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TLR3 essentially promotes protective class I-restricted memory CD8$^+$ T cell responses to *Aspergillus fumigatus* in hematopoietic transplanted patients

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Running title: TLR3 activates CD8$^+$ T cells in aspergillosis

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Abstract

Aspergillus fumigatus is a model fungal pathogen and a common cause of severe infections and diseases. $CD8^+$ T cells are present in the human and murine T cell repertoire to the fungus. However, $CD8^+$ T function in infection as well as the molecular mechanisms that control their priming and differentiation into effector and memory cells in vivo remains elusive. In this study, we report that both $CD4^+$ and $CD8^+$ T cells mediate memory protective responses to the fungus contingent upon the nature of the fungal vaccine. Mechanistically, class I MHC-restricted, $CD8^+$ memory T cells were activated through TLR3 sensing of fungal RNA by cross-presenting dendritic cells. Genetic deficiency of TLR3 was associated with susceptibility to aspergillosis and concomitant failure to activate memory protective $CD8^+$ T cells both in mice and in stem cell-transplanted patients. Thus, TLR3 essentially promotes antifungal memory $CD8^+$ T cell responses and its deficiency is a novel susceptibility factor for aspergillosis in high-risk patients.

Introduction

Toll-like receptor 3 (TLR3) plays a key role in modulating inflammation and innate immunity in the airway. Although best known for recognition of viral double stranded RNA (dsRNA) and its synthetic analog polyinosinic:polycytidylic acid [poly(I:C)],[1] TLR3 also recognizes endogenous ligands[2] including heterologous RNA released from or associated with necrotic cells or generated by in vitro transcription.[3] Thus, TLR3, together with other intracellular signaling proteins,[4] induces or otherwise modulates innate immune responses and inflammation in settings that are not associated with viral dsRNA. TLR3 signaling may also modulate adaptive immune responses by providing cross-priming of cytotoxic T lymphocytes through signaling in DCs[5-6] via type I IFNs[7] and in the absence of $CD4^+$ T cell help.[8] Thus, it is not surprising that individuals with mutations in key TLR3 signaling components have a selective immunodeficiency manifested by recurrent episodes of Herpes simplex virus 1 encephalitis[9-10] or enteroviral myocarditis/cardiomyopathy.[11]

Aspergillus fumigatus is a model fungal pathogen and a common cause of severe infections and diseases. Humans inhale hundreds of conidia everyday without adverse consequences,[12] except for a minority of persons in whom defense systems fail and a life-threatening form of disease can develop. $CD4^+$ and $CD8^+$ T cells are present in the human T cell repertoire to the fungus[13-15] and adoptive transfer of *A. fumigatus*-specific $CD4^+$ T cells conferred protection against invasive fungal infection.[15-16] Recent studies indicate a role for TLR3 in murine aspergillosis. By functioning as an endogenous sensor of fungal RNA,[17] TLR3 mediates expression of the enzyme indoleamine 2,3-dioxygenase (IDO) on both epithelial[18] and DCs,[19] contributing to the local regulation of innate and adaptive inflammation to the fungus. However, the findings that protective memory $CD8^+$ T cells are induced against fungi[20-23] suggest a possible role for TLR3 in activating memory $CD8^+$ T cell-mediated immunity to the fungus.

We evaluated the contribution of TLR3 to the activation of $CD8^+$ T cells to *A. fumigatus* in preclinical models of aspergillosis and in hematopoietic stem cell transplanted (HSCT) patients with a single nucleotide polymorphism (SNP) in *TLR3*. We found that TLR3 on murine and human DCs sorts fungal RNA for activation of class I-restricted protective memory $CD8^+$ T cell responses to the fungus. TLR3 deficiency was associated with severe infection in mice and a *TLR3* SNP resulting in a loss-of-function phenotype of DCs was associated with increased susceptibility to aspergillosis and concomitant failure to activate antifungal $CD8^+$ T cells in HSCT patients.

Materials and methods

Mice

Female C57BL/6 mice (Charles River, Calco, Italy) and homozygous *Tlr3*$^{-/-}$ mice on the C57BL/6 background were bred under pathogen-free conditions in the Animal Facility of the University of Perugia, Perugia, Italy and experiments were performed according to the Italian Approved Animal Welfare Assurance A-3143-01.

Fungal and bacterial strains, infections and treatments

Viable resting and swollen conidia from the *A. fumigatus* Af293 strain were obtained as described.[19] A GFP-expressing strain of *A. fumigatus* (provided by M.M. Moore, Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia, Canada) was used to track viable fungi. Infection and bronchoalveolar lavage (BAL) morphometry (at least 200 cells per cytospin preparation were counted) were performed as described.[19] Mice were monitored for fungal growth (CFU/organ, mean ± SE), histopathology [periodic acid-Schiff (PAS) and Gomori's methenamine silver staining of lung tissue sections] and lung immunofluorescence (see below). Histology sections and cytospin preparations were observed using a BX51 microscope (Olympus, Milan, Italy) and images were captured using a high-resolution DP71 camera (Olympus). Poly(I:C) (Sigma-Aldrich, St. Louis, MO) was given (50 μg) twice i.p. the day of and a day after infection.

Control mice received PBS. Mice were treated with 300 μg of anti-CD4 (GK 1.5) or anti-CD8 (YTS 169) mAbs (provided by L. Boon, Bioceros BV, Netherlands) the day before and one and three days after infection. Control mice received isotype control rat IgG2a mAb (eBR2a) (eBioscience, San Diego, CA). Depletion of the corresponding T cell subsets with this regimen was monitored in each experiment and was consistently between 95 to 98%, lasting for at least 3 days after treatment (data not shown). Mice were infected i.n. with the *Pseudomonas aeruginosa* PAO1 (ATCC BAA-47) strain. Bacterial CFU/organ (mean ± SE) were quantified by plating lung homogenates on trypticase soy agar plates.

Vaccination models

Two vaccination models were used, one using live *A. fumigatus* conidia or purified fungal antigens given with murine CpG oligodeoxynucleotide 1862, the other using fungus-pulsed DCs.[24] In the first model, mice were injected i.n. with 2×10^7 conidia/20 μl saline 14 days before the infection or with 5 μg of the *A. fumigatus* cell wall glucanase Crf1p, known to induce MHC class II-restricted memory antifungal $CD4^+$ Th1 responses[13,25] together with 10 nM CpG in 20 μl saline administered 14, 7 and 3 days before the infection. Mice were immunosuppressed i.p. with 150 mg/kg of cyclophosphamide a day before the infection. In the adoptive transfer model, purified lung $CD11c^+$ DCs were pulsed with viable resting conidia or fungal RNA together with the cationic lipid N-[1-(2,3-dioleoyloxypropyl]-N,N,N,-trimethylammonium methylsulfate (DOTAP) (Boehringer Mannheim, Mannheim, Germany) in serum Opti-MEM medium (Life Technologies) for 2 h, as described.[26] For total RNA isolation, mixing with DOTAP and detection inside the cells, see Supplemental Materials. DCs were adoptively transferred by i.p. injection (5×10^5 cells/each injection) twice, a week apart, before the i.n. infection. For vaccination with fungal RNA, 10 μg fungal RNA + 20 μg DOTAP were administered i.n. three times as above before the infection. A total of 300 μg of anti-CD4, anti-CD8, anti-MHC class I-A (34-5-8S), anti-MHC class II (M5/114)

mAbs (provided by L. Boon) or control rat IgG2a mAb was administered in concomitance with the vaccines.

