γ)–producing cells in the $CD4^+$, $CD8^+$, and $CD4^-CD8^-$ T-cell subsets, determined by intracellular cytokine staining after restimulation of expanded T-cell lines. Median responses of unstimulated controls for $CD4^+$, $CD8^+$, and $CD4^-CD8^-$ T cells were 0.33% (range, 0.03%–4.7%), 0.43% (range, 0%–5.4%), and 0.3% (range, 0%–1.1%), respectively. The expanded cell lines were restimulated with their cognate antigens, and the interferon γ (IFN-γ), interleukin 17 (IL-17), and interleukin 4 (IL-4) response were determined by enzyme-linked immunosorbent spot analysis (*C*), and the GM-CSF response was determined by enzyme-linked immunosorbent assay (*D*). Median responses of unstimulated controls for IFN-γ, IL-17, and IL-4 were 430 spot-forming cells (SFCs)/1×10^6 cells (range, 20–3400 SFCs/1×10^6 cells), 60 SFCs/1×10^6 cells (range, 0–1950 SFCs/1×10^6 cells), and 5 SFCs/1×10^6 cells (range, 0–80 SFCs/1×10^6 cells), respectively, and for GM-CSF, the median response was 7.3 pg/mL (range, 0–51 pg/mL). *E*, Comparison of the specificity of T-cell lines stimulated with single peptide pools or the combination of all 3 peptide pools. Median response of unstimulated controls was 460 SFCs/1×10^6 cells (range, 20–3400 SFCs/1×10^6 cells). Data are shown after subtraction of the unstimulated control. The abbreviation "C + G + P" indicates that all 3 antigens—Crf1, Gel1, and Pmp20—were used for stimulation.

counts and GVHD, and the recovery of Crf1/p41-specific T cells could be longitudinally monitored in a patient with improving fungal lesions. These data are in line with 2 recent publications showing the induction of IFN-γ responses to *A. fumigatus* Crf1 and Gel1 [8, 12] and additionally reveal that Pmp20-specific T cells are also expanding in patients with invasive aspergillosis with a beneficial outcome, suggesting that these peptide-specific T cells help to control the infection. These findings strengthen the use of these candidate antigens for T-cell immunotherapy. We further saw that Pmp20 induced high responses in $CD8^+$ T cells in some donors, which could be particularly relevant for HSCT recipients early during immune recovery, when $CD4^+$ T-cell reconstitution is frequently delayed [24].

To date, there is scarce knowledge about the required T-cell epitopes and their HLA coverage that induce high numbers of polyclonal $CD4^+$ and $CD8^+$ cells effective against mold infections. Similar to the approach used in the development of Epstein-Barr virus–specific T cells [14], we intended to generate a mixed peptide pool containing the most immunogenic epitopes from different *A. fumigatus* proteins. However, as compared to the previously identified Crf1 epitopes in humans [5, 7, 25], we did not find any highly immunogenic T-cell epitopes for Pmp20 and Gel1.

The generation of a T-cell product for adoptive transfer should be fast and simple, to be administered in a timely manner to patients in need. To avoid ex vivo expansion, we compared the CD154 method to the CD137 selection method, which was recently found to comply with GMP but has not yet been used in a clinical setting. The isolated cell number, viability, expansion potential, cytokine profile, and alloreactivity were comparable between all cell lines and independent of the selection method or the antigen. Importantly, our T-cell lines stimulated with all 3 peptide pools remained multispecific. This is in line with our previous findings, in which activation-dependent expression of CD154 allowed selection of T cells specific for 3 viral and 2 fungal pathogens [16]. We isolated a median of 0.25% of the starting cell fraction. A clinical scale stimulation starting with 1×10^9 PBMCs would therefore yield

