SUPPLEMENTARY APPENDIX

Induction of *A. fumigatus*-specific CD4-positive T cells in patients recovering from invasive aspergillosis

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Online supplementary methods

Aspergillus antigens and synthetic peptides

Overlapping peptides of the *Aspergillus* proteins Crf1 and Catalase1, consisting of 15mer peptides with an 11 amino acid overlap, were synthesized by JPT Peptide Technologies (Berlin, Germany). Peptides were divided in a complete peptide pool consisting of all overlapping peptides, subpools of 8 to 12 peptides, and 96 single peptides for Crf1 and 180 single peptides for Catalase1. For additional analyses we used overlapping peptides of the *Aspergillus* proteins Aspf1, Aspf2, Aspf3 and Aspf4, also synthesized by JPT Peptide Technologies. To confirm specificity of the T cell clones synthetic peptides were made at the Leiden University Medical Center (LUMC, Leiden, Netherlands).

For the production of Catalase1 recombinant protein three Catalase1 fragments were generated with a 12aa overlap. The fragments were cloned into pDon221 and following sequencing cloned into Gateway (Invitrogen) bacterial expression vector pDEST17. Vectors were transformed into *Escherichia coli* BL21 and protein expression was induced by 1 mM IPTG (Promega). Recombinant protein was isolated by purification of inclusion bodies.

For the preparation of *Aspergillus* crude extract, conidia of *A. fumigatus* strain CBS 144.89 (10^6/ml) were cultured in 70 ml of liquid complete medium, and gently shaken at 37°C overnight. The mycelium was ground in the presence of liquid nitrogen and protein extraction buffer was added. Protein content was measured by the Bio-Rad technique. Furthermore, commercially available crude extracts of the *A. fumigatus* strains CBS 192.65 (HAL Allergy, Leiden, Netherlands) and *A. fumigatus* strain CBS 545.65 (Allergon, Ängelholm, Sweden) were used.

Flowcytometry

In some patients we performed intracellular staining with PerCP-Cy5.5 labeled anti-IL-4 (BD/Pharmingen), PE-labeled anti-IL-5 (BD/Pharmingen) and Brilliant Violet 605-labeled anti-IL-17 (ITK Biolegend, Uithoorn, Netherlands), combined with fluorescein isothiocyanate (FITC)-labeled anti-CD154 (BD/Pharmingen). In those experiments, cells were after restimulation analyzed on the LSR II (BD).

Determination of Aspergillus reactivity and HLA-restriction

To determine *Aspergillus* reactivity, peptide specific clones were stimulated overnight with autologous EBV-LCL (R:S ratio 1:4) loaded with recombinant protein (100 μg/ml) or with autologous monocyte derived DC preloaded with *Aspergillus* crude extract (R:S ratio 1:4). Briefly, for the generation of immature DC we isolated monocytes from PBMC by MACS using anti-CD14 coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and CD14+ cells ($1x10^6$) were cultured in 24-well plates in 1 ml of IMDM supplemented with 10% human serum, 500 IU/ml IL-4 (Schering-Plough) and 100 ng/ml GM-CSF (Novartis). After 2 days, immature DC were preloaded with *Aspergillus* crude extract (50 μg/ml) for 4 hours, and then matured by adding 100 ng/ml GM-CSF, 10 ng/ml TNFα (Cellgenix, Freiburg, Germany), 10 ng/ml IL1-β (Cellgenix), 10 ng/ml IL-6 (Cellgenix), 1 μg/ml prostaglandin E_2 (Sigma-Aldrich) and 500 IU/ml IFNγ (Boehringer-Ingelheim, Alkmaar, Netherlands) for 3 days.

To determine the HLA-restriction of the T cell clones, autologous EBV-LCL or autologous PBMC were loaded with the single peptide (10^{-6}M) and incubated for 2 hours. After washing the stimulator cells, saturating concentrations of mAbs specific for HLA-class II (PdV5.2), HLA-DR (B8.11.2), HLA-DQ (SPVL3) or HLA-DP (B7.21) were added and incubated for 1