Fluorescence microscopy and morphometric analysis

Mice were perfused with 2 ml of cold PBS and 1:1 OCT (Cryomount Histolab, Gothenburg, Sweden). The lung was snap frozen in OCT and 5-µm sections were performed with a semiautomatic cryostat (MC4000; Histo-Line Laboratories, Milan, Italy). Rehydrated sections were fixed in 4% paraformaldehyde, blocked with 5% BSA and stained with a combination of Alexa Fluor 488 anti-mouse CD11c (N418, BioLegend, Campoverde Srl, Milan, Italy) and anti-mouse CD3 (17A2, BioLegend) mAbs at room temperature for 1h. 4'-6-Diamino-2-phenylindole (DAPI, Molecular Probes, Invitrogen, Milan, Italy) was used to counterstain tissues and to detect nuclei. Images were acquired using a fluorescence microscope (BX51 Olympus) with a 40× objective and the analySIS image processing software (Olympus).

Generation of bone marrow chimeras

Bone marrow chimeras were generated as described[18] (detailed in the Supplemental Materials).

Cell preparation and cultures

Murine lung cells were isolated as described[24] (detailed in the Supplemental Materials). $CD4^+$ or $CD8^+$ T cells were isolated after incubation with FITC-labeled anti-CD4 or anti-CD8, followed by anti-FITC MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and DCs were isolated with MicroBeads (Miltenyi Biotec) conjugated to hamster anti-mouse CD11c mAbs (N418) before magnetic cell sorting.[24] Upon written informed consent, human PBMCs were isolated using Histopaque®-1077 (Sigma-Aldrich) according to manufacturer's instructions. Human $CD141^+$ $(BDCA\text{-}3)^+$ DCs were isolated from total PBMCs using the CD141 (BDCA-3) MicroBead Kit (Miltenyi Biotec) according to manufacturer's instructions (see the Supplemental Materials for purity and phenotypic analysis). Murine or human cells (10^6 cells/ml) were pulsed with poly(I:C)

(50 μg/ml), viable conidia of *A. fumigatus* (5:1 cell:fungus ratio), fungal RNA (10 μg) + DOTAP (20 μg) or DOTAP alone for 18 h before RNA extraction and flow cytometry. Amphotericin B (Sigma-Aldrich) (2.5 μg/ml) was added after 2 h of pulsing to prevent fungal overgrowth.

Flow cytometry

Directly conjugated Abs were purchased from BD Pharmingen (San Diego, CA) (see Supplemental Materials for details). Cells were analyzed with a FACScan flow cytofluorometer (Becton Dickinson, San Jose, CA) equipped with CellQuest^{TM} software.

Uptake tracking of GFP-expressing *A. fumigatus* in vivo

Single cell suspensions were prepared from lungs or draining lymph nodes depleted of T and B cells. Cells were immunostained with the CD11c markers and analyzed by flow cytometry. For the phagocytosis assay, see Supplemental Materials.

Antigen presentation in vitro, cytotoxic and conidiocidal assays

A panel of Ag presentation inhibitors (all from Sigma-Aldrich) at their optimum concentrations was added to DCs for 120 min prior to the 2 h pulsing with *A. fumigatus* conidia or RNA + DOTAP. We used 5 μg/ml chloroquine, 100 nM bafilomycin A, 5 μg/ml brefeldin A and 50 μM lactacystin. After pulsing, DCs were fixed in 0.4% paraformaldehyde (Sigma-Aldrich) and extensively washed. DCs (1×10^5) were incubated with $CD4^+$ or $CD8^+$ T cells (5×10^5), purified from lungs a week after infection for lymphoproliferation (after 72 h incubation), gene expression, supernatant collection and cytolytic activity (after 24 h incubation). DNA synthesis was measured by ^{3}H-thymidine labeling (Amersham Biosciences, Milan, Italy) for 6 h. Cytolytic activity was tested using a standard ^{51}Cr-release assay[15] (see Supplemental Materials for details). Live or heat-inactivated (85°C for 30 min) conidia of *A. fumigatus* were labeled with FUN-1 (5 μM, Molecular Probes, Eugene, OR). Because lymphocytes take up FUN-1, cells could not be added directly in this assay. FUN-1-stained live conidia were incubated overnight in culture medium alone or with medium

from naïve $CD8^+$ T cells or cells from infected mice either unexposed or exposed to DCs pulsed with conidia or fungal RNA and were examined under fluorescence microscopy. Metabolically active conidia accumulate orange fluorescence in vacuoles, while dormant and dead conidia stain green.

Proliferative activity of human T lymphocyte clones

Limiting dilution and proliferation assays were performed as described[15] (detailed in the Supplemental Materials).

Western blotting

Blots of cell lysates were incubated with rabbit polyclonal Abs recognizing phospho-IRF3 (Ser396, 4D4G) followed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG, as per the manufacturer's instruction (Cell Signaling Technology, Danvers, MA). Immunoblotting for IDO was performed with rabbit polyclonal IDO-specific antibody on total lung cells.[18] Scanning densitometry was done on a Scion Image apparatus. The pixel density of bands was normalized against total protein or β-tubulin.

ELISA and real-time PCR

The level of cytokines in culture supernatants of DCs was determined by Kit ELISA (R&D Systems, Milan, Italy) at 24 h. The detection limits of the assays were <10 pg/ml for IL-12p70, <3 pg/ml for IL-10, <30 pg/ml for IL-23, <10 pg/ml for IL-17A and <10 pg/ml for IFN-γ. Cell lysis, RNA extraction and real-time PCR were performed as described.[18-19] Amplification efficiencies were validated and normalized against *Gapdh* or *ACTB*. Each data point was examined for integrity by analysis of the amplification plot. The mRNA-normalized data were expressed as relative mRNA expression in treated cells compared to that of unstimulated cells. For details, see Supplemental Materials.

Patients

The genetic study included 223 patients undergoing allogeneic HSCT at the University of Perugia, Italy, between 2003 and 2010, and respective donors. Patient characteristics are summarized in Table S1. Grafts consisted of immunoselected $CD34^+$ peripheral blood cells in all cases and transplantation procedures, antifungal prophylaxis and surveillance for fungal infection were performed as described[27] (for details, see Supplemental Materials). Probable/proven fungal infection was defined according to the revised standard criteria from the European Organization for Research and Treatment of Cancer/Mycology Study Group. Study approval was provided by the local ethics committee and informed written consent was obtained from all participants in accordance with the Declaration of Helsinki.

SNP genotyping

SNPs were selected from a literature review and public databases based on three selection criteria: i) published evidence of association with human diseases, ii) localization to the promoter, untranslated or coding regions, and (iii) minor allele frequencies higher than 5% in the Caucasian population. Two TLR3 SNPs complied with the selection criteria: +1234C/T (L412F, rs3775291) and +95C/A (rs3775296). Genotyping was performed as described[27] (for details and primer sequences, see Supplemental Materials).

Statistical analysis

Student's T-test or analysis of variance (ANOVA) with Bonferroni's adjustment were used to determine statistical significance ($P<0.05$). Data reported are either from one out of three to five independent experiments (western blotting and RT–PCR) or pooled from three to five experiments, otherwise. The in vivo groups consisted of 6–8 mice/group. Data were analyzed by GraphPad Prism 4.03 program (GraphPad Software, San Diego, CA). For details on the statistical analysis of genetic associations, see Supplemental Materials.