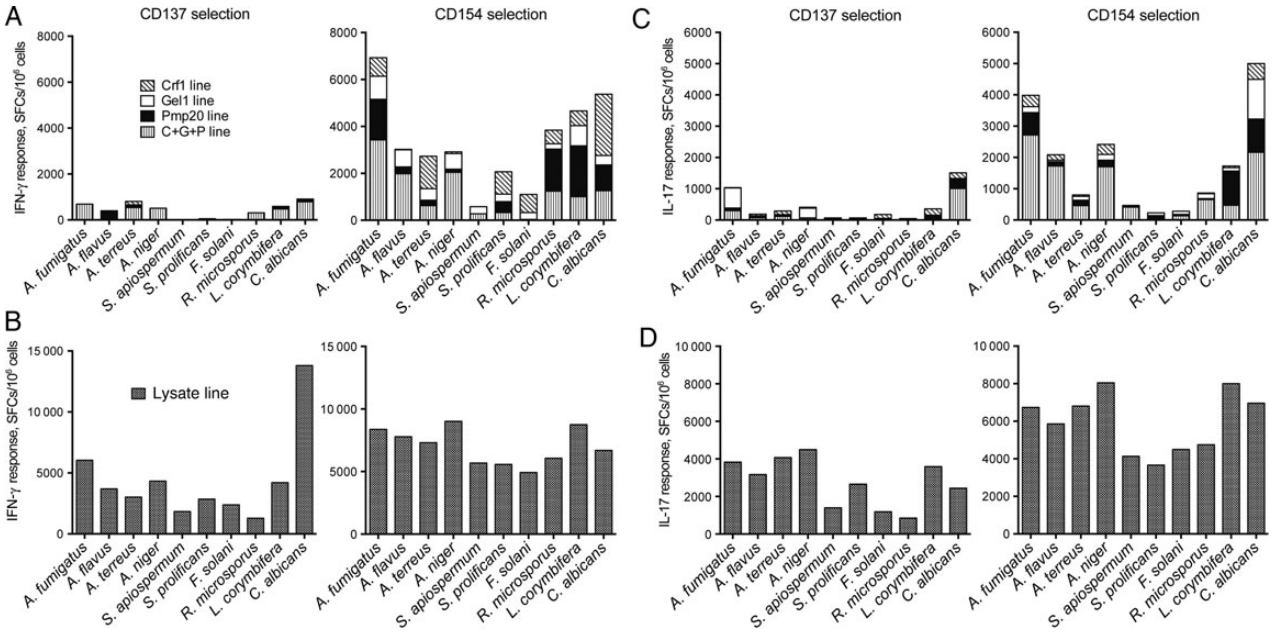

Figure 5. The interferon γ (IFN-γ) and interleukin-17 (IL-17) response of T-cell lines generated by CD137 and CD154 selection to different molds and yeast. The IFN-γ (*A* and *B*) and interleukin 17 (IL-17; *C* and *D*) responses of peptide pool–specific (*A* and *C*) or lysate-specific (*B* and *D*) T-cell lines after stimulation with different heat-inactivated molds and yeast were determined by enzyme-linked immunosorbent spot analysis. Median responses of unstimulated controls for IFN-γ and IL-17 were 430 spot-forming cells (SFCs)/1×10^6 cells (range, 20–3400 SFCs/1×10^6 cells) and 60 SFCs/1×10^6 cells (range, 0–1950 SFCs/1×10^6 cells), respectively. The median responses are shown after subtraction of values for the unstimulated controls (n = 4). Abbreviations: *A. flavus*, *Aspergillus flavus*; *A. fumigatus*, *Aspergillus fumigatus*; *A. niger*, *Aspergillus niger*; *A. terreus*, *Aspergillus terreus*; *C. albicans*, *Candida albicans*; *F. solani*, *Fusarium solani*; *L. corymbifera*, *Lichtheimia corymbifera*; *R. microsporus*, *Rhizopus microsporus*; *S. apiospermum*, *Scedosporium apiospermum*; *S. prolificans*, *Scedosporium prolificans*.