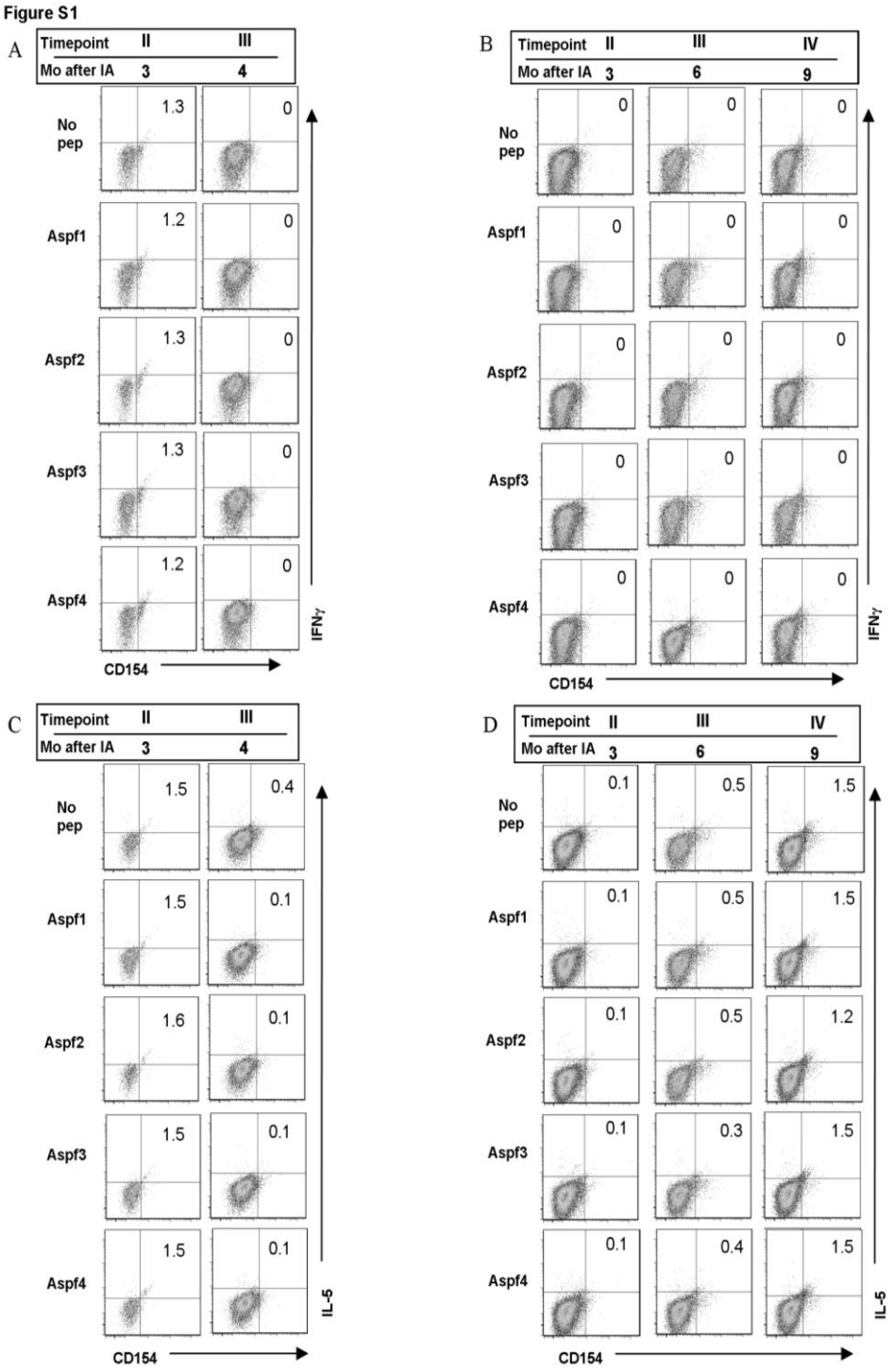
hour. Subsequently, T cells were added in a R:S ratio of 1:4 and incubated ON. Supernatant was collected and analyzed using standard ELISA. Further determination of the HLA-restriction was analyzed using the HLA-typed EBV-LCL panel, containing partially matched allogeneic EBV-LCL. The T cell clones and EBV-LCL (R:S ratio 1:4) were incubated ON with the complete overlapping peptide pool (10^{-6}M) and the supernatant was tested for cytokine production in ELISA. In case of unclear results, the HLA-restriction of the T cell clones was determined by loading the peptide pool on HLA-class II negative Hela cells that were transduced with the HLA-DR, -DQ or -DP molecules expressed by the individuals the T cell clones were derived from.