Results

Tlr3$^{-/-}$ mice are highly susceptible to pulmonary aspergillosis

We evaluated resistance to primary pulmonary aspergillosis and reinfection in *Tlr3*$^{-/-}$ and C57BL/6 mice. In the primary infection, fungal growth was higher in the lungs of *Tlr3*$^{-/-}$ than control mice throughout the course of the infection (Figure 1A) and was associated with higher and persistent neutrophil recruitment and infiltration in the lung parenchyma and BAL fluids (inset of Figure 1B also showing numerous hyphae) and presence of peribronchiolar lymphocyte infiltrations (arrows in Figure 1B and immunofluorescence staining). Fungal dissemination was also observed in the brain of *Tlr3*$^{-/-}$ mice (Figure 1A), more than 85% of which survived the infection (data not shown), if not immunosuppressed (Figure 2C). Stimulating TLR3 with the poly(I:C) agonist significantly reduced both the fungal growth and inflammation in the lungs of C57BL/6 mice (Supplemental Figure 1A-B), a finding further supporting that TLR3 provides protection in infection. Gene expression analysis of the lung confirmed the higher and more persistent inflammatory response in *Tlr3*$^{-/-}$ than control mice, as revealed by the higher mRNA expression of *Cxcl1*, *Cxcl2* and *Mpo* genes as well as of genes for inflammatory cytokines, such as *Il1b* (Figure 1C). The levels of *Ifna1* and *Ifnb1* were instead lower in *Tlr3*$^{-/-}$ mice (Figure 1C). In addition, the conidiocidal activity of lung cells was half reduced in *Tlr3*$^{-/-}$ mice compared to the C57BL/6 control (data not shown). Thus, TLR3 contributes to the effector activity of lung cells as well as to protection from unintended inflammation.

Because *A. fumigatus* is sensed by TLR3 on both epithelial[17] and DCs,[19] we determined the relative contribution of either type of cells to the TLR3-mediated protection by infecting chimeric mice with TLR3-deficient hematopoietic or nonhematopoietic cells. TLR3 deficiency in myeloid, more than epithelial cells, greatly impaired resistance to the fungus, as revealed by the inability to control fungal growth and restrict inflammation (Supplemental Figure 2). Accordingly, DC cytokines were altered in *Tlr3*$^{-/-}$ mice, in that levels of IL-12p70 were lower and those of IL-23 higher than in C57BL/6 mice (Supplemental Figure 3). Thus, the myeloid-DC compartment is dysfunctional in *Tlr3*$^{-/-}$ mice and this would predict altered T cell priming in infection.

$CD8^+$ T cells do not expand in *Tlr3*$^{-/-}$ mice upon infection

We evaluated the priming of $CD4^+$ or $CD8^+$ T cells in *Tlr3*$^{-/-}$ or C57BL/6 mice by assessing the expansion of either T cell subset in infection. Both subsets expanded in the lung and the draining lymph nodes of infected C57BL/6 mice, whereas $CD4^+$ but not $CD8^+$ T cells expanded in the lung and less in the lymph nodes of *Tlr3*$^{-/-}$ mice (Supplemental Figure 4). Not only were $CD8^+$ T cells defective, but $CD4^+$ T helper (Th) cell subsets were also abnormally activated in *Tlr3*$^{-/-}$ mice, as judged by the decreased expression of *Tbet*/*Foxp3* specific transcripts in cells from draining lymph nodes (Figure 1D) and the production of IFN-γ/IL-10 in the lung (Figure 1E) and the increased expression of the *Rorc* transcript (Figure 1D) and the corresponding cytokine IL-17A (Figure 1E). Thus, besides being required for the expansion of $CD8^+$ T cells, TLR3 is also required for the proper $CD4^+$ Th balance in infection. We know that the activation of IDO via the TLR3/TRIF pathway confers tolerogenic and Th1-activating potential to lung DCs in murine aspergillosis.[18-19] Consistently, the enzyme was not induced in infection in the lung of *Tlr3*$^{-/-}$ mice, despite being expressed at the basal level (Supplemental Figure 5). Thus, TLR3 likely contributes, through IDO, to the promotion of protective tolerance to the fungus in infection as well as to the down-regulation of Th17 responses, as already shown in other lung infections.[28]

***Tlr3*$^{-/-}$ mice fail to develop MHC class I-restricted $CD8^+$ T cell responses upon vaccination**

To define the contribution of $CD8^+$ T cells in infection, we evaluated fungal growth and histopathology of infected C57BL/6 mice in conditions of $CD4^+$ or $CD8^+$ T cell depletion. Both $CD4^+$ and $CD8^+$ T cell depletion impaired antifungal resistance (Figure 2A and B), even though with interesting differences. At variance with $CD4^+$ T cell depletion, $CD8^+$ T cell depletion did not adversely affect antifungal resistance early in infection as it did late, as revealed by the increased fungal burden and concomitant inflammatory pathology in the lung. This finding suggests that $CD8^+$ T cells, somehow dispensable in the early phase of the infection, are instead needed for optimal clearance of the fungus late in infection, as already demonstrated in other fungal

infections,[22] and points to a role for $CD8^+$ T cells in adaptive antifungal immunity. To assess this role, we resorted to well-established experimental models of vaccine-induced resistance.[24] Mice were treated with conidia or the protective recombinant fungal antigen Crf1p[24] along with CpG as adjuvant and assessed for resistance to subsequent infection in terms of survival, fungal growth and pattern of cytokine gene expression. In contrast to control mice, *Tlr3*$^{-/-}$ mice failed to develop vaccine-induced resistance in response to conidia, as revealed by the inability to survive infection (Figure 2C), restrict the fungal growth (Figure 2D) and produce protective IFN-γ and IL-10 (Figure 2E). Surprisingly, however, they developed full resistance upon Crf1p vaccination (Figure 2C-E).

The ability of TLR3 signaling to provide cross-priming of $CD8^+$ T cells[5] in the relative absence of $CD4^+$ T cell help[8] prompted us to evaluate whether, similarly to what described for other fungi,[20-21] exogenous fungal antigens would activate memory $CD8^+$ T cells in a MHC class I-restricted manner and in the relative absence of Th cells. For this purpose, we evaluated vaccine-induced resistance in conditions of CD4, CD8, and MHC class I or class II ablation by means of specific neutralizing antibodies, thus avoiding residual T helper activity of genetically deficient mice. We found that blocking CD4 or class II antigens abrogated the resistance induced by vaccination with Crf1p in both C57BL/6 (Figure 2F and G) and *Tlr3*$^{-/-}$ (Figure 2H and I) mice, whereas blocking CD8 or class I antigens abrogated the resistance induced by vaccination with conidia in C57BL/6 mice, as revealed by the survival (Figure 2F), the fungal growth (Figure 2G) and lung histopathology (Supplemental Figure 6). These results indicate that both $CD4^+$ and $CD8^+$ T cells mediate memory responses to the fungus, contingent upon the nature of the fungal vaccine, and that $CD8^+$- but not $CD4^+$-dependent memory responses to the fungus are defective in the absence of TLR3.