around 2×10^6–3×10^6 cells, which is comparable to the numbers used for antiviral T-cell transfer [13, 26, 27], suggesting that the number of isolated $CD137^+$ or $CD154^+$ T cells could be enough for the reconstitution of fungus-specific immunity

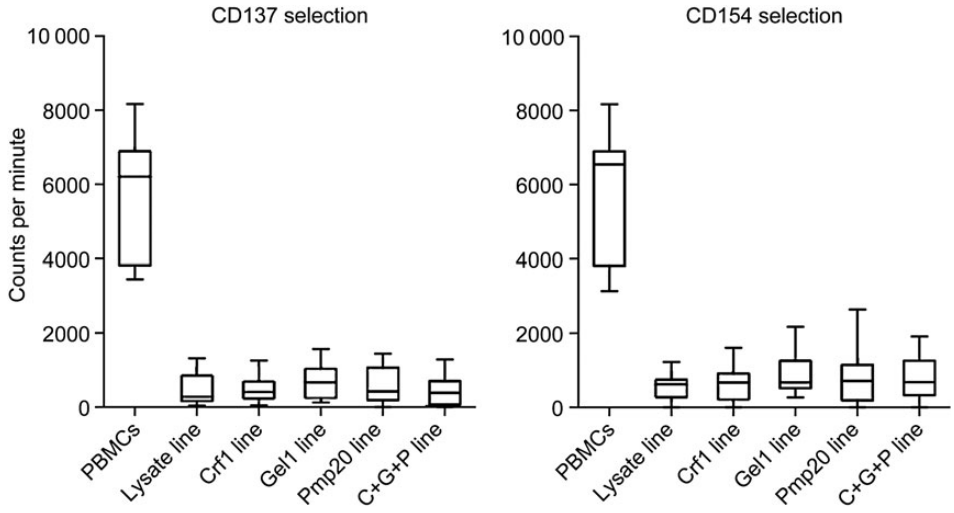

Figure 6. *Aspergillus fumigatus*–specific T-cell lines have significantly reduced alloreactivity. CD137 or CD154-selected cell lines or autologous peripheral blood mononuclear cells (PBMCs) were coincubated with γ-irradiated, partially mismatched third-party dendritic cells, and the alloreactivity was determined on the basis of ^{3}H-thymidine incorporation in proliferating cells ($P \leq .01$ for all lines, by 2-way analysis of variance with the Dunn multiple comparisons test). Median responses of unstimulated controls for PBMC and cell lines were 751 counts per minute (range, 336–1043 counts per minute) and 473 counts per minute (range, 272–1294 counts per minute), respectively. Data are shown after subtraction of values for the unstimulated control (n = 14).

without the need of ex vivo expansion. These cells could be particularly suitable for lymphopenic patients after neutrophil engraftment, to support the effector functions of the neutrophils and macrophages to prevent or treat invasive aspergillosis [28].

In line with the protective immunity of T_h1 cells, the cytokine profile of CD137 and CD154-selected cell lines was dominated by the T_h1 cytokines IFN-γ and GM-CSF. The cell lines additionally produced IL-17, which is in general regarded as an important cytokine in antifungal immunity [28]. In vitro–stimulated PBMCs showed very low IL-17 production, indicating that the culture conditions during expansion probably favored proliferation of the T_h17 subset. Direct infusion of the isolated cells should circumvent this imbalance. We furthermore excluded the presence of regulatory T cells, as previous results have postulated their enrichment after CD137 selection [29, 30], and of T_h2 cells, which are associated with antiinflammatory responses [23, 28].

The percentage of cells expressing the activation marker was significantly higher for CD137 selection than for CD154 selection, with median values of 48.8% and 2.9%, respectively. This is probably due to low expression and fast downregulation of CD154 leading to an underestimation of positive cells [16] and to unspecific expression of CD137 causing overestimation, indicating that activation marker expression cannot be equated with antigen specificity. Compared with CD154 selection, which strongly favors enrichment of $CD4^+$ T cells [17], CD137-selected cells were more diverse and additionally included high numbers of natural killer cells and γδ T cells. These cells are increasingly recognized to contribute to antifungal immunity [31–36], and recent studies in patients with solid tumors or hematological malignancies demonstrated their safety [37].

In the last decade, infections with other *Aspergillus* and *Mucorales* species have increasingly been reported [38–40]. We have previously shown that $CD4^+$ T cells specific for the *A. fumigatus* Crf1/p41 epitope confer cross-reactivity to *C. albicans* [5], and a potential cross-reactivity of Gel1 was suggested in a mouse model [6]. In particular, T-cell lines specific for all 3 antigens and selected by CD154 expression not only efficiently recognized naturally processed *A. fumigatus*, but also cross-reacted to different clinically relevant *Aspergillus* species and the *Mucorales* species, *Rhizopus microsporus*, and *Lichtheimia corymbifera*, suggesting that adoptively transferred T cells could very likely protect the recipients against a variety of fungal infections. It remains unclear, however, which epitopes confer cross-reactivity, as BLAST analysis indicated only limited genetic similarity between these fungi.

Compared with the peptide pools, *A. fumigatus* lysate–specific lines cross-reacted to all tested molds and yeast. This may be explained by the fact that lysate contains various cell wall components that are less diverse between different fungal species, compared with protein antigens [41]. On the other hand, some cell wall components, such as galactosaminogalactan, are able to actively inhibit the induction of protective responses [42]. This might also explain the high level of IL-10 secretion in PBMCs stimulated with fungal lysate, which could be mediated by T cells as well as monocytes, and the higher IL-4 response in lysate-specific as compared to peptide-specific lines. The immune response induced by lysate is therefore less predictable and could potentially reduce the efficacy of a T-cell product. By contrast, the combination of all 3 peptide pools is highly suitable for the generation of mold-specific T cells, because it probably covers a wider range of HLA types, induces higher numbers of polyclonal $CD4^+$ and $CD8^+$ T cells, confers cross-reactivity to different fungi, and appears to be safe.

In conclusion, T cells specific for *A. fumigatus* Crf1, Gel1, and Pmp20 selected by activation-dependent CD137 or CD154 expression may form the basis for adoptive T-cell transfer for prophylaxis in high-risk patients or for treatment in patients with refractory mold infections.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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C. S., J. N., and C. B. performed the experiments; J. P. provided the patient samples; C. S. and N. K. designed the research; and C. S., M. B., M. T., and N. K. wrote the manuscript.

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Potential conflicts of interests. All authors: No reported conflicts.

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References

1. Gooley TA, Chien JW, Pergam SA, et al. Reduced mortality after allogeneic hematopoietic-cell transplantation. N Engl J Med **2010**; 363:2091–101.
2. Marr KA. Fungal infections in hematopoietic stem cell transplant recipients. Med Mycol **2008**; 46:293–302.
3. Perruccio K, Tosti A, Burchielli E, et al. Transferring functional immune responses to pathogens after haploidentical hematopoietic transplantation. Blood **2005**; 106:4397–406.
4. Nierman WC, Pain A, Anderson MJ, et al. Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. Nature **2005**; 438:1151–6.