Migratory $CCR7^+$ DCs are defective in *Tlr3*$^{-/-}$ mice

Lung DCs transport *A. fumigatus* conidia or hyphae to the draining lymph nodes where T cell priming occurs.[29] To assess whether the defective $CD8^+$ T cell expansion in *Tlr3*$^{-/-}$ infected mice

could result from defective priming in the draining lymph nodes, we infected *Tlr3*$^{-/-}$ and C57BL/6 mice with a GFP-expressing strain of *A. fumigatus* and investigated migration of GFP^{+} DCs from the lung to the draining lymph nodes. We found that a population of CD11c^{high} DCs expressed GFP early in the lungs of C57BL/6 (Figure 3A) and, to a lesser extent, in *Tlr3*$^{-/-}$ mice in which a population of CD11c^{low} cells (also CD11b^{high}, data not shown) expressed GFP (Figure 3B). While still present in the lung, GFP^{+} DCs also accumulated in the draining lymph nodes of C57BL/6 (Figure 3A) but not *Tlr3*$^{-/-}$ mice (Figure 3B). Thus, DCs responsible for T cell priming in the draining lymph nodes are defective in *Tlr3*$^{-/-}$ mice. This was confirmed by flow cytometry analysis of lung DCs showing a decreased number of CD103^{+} cells that poorly express both CD8α and CD11b (Figure 3C), as reported.[30]

As CCR7, known to be crucially required for CD8^{+} T cell priming in the lymph nodes,[31] is up-regulated in DCs upon conidia internalization,[32] we have assessed phagocytosis and *Ccr7* expression in *Tlr3*$^{-/-}$ DCs. We found that, while the phagocytosis percentage was not different (data not shown), the ability to internalize more than one conidia was greatly impaired in *Tlr3*$^{-/-}$ DCs, as revealed by the decreased phagocytic index compared to DCs from C57BL/6 mice (Figure 3D). *Tlr3*$^{-/-}$ DCs also failed to up-regulate *Ccr7* expression upon phagocytosis in a manner similar to control DCs upon blocking phagocytosis (Figure 3E). Thus, TLR3 contributes to *Ccr7* expression of lung DCs upon conidia phagocytosis, likely conditioning their migratory capacity and priming efficiency.

Recognition of fungal RNA is defective in *Tlr3*$^{-/-}$ DCs

TLR3 is present within endocytic vesicles of cross-presenting DC subsets[6] where it is activated by microbial dsRNA[1,33] or endogenous mRNA.[2] Fungal RNA is known to activate DCs for vaccine-induced resistance[26] and is recognized by TLR3.[17] DCs transfected with fungal RNA expressed immunogenic fungal antigens,[26] a finding indicating that newly synthesized fungal antigens are originated from RNA translation. This was confirmed in experiments in which vaccination of mice

with fungal RNA and the cationic lipid DOTAP – known to target dsRNA to endosomal TLR3 and to direct antigens into the class I MHC antigen-presentation pathway[34] – resulted in the induction of fungus-specific $CD8^+$ T cell-mediated resistance (Supplemental Figure 7). Based on these assumptions, we comparatively assessed purified DCs from the lung of *Tlr3*$^{-/-}$ and C57BL/6 mice for functional responses to *A. fumigatus* conidia or fungal RNA in terms of signaling events downstream TLR3, such as phosphorylation of IFN regulatory factor 3 (IRF3), cytokine gene expression (type I IFNs), maturation (CD80, CD86 and MHC class II expression), ability to prime antigen-specific $CD4^+$ or $CD8^+$ T cells in vitro as well as ability to confer resistance upon adoptive transfer in vivo. Fungal RNA potently induced IRF3 phosphorylation in C57BL/6 DCs, an activity also observed with swollen conidia, but not with resting conidia or DOTAP nor in *Tlr3*$^{-/-}$ DCs (Figure 4A). Fungal RNA also potently activated gene expression of type I IFNs (Figure 4B) and induced DC maturation (Figure 4C) in a TLR3-dependent manner. Although conidia similarly activated DCs, the residual activity observed in *Tlr3*$^{-/-}$ DCs suggests the participation of other TLRs and/or intracellular RNA sensors in response to conidia. This was confirmed by the ability of conidia-pulsed DCs to prime $CD4^+$ T cells in both C57BL/6 (Figure 4D and E) and *Tlr3*$^{-/-}$ mice (Figure 4F and G) and, similar to RNA-pulsed DCs, to activate $CD8^+$ T cells. Indeed, conidia-pulsed DCs primed both T cell subsets from C57BL/6 mice for proliferation (Figure 4D) and *Ifng* expression (Figure 4E) and primed $CD8^+$ T cells for *Prf1* gene expression (Figure 4E). In contrast, RNA-pulsed DCs primed only $CD8^+$ T cells for proliferation (Figure 4D), *Ifng* and *Prf1* gene expression (Figure 4E), as well as for cytolytic activity against fungus-pulsed DCs (Figure 4H) or against the fungus itself (Figure 4I). Of interest, $CD8^+$ T cell priming by RNA-pulsed DCs was abrogated in the presence of known inhibitors of the TLR3-dependent antigen presentation pathway[11] – bafilomycin A1 and chloroquine – and of the cytosolic, class I-dependent antigen presentation pathway[35] – brefeldin A and lactacystin – (Figure 4J). These findings indicate that both the endosomal/lysosomal and proteasomal degradation pathways are involved in antigen processing after RNA transfection. Finally, fungus- or RNA-pulsed DCs isolated from *Tlr3*$^{-/-}$ mice failed to

confer protection upon adoptive transfer in vivo in C57BL/6 mice. However, fungus- or RNA-pulsed DCs from C57BL/6 mice also failed to confer protection upon transfer in *Tlr3*$^{-/-}$ mice (Figure 4K), a finding suggesting that host DCs cross-present fungal antigens from migratory donor DCs, as proposed.[36]

A *TLR3* SNP associates with increased risk of invasive aspergillosis in HSCT recipients

Given the above results, we investigated whether genetic variants affecting the function of TLR3 might influence susceptibility to aspergillosis in patients with predisposing conditions. To this purpose, we performed a genetic analysis of selected SNPs in *TLR3* in a cohort of 223 consecutive HSCT recipients and respective donors (Table S1). To estimate risk of infection according to patient or donor *TLR3* genotypes, we determined cumulative incidences of aspergillosis among transplant recipients at 12 months following transplantation. The +1234C/T (L412F) SNP did not influence susceptibility to infection when present in either patients [22.1% for CC and 20.3% for CT + TT; P=0.73] or donors [24.5% for CC, 15.9% for CT + TT; P=0.19]. In contrast, the +95C/A SNP was found to significantly increase risk of aspergillosis when present in donors [14.8% for CC and 30.2% for CA + AA; P=0.01] (Figure 5B), but not in patients (22.2% for CC and 19.9% for CA + AA; P=0.89) (Figure 5A). Thus, recapitulating the murine findings, the specific contribution of the *TLR3* +95C/A SNP to the infection appears to rely on its presence on myeloid cells. Moreover, the genetic association remained significant after correction for clinical co-variables, namely transplant matching, graft-versus-host disease (GVHD) and antifungal prophylaxis. In the multivariate model, donor *TLR3* +95C/A SNP (CA + AA genotype) independently increased risk of IA [adjusted hazard ratio (HR), 2.41; 95% confidence interval (CI), 1.27–4.58; P=0.007). No statistically significant influence of the tested SNPs was observed on patient survival (data not shown).

The *TLR3* +95C/A SNP leads to defective CD8^{+} T cell activation by DCs

Human CD141$^+$ (BDCA-3)$^+$ DCs are known to represent the equivalent of mouse DCs that cross-present antigens to CD8$^+$ T cells upon treatment with poly(I:C).[37] We purified BDCA-3$^+$ DCs from individuals bearing distinct *TLR3* +95C/A genotypes and assessed *TLR3* expression upon stimulation with poly(I:C), *A. fumigatus* conidia or fungal RNA. Phenotypic analysis revealed that BDCA-3$^+$ DCs were CD11c$^+$, CD11b$^-$, CD1a$^-$, CD86$^+$ and CD80$^-$ (Supplemental Figure 8), as described.[38] We found that *TLR3* mRNA expression in WT BDCA-3$^+$ cells was strongly induced following pulsing with each stimulus (Figure 6A). In contrast, BDCA-3$^+$ cells bearing the +95C/A SNP displayed instead a marked decrease in *TLR3* expression and responsiveness, a defect found to be genotype dose-dependent. We also found that expression of both *IFNA1* and *IFNB1* genes was triggered by all the stimuli in WT BDCA-3$^+$ DCs, but not in *TLR3* polymorphic cells (Figure 6B). Finally, on assessing the ability of BDCA-3$^+$ DCs to prime CD4$^+$ or CD8$^+$ T cells for proliferation, we found that antigen-specific (Figure 6C) and, to a lesser extent, polyclonal (Figure 6D) CD8$^+$ T cell proliferation were mostly affected by the *TLR3* +95C/A SNP compared to CD4$^+$ T cells. Given that TLR3 is expressed on CD8$^+$ T cells (ref. 39 and Supplemental Figure 9), we cannot rule out the contribution of direct TLR3 signaling to the defective proliferative activity of CD8$^+$ T cells. Altogether, these results point to an essential role for TLR3 in the activation of memory protective CD8$^+$ T cell responses to *A. fumigatus* in mice and humans.