5. Stuehler C, Khanna N, Bozza S, et al. Cross-protective TH1 immunity against *Aspergillus fumigatus* and Candida albicans. Blood **2011**; 117:5881–91.
6. Bozza S, Clavaud C, Giovannini G, et al. Immune sensing of *Aspergillus fumigatus* proteins, glycolipids, and polysaccharides and the impact on Th immunity and vaccination. J Immunol **2009**; 183:2407–14.
7. Jolink H, Meijssen IC, Hagedoorn RS, et al. Characterization of the T-cell-mediated immune response against the *Aspergillus fumigatus* proteins Crf1 and catalase 1 in healthy individuals. J Infect Dis **2013**; 208:847–56.
8. Potenza L, Vallerini D, Barozzi P, et al. Characterization of specific immune responses to different Aspergillus antigens during the course of invasive Aspergillosis in hematologic patients. PLoS One **2013**; 8: e74326.
9. Bacher P, Kniemeyer O, Teutschbein J, et al. Identification of immunogenic antigens from *Aspergillus fumigatus* by direct multiparameter characterization of specific conventional and regulatory CD4+ T cells. J Immunol **2014**; 193:3332–43.
10. Chaudhary N, Staab JF, Marr KA. Healthy human T-Cell Responses to *Aspergillus fumigatus* antigens. PLoS One **2010**; 5:e9036.
11. Diaz-Arevalo D, Bagramyan K, Hong TB, Ito JI, Kalkum M. CD4+ T cells mediate the protective effect of the recombinant Asp f3-based anti-aspergillosis vaccine. Infect Immun **2011**; 79:2257–66.
12. Jolink H, Hagedoorn RS, Lagendijk EL, et al. Induction of *A. fumigatus*-specific CD4+ T-cells in patients recovering from invasive aspergillosis. Haematologica **2014**; 99:1255–63.
13. Feuchtinger T, Opherk K, Bethge WA, et al. Adoptive transfer of pp65-specific T cells for the treatment of chemorefractory cytomegalovirus disease or reactivation after haploidentical and matched unrelated stem cell transplantation. Blood **2010**; 116:4360–7.
14. Moosmann A, Bigalke I, Tischer J, et al. Effective and long-term control of EBV PTLD after transfer of peptide-selected T cells. Blood **2010**; 115:2960–70.
15. Peggs KS, Thomson K, Samuel E, et al. Directly selected cytomegalovirus-reactive donor T cells confer rapid and safe systemic reconstitution of virus-specific immunity following stem cell transplantation. Clin Infect Dis **2011**; 52:49–57.
16. Khanna N, Stuehler C, Conrad B, et al. Generation of a multipathogen-specific T-cell product for adoptive immunotherapy based on activation-dependent expression of CD154. Blood **2011**; 118:1121–31.
17. Frentsch M, Arbach O, Kirchhoff D, et al. Direct access to CD4+ T cells specific for defined antigens according to CD154 expression. Nat Med **2005**; 11:1118–24.
18. Wolfl M, Kuball J, Ho WY, et al. Activation-induced expression of CD137 permits detection, isolation, and expansion of the full repertoire of CD8+ T cells responding to antigen without requiring knowledge of epitope specificities. Blood **2007**; 110:201–10.
19. Beck O, Topp MS, Koehl U, et al. Generation of highly purified and functionally active human TH1 cells against *Aspergillus fumigatus*. Blood **2006**; 107:2562–9.
20. Carvalho A, De Luca A, Bozza S, et al. TLR3 essentially promotes protective class I-restricted memory CD8(+) T-cell responses to *Aspergillus fumigatus* in hematopoietic transplanted patients. Blood **2012**; 119:967–77.
21. Nanjappa SG, Heninger E, Wuthrich M, Sullivan T, Klein B. Protective antifungal memory CD8(+) T cells are maintained in the absence of CD4(+) T cell help and cognate antigen in mice. J Clin Invest **2012**; 122:987–99.
22. Bozza S, Gaziano R, Lipford GB, et al. Vaccination of mice against invasive aspergillosis with recombinant Aspergillus proteins and CpG oligodeoxynucleotides as adjuvants. Microbes Infect **2002**; 4:1281–90.
23. 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.
24. Seggewiss R, Einsele H. Immune reconstitution after allogeneic transplantation and expanding options for immunomodulation: an update. Blood **2010**; 115:3861–8.
25. Ramadan G, Davies B, Kurup VP, Keever-Taylor CA. Generation of Th1T cell responses directed to a HLA Class II restricted epitope from the Aspergillus f16 allergen. Clin Exp Immunol **2005**; 139: 257–67.
26. Feuchtinger T, Matthes-Martin S, Richard C, et al. Safe adoptive transfer of virus-specific T-cell immunity for the treatment of systemic adenovirus infection after allogeneic stem cell transplantation. Br J Haematol **2006**; 134:64–76.
27. Icheva V, Kayser S, Wolff D, et al. Adoptive transfer of epstein-barr virus (EBV) nuclear antigen 1-specific t cells as treatment for EBV reactivation and lymphoproliferative disorders after allogeneic stem-cell transplantation. J Clin Oncol **2013**; 31:39–48.
28. Romani L. Immunity to fungal infections. Nat Rev Immunol **2011**; 11:275–88.
29. Goldstein MJ, Kohrt HE, Houot R, et al. Adoptive cell therapy for lymphoma with CD4T cells depleted of CD137-expressing regulatory T cells. Cancer Res **2012**; 72:1239–47.
30. Snell LM, Lin GH, McPherson AJ, Moraes TJ, Watts TH. T-cell intrinsic effects of GITR and 4–1BB during viral infection and cancer immunotherapy. Immunol Rev **2011**; 244:197–217.
31. Bouzani M, Ok M, McCormick A, et al. Human NK cells display important antifungal activity against *Aspergillus fumigatus*, which is directly mediated by IFN-gamma release. J Immunol **2011**; 187:1369–76.
32. Dejima T, Shibata K, Yamada H, et al. Protective role of naturally occurring interleukin-17A-producing gammadelta T cells in the lung at the early stage of systemic candidiasis in mice. Infect Immun **2011**; 79:4503–10.
33. Fenoglio D, Poggi A, Catellani S, et al. Vdelta1T lymphocytes producing IFN-gamma and IL-17 are expanded in HIV-1-infected patients and respond to Candida albicans. Blood **2009**; 113:6611–8.
34. Ramadan G. In vitro expansion of human gammadelta and CD56(+) T-cells by Aspergillus-antigen loaded fast dendritic cells in the presence of exogenous interleukin-12. Immunopharmacol Immunotoxicol **2012**; 34:309–16.
35. Schmidt S, Tramsen L, Hanisch M, et al. Human natural killer cells exhibit direct activity against *Aspergillus fumigatus* hyphae, but not against resting conidia. J Infect Dis **2011**; 203:430–5.
36. van de Veerdonk FL, Netea MG. T-cell subsets and antifungal host defenses. Curr Fungal Infect Rep **2010**; 4:238–43.
37. Locatelli F, Merli P, Rutella S. At the Bedside: Innate immunity as an immunotherapy tool for hematological malignancies. J Leukoc Biol **2013**; 94:1141–57.
38. Kontoyiannis DP, Marr KA, Park BJ, et al. Prospective surveillance for invasive fungal infections in hematopoietic stem cell transplant recipients, 2001–2006: overview of the Transplant-Associated Infection Surveillance Network (TRANSNET) Database. Clin Infect Dis **2010**; 50:1091–100.
39. Roden MM, Zaoutis TE, Buchanan WL, et al. Epidemiology and outcome of zygomycosis: a review of 929 reported cases. Clin Infect Dis **2005**; 41:634–53.
40. Skiada A, Pagano L, Groll A, et al. Zygomycosis in Europe: analysis of 230 cases accrued by the registry of the European Confederation of Medical Mycology (ECMM) Working Group on Zygomycosis between 2005 and 2007. Clin Microbiol Infect **2011**; 17:1859–67.
41. van de Veerdonk FL, Kullberg BJ, van der Meer JW, Gow NA, Netea MG. Host-microbe interactions: innate pattern recognition of fungal pathogens. Curr Opin Microbiol **2008**; 11:305–12.
42. Gresnigt MS, Bozza S, Becker KL, et al. A polysaccharide virulence factor from *Aspergillus fumigatus* elicits anti-inflammatory effects through induction of Interleukin-1 receptor antagonist. PLoS Pathog **2014**; 10: e1003936.