Discussion

The ability of exogenous fungal antigens to activate memory protective CD8$^+$ cells without CD4$^+$ T-cell help ("helpless") is of relevance for both basic and clinical mycology. This demands for a better definition of cells and molecules that control CD8$^+$ T cell priming and differentiation into effector and memory cells in vivo. The major findings to emerge from these studies are that TLR3 signaling in DCs pulsed with *A. fumigatus* conidia or RNA efficiently prime resting CD8$^+$ T cells in vivo and in vitro for memory responses to the fungus by a mechanism that appears to involve acidic

endosomal vesicles as well as cytosolic processing. Susceptibility to aspergillosis was increased in conditions of TLR3 deficiency both in mice and humans, a finding pointing to a previously unrecognized role for the TLR3-dependent antigen recognition pathway in aspergillosis. Similarly to what was observed with other fungal pathogens,[20-21,40] by producing effector cytokines such as IFN-γ, and exhibiting cytotoxic activity against fungus-laden cells or the fungus itself, $CD8^+$ T cells provided vaccine-induced resistance likely by restricting the fungal growth and terminating the cellular response in infection.

Both $CD4^+$ T cells[13-15] and $CD8^+$ T cells[13,25,41] are present in the murine and human T cell repertoire to the fungus. However, the role of $CD8^+$ T cells in infection, the specific fungal antigens they recognize and mechanisms underlying their activation are unknown. Our study provides evidence that both $CD4^+$ and $CD8^+$ T cells mediate memory responses to the fungus, contingent upon the nature of the fungal vaccine. Indeed, the cell wall glucanase Crf1p, known to be presented by three common human MHC class II alleles and to induce memory $CD4^+$ Th1 cells to the fungus,[42] activated antifungal memory $CD4^+$ Th1 cells independently of TLR3. We have evidence that recognition of Crf1p and $CD4^+$ Th1 cell activation occur via MyD88 (unpublished observation), a finding suggesting that TLRs, other than TLR3, are involved in fungal antigen sampling and signaling for activation of the CD4 arm of the memory immune response to the fungus. However, the finding that *Tlr3*$^{-/-}$ mice failed to mount an efficient memory response to the fungus, while retaining the ability to activate memory fungal-antigen specific $CD4^+$ T cell responses, points to either a superior activity of the CD8-dependent mechanisms in immune memory to the fungus or a dysfunctional fungal processing in conditions of TLR3 deficiency.

TLR3 is a crucial "danger" signaling receptor that senses endogenous mRNA released by necrotic cells.[3] Thus, it is possible that TLR3 is activated in infection by sensing host damage. However, in this case, $CD8\alpha^+$ DCs are the solely responsible for cross-presenting antigens from apoptotic host cells.[43] In addition, we have recently shown that TLR3 restrained rather than promoted danger-dependent inflammation in aspergillosis.[17] Thus, sensing fungal RNA is the likely

function of TLR3 in infection. By migrating from early endosomes to LAMP1$^+$ endosomes upon stimulation with dsRNA, TLR3 mediates the activation of DCs for the cross-presentation of exogenous antigens to CD8$^+$ T cells.[6] Thus, TLR3-specific adjuvants are used to tailor CD8$^+$ T cell immune responses independently of CD4 help.[44] We have tested the adjuvant activity of TLR3 and found that administration of Crf1p with poly(I:C) skewed T cell memory from class II-dependent CD4$^+$ T cells – obtained with CpG known to block cross-presentation[43]– to class I-dependent CD8$^+$ T cells (data not shown). This finding highlights the importance of adjuvants in skewing antifungal memory responses to candidate subunit fungal vaccines. However, the function of fungal RNA may go beyond its adjuvant activity as RNA-transfected DCs also originate fungal antigens.[26] As in viral infection,[45] the requirement of host translational machinery could enhance the efficiency of class I presentation. We show that these antigens are processed via both the TLR3-dependent endosomal/lysosomal and class I-dependent proteasomal degradation pathways. It is also likely that direct presentation of fungal antigens, newly synthesized from endogenous RNA produced after fungal internalization and degradation, occurs in infection through cytosolic receptors other than the endosomal TLR3. Consistent with the findings obtained with conidia, apparently many routes of antigen sampling and presentation are used by DCs confronted with the fungus.

TLR3 may further optimize CD8$^+$ T cell activation by facilitating CCR7 expression on DCs through unimpaired phagocytosis. Despite the fact that CCR7 deficiency does not impair fungal clearance in primary aspergillosis,[46] CCR7$^+$ DCs are required for the priming of CD8$^+$ T cells in the draining lymph node and this finding highlights the interesting difference with CD4$^+$ T cell anti-*A. fumigatus* priming that was reported to be mediated by migratory CCR2$^+$ inflammatory monocytes.[47] However, the role of TLR3 may go beyond regulation of adaptive memory to include regulation of inflammation and tolerance. The defective IDO activation likely contributed to the inability of these mice to promote tolerogenic responses to the fungus that are required to limit inflammation in infection.[18] Indeed, IL-17A-producing Th17 cells were expanded in *Tlr3*$^{-/-}$ mice and likely contributed to inflammatory pathology, as suggested.[33] Besides the ability of type I IFNs

to inhibit Th17 development,[48] it is interesting that, disabling lymph node priming, as in CCR7 deficiency,[49] promoted IL-17A–mediated lung pathology via abnormal T cell sensitization in ectopic lymph node formation.[50] Both defective *Ccr7* expression and peribronchiolar lymphoid tissues were observed in *Tlr3*$^{-/-}$ mice.

In conclusion, this study discloses the contribution of TLR3 to CD8^{+} T cell memory responses to *A. fumigatus* and identifies a *TLR3* SNP leading to a loss-of-function phenotype of DCs that is associated with susceptibility to infection and concomitant failure to activate antifungal CD8^{+} T cells in HSCT patients. Our findings may offer a plausible explanation for the increased susceptibility to aspergillosis after CMV reactivation in HSCT patients and predict that, in addition to *TLR9*,[51] *TLR3* SNPs are also a likely risk factor for infection by CMV that, incidentally, shares with other members of the Herpes virus family, the ability to subvert endogenous MHC class I pathway via TRIF degradation.[52] Regardless of possible differences between viral and fungal cross-presentation pathways, our findings suggest that a defective antigen cross-presentation pathway may predispose to both viral and fungal infections in HSCT patients and point to novel therapeutic interventions.

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Authorship

Contribution: A.C., F.A., A.V., J. P. L. and L.R. designed research; A.D.L., S.B., C.C., C.D.A., S.M., K.P, R.G.I., A.P. and F.F. performed the experiments; A.C. and L.R. wrote the manuscript.

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References

1. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature*. 2001;413(6857):732-738.

2. Kariko K, Ni H, Capodici J, Lamphier M, Weissman D. mRNA is an endogenous ligand for Toll-like receptor 3. *J Biol Chem*. 2004;279(13):12542-12550.

3. Cavassani KA, Ishii M, Wen H, et al. TLR3 is an endogenous sensor of tissue necrosis during acute inflammatory events. *J Exp Med*. 2008;205(11):2609-2621.

4. Wang Y, Cella M, Gilfillan S, Colonna M. Cutting edge: polyinosinic:polycytidylic acid boosts the generation of memory CD8 T cells through melanoma differentiation-associated protein 5 expressed in stromal cells. *J Immunol*. 2010;184(6):2751-2755.

5. Schulz O, Diebold SS, Chen M, et al. Toll-like receptor 3 promotes cross-priming to virus-infected cells. *Nature*. 2005;433(7028):887-892.

6. Jelinek I, Leonard JN, Price GE, et al. TLR3-specific double-stranded RNA oligonucleotide adjuvants induce dendritic cell cross-presentation, CTL responses, and antiviral protection. *J Immunol*. 2011;186(4):2422-2429.

7. Le Bon A, Etchart N, Rossmann C, et al. Cross-priming of CD8+ T cells stimulated by virus-induced type I interferon. *Nat Immunol*. 2003;4(10):1009-1015.

8. Hervas-Stubbs S, Olivier A, Boisgerault F, Thieblemont N, Leclerc C. TLR3 ligand stimulates fully functional memory CD8+ T cells in the absence of CD4+ T-cell help. *Blood*. 2007;109(12):5318-5326.

9. Perez de Diego R, Sancho-Shimizu V, Lorenzo L, et al. Human TRAF3 adaptor molecule deficiency leads to impaired Toll-like receptor 3 response and susceptibility to herpes simplex encephalitis. *Immunity*. 2010;33(3):400-411.

10. Zhang SY, Jouanguy E, Ugolini S, et al. TLR3 deficiency in patients with herpes simplex encephalitis. *Science*. 2007;317(5844):1522-1527.

11. Gorbea C, Makar KA, Pauschinger M, et al. A role for Toll-like receptor 3 variants in host susceptibility to enteroviral myocarditis and dilated cardiomyopathy. *J Biol Chem*. 2010;285(30):23208-23223.

12. Aimanianda V, Bayry J, Bozza S, et al. Surface hydrophobin prevents immune recognition of airborne fungal spores. *Nature*. 2009;460(7259):1117-1121.

13. Chaudhary N, Staab JF, Marr KA. Healthy human T-Cell Responses to *Aspergillus fumigatus* antigens. *PLoS One*. 2010;5(2):e9036.

14. 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(13):4521-4528.

15. Perruccio K, Tosti A, Burchielli E, et al. Transferring functional immune responses to pathogens after haploidentical hematopoietic transplantation. *Blood*. 2005;106(13):4397-4406.

16. Beck O, Topp MS, Koehl U, et al. Generation of highly purified and functionally active human TH1 cells against *Aspergillus fumigatus*. *Blood*. 2006;107(6):2562-2569.

17. Sorci G, Giovannini G, Riuzzi F, et al. The danger signal S100B integrates pathogen- and danger-sensing pathways to restrain inflammation. *PLoS Pathog*. 2011;7(3):e1001315.

18. de Luca A, Bozza S, Zelante T, et al. Non-hematopoietic cells contribute to protective tolerance to *Aspergillus fumigatus* via a TRIF pathway converging on IDO. *Cell Mol Immunol*. 2010;7(6):459-470.

19. Bonifazi P, D'Angelo C, Zagarella S, et al. Intranasally delivered siRNA targeting PI3K/Akt/mTOR inflammatory pathways protects from aspergillosis. *Mucosal Immunol*. 2010;3(2):193-205.

20. Wuthrich M, Filutowicz HI, Warner T, Deepe GS, Jr., Klein BS. Vaccine immunity to pathogenic fungi overcomes the requirement for CD4 help in exogenous antigen presentation to CD8+ T cells: implications for vaccine development in immune-deficient hosts. *J Exp Med*. 2003;197(11):1405-1416.

21. Lindell DM, Moore TA, McDonald RA, Toews GB, Huffnagle GB. Generation of antifungal effector CD8+ T cells in the absence of CD4+ T cells during *Cryptococcus neoformans* infection. *J Immunol*. 2005;174(12):7920-7928.

22. Deepe GS, Jr. Role of CD8+ T cells in host resistance to systemic infection with *Histoplasma capsulatum* in mice. *J Immunol*. 1994;152(7):3491-3500.

23. Huffnagle GB, Yates JL, Lipscomb MF. Immunity to a pulmonary *Cryptococcus neoformans* infection requires both CD4+ and CD8+ T cells. *J Exp Med*. 1991;173(4):793-800.

24. 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(4):2407-2414.

25. 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* f16 allergen. *Clin Exp Immunol*. 2005;140(1):81-91.

26. Bozza S, Perruccio K, Montagnoli C, et al. A dendritic cell vaccine against invasive aspergillosis in allogeneic hematopoietic transplantation. *Blood*. 2003;102(10):3807-3814.

27. Cunha C, Di Ianni M, Bozza S, et al. Dectin-1 Y238X polymorphism associates with susceptibility to invasive aspergillosis in hematopoietic transplantation through impairment of both recipient- and donor-dependent mechanisms of antifungal immunity. *Blood*. 2010;116(24):5394-5402.

28. Joshi AD, Schaller MA, Lukacs NW, Kunkel SL, Hogaboam CM. TLR3 modulates immunopathology during a *Schistosoma mansoni* egg-driven Th2 response in the lung. *Eur J Immunol*. 2008;38(12):3436-3449.

29. 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(13):1281-1290.

30. del Rio ML, Rodriguez-Barbosa JI, Kremmer E, Forster R. CD103- and CD103+ bronchial lymph node dendritic cells are specialized in presenting and cross-presenting innocuous antigen to CD4+ and CD8+ T cells. *J Immunol*. 2007;178(11):6861-6866.

31. Heer AK, Harris NL, Kopf M, Marsland BJ. CD4+ and CD8+ T cells exhibit differential requirements for CCR7-mediated antigen transport during influenza infection. *J Immunol*. 2008;181(10):6984-6994.

32. Gafa V, Lande R, Gagliardi MC, et al. Human dendritic cells following *Aspergillus fumigatus* infection express the CCR7 receptor and a differential pattern of interleukin-12 (IL-12), IL-23, and IL-27 cytokines, which lead to a Th1 response. *Infect Immun*. 2006;74(3):1480-1489.

33. Aksoy E, Zouain CS, Vanhoutte F, et al. Double-stranded RNAs from the helminth parasite *Schistosoma* activate TLR3 in dendritic cells. *J Biol Chem*. 2005;280(1):277-283.

34. Walker C, Selby M, Erickson A, Cataldo D, Valensi JP, Van Nest GV. Cationic lipids direct a viral glycoprotein into the class I major histocompatibility complex antigen-presentation pathway. *Proc Natl Acad Sci U S A*. 1992;89(17):7915-7918.

35. Amigorena S, Savina A. Intracellular mechanisms of antigen cross presentation in dendritic cells. *Curr Opin Immunol*. 2010;22(1):109-117.

36. Allan RS, Waithman J, Bedoui S, et al. Migratory dendritic cells transfer antigen to a lymph node-resident dendritic cell population for efficient CTL priming. *Immunity*. 2006;25(1):153-162.

37. Jongbloed SL, Kassianos AJ, McDonald KJ, et al. Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. *J Exp Med*. 2010;207(6):1247-1260.

38. Poulin LF, Salio M, Griessinger E, et al. Characterization of human DNGR-1+ BDCA3+ leukocytes as putative equivalents of mouse CD8alpha+ dendritic cells. *J Exp Med*. 2010;207(6):1261-1271.

39. Edwards AD, Diebold SS, Slack EM, et al. Toll-like receptor expression in murine DC subsets: lack of TLR7 expression by CD8 alpha+ DC correlates with unresponsiveness to imidazoquinolines. *Eur J Immunol*. 2003;33(4):827-833.

40. Lin JS, Yang CW, Wang DW, Wu-Hsieh BA. Dendritic cells cross-present exogenous fungal antigens to stimulate a protective CD8 T cell response in infection by *Histoplasma capsulatum*. *J Immunol*. 2005;174(10):6282-6291.

41. Templeton SP, Buskirk AD, Law B, Green BJ, Beezhold DH. Role of germination in murine airway CD8 T-cell responses to *Aspergillus* conidia. *PLoS One*. 2011;6(4):e18777.

42. Stuehler C, Khanna N, Bozza S, et al. Cross-protective TH1 immunity against *Aspergillus fumigatus* and *Candida albicans*. *Blood*. 2011;117(22):5881-5891.

43. Schnorrer P, Behrens GM, Wilson NS, et al. The dominant role of CD8+ dendritic cells in cross-presentation is not dictated by antigen capture. *Proc Natl Acad Sci U S A*. 2006;103(28):10729-10734.

44. Zaks K, Jordan M, Guth A, et al. Efficient immunization and cross-priming by vaccine adjuvants containing TLR3 or TLR9 agonists complexed to cationic liposomes. *J Immunol*. 2006;176(12):7335-7345.

45. Schubert U, Anton LC, Gibbs J, Norbury CC, Yewdell JW, Bennink JR. Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature*. 2000;404(6779):770-774.

46. Hartigan AJ, Westwick J, Jarai G, Hogaboam CM. CCR7 deficiency on dendritic cells enhances fungal clearance in a murine model of pulmonary invasive aspergillosis. *J Immunol*. 2009;183(8):5171-5179.

47. Hohl TM, Rivera A, Lipuma L, et al. Inflammatory monocytes facilitate adaptive CD4 T cell responses during respiratory fungal infection. *Cell Host Microbe*. 2009;6(5):470-481.

48. Shinohara ML, Kim JH, Garcia VA, Cantor H. Engagement of the type I interferon receptor on dendritic cells inhibits T helper 17 cell development: role of intracellular osteopontin. *Immunity*. 2008;29(1):68-78.

49. Worbs T, Mempel TR, Bolter J, von Andrian UH, Forster R. CCR7 ligands stimulate the intranodal motility of T lymphocytes in vivo. *J Exp Med*. 2007;204(3):489-495.

50. Kallal LE, Hartigan AJ, Hogaboam CM, Schaller MA, Lukacs NW. Inefficient lymph node sensitization during respiratory viral infection promotes IL-17-mediated lung pathology. *J Immunol*. 2010;185(7):4137-4147.

51. Carvalho A, Cunha C, Carotti A, et al. Polymorphisms in Toll-like receptor genes and susceptibility to infections in allogeneic stem cell transplantation. *Exp Hematol*. 2009;37(9):1022-1029.

52. Ahmad H, Gubbels R, Ehlers E, et al. Kaposi sarcoma-associated herpesvirus degrades cellular Toll-interleukin-1 receptor domain-containing adaptor-inducing beta-interferon (TRIF). *J Biol Chem*. 2011;286(10):7865-7872.

Figure legends

Figure 1. *Tlr3*$^{-/-}$ mice are highly susceptible to pulmonary aspergillosis. C57BL/6 or *Tlr3*$^{-/-}$ mice were infected i.n. with live *A. fumigatus* conidia (6-8 mice/group). (A) Fungal growth (mean CFU ± SE) in the lungs and brains of infected mice was assessed at different days post-infection (dpi). (B) Lung histology (PAS staining), BAL morphometry [%, mean ± SD, of MNC or polymorphonuclear (PMN) cells] and immunofluorescence of infected mice at different dpi. Note the sustained inflammatory cell recruitment in the lungs and BAL (May-Grünwald Giemsa staining in the inset) of *Tlr3*$^{-/-}$ mice as well as the presence of peribronchiolar lymphocyte infiltrates (at 3 dpi) (arrows in PAS staining and immunofluorescence images). Cell surface markers used for DC and T cell visualization were CD11c (Green-Alexa 488) and CD3 epsilon followed by PE-secondary antibody, respectively. Cell nuclei were stained blue with DAPI. Representative images of two independent experiments were acquired with a 40× objective. Bars in histology sections indicate magnifications. (C) Lung total RNA from infected mice was extracted from naïve (0) or at 1, 3 and 7 dpi and the relative expression of *Cxcl1*, *Cxcl2*, *Mpo*, *Il1b*, *Ifna1* and *Ifnb1* was assessed by RT-PCR. (D) Total RNA was extracted from purified CD4^{+} T cells from the draining lymph nodes of naïve or infected mice at 7 dpi and the relative expression of *Tbet*, *Rorc* and *Foxp3* was assessed by RT-PCR. (E) Total lung homogenates at 3 and 7 dpi were assessed for levels of IFN-γ, IL-17A and IL-10 by specific ELISA (mean values ± SD). Data are pooled from 3 independent experiments. *, P<0.05; **, P<0.01; ***, P<0.001; infected vs. naïve mice (D) or *Tlr3*$^{-/-}$ vs. C57BL/6 mice (A, C and E).

Figure 2. *Tlr3*$^{-/-}$ mice fail to develop MHC class I-restricted CD8^{+} T cell responses upon vaccination. (A) C57BL/6 mice were treated with a total of 300 µg of anti-CD4 or anti-CD8 antibody the day before, 1 or 3 days after the i.n. infection with live *A. fumigatus* conidia (6-8 mice/group). None, mice receiving isotype control antibody. Depletion of the corresponding T cell

subsets with this regimen was between 95 to 98% at 3 and 7 days post-infection (dpi). (B) PAS staining of the lungs from mice treated as above. Note the presence of fungi (Gomori's methenamine silver staining in the inset) and the sustained inflammation in the lungs of CD4-depleted mice throughout the infection, as opposed to the increased inflammation and fungal growth only observed at 7 dpi in CD8-depleted mice. Bars indicate magnifications. (C) Survival (%), (D) fungal growth (CFU, mean ± SE) and (E) relative lung expression of *Ifng*, *Il17a*, *Il4* and *Il10* by RT-PCR in C57BL/6 or *Tlr3*$^{-/-}$ mice vaccinated with *A. fumigatus* conidia 14 days before (blue color) or with the recombinant fungal antigen Crf1p + CpG 14, 7 and 3 days (red color) before subsequent i.n. infection with live conidia (6-8 mice/group). Mice were given cyclophosphamide a day before the infection. Fungal growth and gene expression were assessed at 3 dpi. CpG alone failed to induce vaccine-induced resistance, as already reported.[24] (F, H) Survival and (G, I) fungal growth (at 3 dpi) in mice treated with anti-CD4, anti-CD8, anti-MHC class I or anti-MHC class II mAbs in concomitance with *A. fumigatus* conidia vaccination (blue color) or Crf1p + CpG vaccination (red color). Treatment with anti-CD4 alone, but not anti-CD8, anti-MHC class I-A or anti-MHC class II mAbs alone, further increased the susceptibility to the infection in cyclophosphamide-treated mice compared to control mice. Ct, infected unvaccinated mice. None, vaccinated mice treated with an isotype control antibody. Bars indicate magnifications. Data are representative from at least 3 independent experiments. *, P<0.05; **, P<0.01; ***, P<0.001; vaccinated vs. unvaccinated mice (D and E) or treated vs. untreated mice (A, G and I).

Figure 3. Migratory CCR7^{+} DCs are defective in *Tlr3*$^{-/-}$ mice. (A) C57BL/6 or (B) *Tlr3*$^{-/-}$ mice were infected i.n. with GFP-expressing *A. fumigatus* conidia and the numbers of GFP^{+} CD11c^{+} cells were assessed by flow cytometry on T- and B-cell depleted lungs or draining lymph nodes at different dpi. Numbers (mean values ± SD, *n* = 3) refer to % positive cells on gated CD11c^{+} cells. Shown is one representative experiment. (C) Phenotype of CD11c^{+} DCs from naïve C57BL/6 or *Tlr3*$^{-/-}$ mice. Flow cytometry was performed on gated CD11c^{+} DCs enriched from lungs. Numbers

(mean values ± SD, *n* = 3) refer to % positive $CD103^+$ cells. Histograms refer to cells expressing CD8α or CD11b on gated $CD103^+$ cells from one representative experiment. Isotype matched rat IgG2a (CD8α) or IgG2b (CD11b) control staining is shown in the blue histograms. (D) Phagocytic index (*n* = 3) and (E) relative expression of *Ccr7* (RT-PCR, mean values ± SD of at least 3 independent experiments assessed in triplicates) by purified lung DCs. Monolayers of DCs were added of live GFP-expressing conidia for 2 h at 37°C. Cytochalasin D was added for 1 h at 37°C before phagocytosis. Phagocytosis was quantified via phase contrast and fluorescence microscopy using the ×100 objective after quenching the fluorescence of bound uningested conidia with trypan blue and the DCs were fixed in 1% paraformaldehyde (shown are representative microscopy images of 2 independent experiments). Ct, control, unexposed cells.

Figure 4. Recognition of fungal RNA is defective in *Tlr3*$^{-/-}$ DCs. C57BL/6 or *Tlr3*$^{-/-}$ lung DCs from naïve mice were left unpulsed (None) or pulsed with resting (RC) or swollen (SC) *A. fumigatus* conidia, DOTAP alone or fungal RNA + DOTAP for 2 h. (A) Phosphorylation of IRF3 (p-IRF3) in DCs pulsed as above. Shown is a representative Western blot out of 3 independent experiments. The IRF3 bands were quantified and the ratios of phosphorylated to total IRF3 are shown. (B) Relative expression of *Ifna1* and *Ifnb1* (RT-PCR) and (C) expression of CD80, CD86 and MHC class II (flow cytometry) on pulsed DCs (mean values ± SD of at least 3 independent experiments assessed in triplicates). Flow cytometry was performed 6 h after pulsing and results are expressed as % positive cells. (D, F) T cell proliferation induced by conidia- or RNA-pulsed DCs in $CD4^+$ or $CD8^+$ T cells purified from lungs of (D) C57BL/6 or (F) *Tlr3*$^{-/-}$ mice, a week after the i.n. infection and assessed for lymphoproliferation after 72 h co-culture with pulsed-DCs from naïve mice. DNA synthesis was measured by ^{3}H-thymidine uptake (cpm, counts per minute). Ct, pulsed DCs alone. (E, G) Relative expression of *Ifng* and *Prf1* by RT-PCR in $CD4^+$ and $CD8^+$ T cells exposed to conidia- or fungal RNA-pulsed DCs for 24 h from (E) C57BL/6 or (G) *Tlr3*$^{-/-}$ mice. (H) Cytolytic activity of $CD8^+$ T cells from C57BL/6 mice, obtained as in D, against conidia-pulsed

DCs at different effector cell to target cell (E:T) ratios. Shown is the % specific cytotoxic activity determined by a standard 4h ^{51}Cr-release assay. (I) Conidiocidal activity of culture supernatants from CD8$^+$ T cells exposed as in H. Live conidia were labeled with the fluorescent molecular stain FUN-1 and incubated overnight with culture medium from cultured CD8$^+$ T cells before examination by fluorescence microscopy. Metabolically active conidia accumulate orange fluorescence in vacuoles, while dormant and dead conidia stain green. Shown are representative fluorescence microscopy images of 3 independent experiments. (J) CD8$^+$ T cell proliferation induced by RNA-pulsed C57BL/6 DCs in the presence or not (–) of inhibitors targeting class II (bafilomycin A1 and chloroquine) or class I (brefeldin A and lactacystin) antigen presentation pathways. Ct, CD8$^+$ T cells alone. (K) Adoptive transfer of C57BL/6 or *Tlr3*$^{-/-}$ DCs pulsed with conidia or fungal RNA into C57BL/6 or *Tlr3*$^{-/-}$ mice. DCs were adoptively transferred by i.p. injection twice, a week apart, before the infection. Fungal growth in the lungs (mean CFU ± SE, representative of at least 3 independent experiments) was assessed 3 days after the i.n. infection. In vivo data are from 4 independent experiments assessed in triplicates. *, P<0.05; **, P<0.01; ***, P<0.001, *Tlr3*$^{-/-}$ vs. C57BL/6 mice (B and C), antigen-pulsed DC-primed vs. unpulsed DC-primed (None) T cells (D, E, F and G), stimulated vs. unstimulated CD8$^+$ T cells (H), inhibitors vs. no inhibitors (-) (J) and DC-treated vs. untreated (None) mice (K).

Figure 5. A *TLR3* SNP associates with increased risk of invasive aspergillosis in stem cell transplanted recipients. The *TLR3* +95C/A genotype of 223 consecutive hematological patients undergoing allogeneic stem cell transplantation and respective donors was determined by the bidirectional PCR amplification of specific alleles (Bi-PASA) method and correlated with the incidence of invasive aspergillosis. (A) Cumulative incidence of invasive aspergillosis at 12 months according to patient +95C/A genotype: CC (22.2%, n=123) and CA + AA (19.9%, n=66), P=0.70 (B) Cumulative incidence of invasive aspergillosis at 12 months according to donor +95C/A genotype: CC (14.8%, n=109) and CA + AA (30.2%, n=80), P=0.01.

Figure 6. The +95C/A SNP in *TLR3* associates with defective $CD8^+$ T cells by peripheral DCs. Human $CD141^+$ ($BDCA\text{-}3^+$) DCs were isolated from peripheral blood of healthy volunteers bearing distinct *TLR3* +95C/A genotypes (CC, CA and AA) and were either left untreated or pulsed with poly(I:C), *A. fumigatus* conidia or fungal RNA. Relative expression of (A) *TLR3* and (B) type I IFNs (*IFNA1* and *IFNB1*) was assessed by RT-PCR. (C) Frequencies of antigen-specific $CD4^+$ or $CD8^+$ T cell clones were obtained by limiting dilution assays. Antigen-specific proliferation of T cell clones was assessed after co-culture of responder $CD4^+$ and $CD8^+$ T cells with $BDCA\text{-}3^+$ DCs pulsed with *A. fumigatus* conidia or fungal RNA and measured by ^{3}H-thymidine incorporation. Shown are % $CD4^+$ or $CD8^+$ T cell clones/10^6 cells. (D) Purified T cell subsets were stimulated with 0.1% PHA in the presence of autologous PBMCs. DNA synthesis was measured by ^{3}H-thymidine uptake (cpm, counts per minute). None, untreated DCs. Ct, T cells alone. Data are mean values ± SD of at least 3 independent samples for each +95C/A genotype assessed in triplicates. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; treated vs. untreated (None) $BDCA\text{-}3^+$ DCs.