Supplementary table S1. Identified Crf1 and Catalase1 epitopes in patients with invasive aspergillosis

Epitope	aa sequence	Clone	Cytokine production	Vβ‡§	HLA-restriction	*Aspergillus* extract	Catalase1 rec protein*
Crf1							
aa 57-67	EVTAGKVPVGP	MLF 77	IFNγ	7.2	DRB1*11	yes	n/a
aa 57-67	EVTAGKVPVGP	ACD 11, JHF 31	IL-17, IFNγ	17, 13.2	DQB1*0301	yes	n/a
aa 89-99	**FFFGKAEVVMK**	ACD 4	IFNγ	u.k.	DRB1*0101	yes	n/a
aa 122-132	VLESDDLDEVD	NKB 14	IFNγ	2	DQB1*0202	yes	n/a
aa 161-175	**HTYTIDWTKDAVTWS**	FBV 16	IFNγ	u.k.	DRB3*01	yes	n/a
aa 165-179	**IDWTKDAVTWSIDGA**	FBV 1	IFNγ	2	DPB1*0101	yes	n/a
aa 169-183	KDAVTWSIDGAVVRT	MLF 3	IFNγ	u.k.	DRB1*03	yes	n/a
aa 217–231	GTIEWAGGLTDYSAG	JHF 21	IFNγ	2	DQB1*0301	yes	n/a
aa 233-243	**YTMYVKSVRIE**	NKB 6, JHF 22	IFNγ, IFNγ	8, 13.1	DRB1*0701	yes	n/a
aa 237-247	VKSVRIENANP	JHF 25	IFNγ	u.k.	DRB4*0103	yes	n/a
Catalase1							
aa 117-127	**DFSNITAASFL**	NKB 10	IFNγ	2	DRB1*0701	no	no
aa 137-147	FVRFSTVAGSR	JHF 5	IFNγ	3	DRB1*0408	no	yes
aa 333-347	DPTKIVPEEFVPITK	ACD 3	IFNγ	3	DPB1*0401/DPB1*0402	yes	yes
aa 345-355	**GKMQLNRNPRN**	ACD 1, ACD 5	IFNγ, IFNγ	12, u.k.	DRB3*02	yes	yes
aa 497-507	**IRFENANVKSP**	TPA 6	IL-17	22	DRB1*15	no	yes
aa 705-719	**DDFANDLKEGLRTFK**	FBV 9	IL-4	2	DRB1*0301	no	no

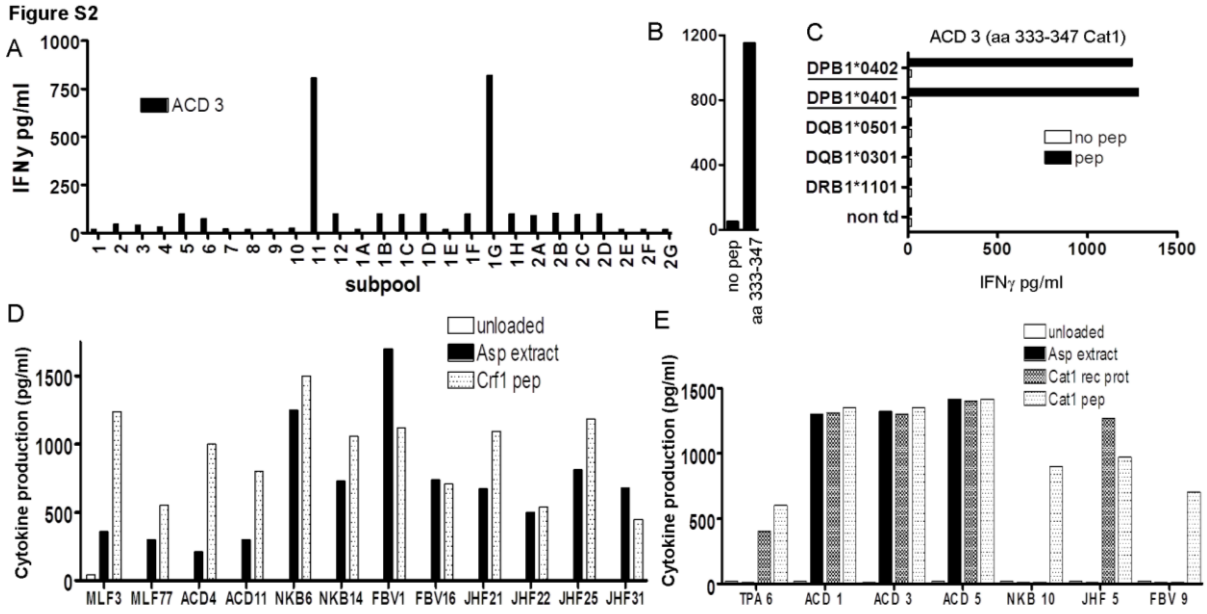
* n/a: not applicable
‡ Vβ analysis by TCR Vβ monoclonal antibodies
§ u.k.: Vβ unknown when not present in TCR Vβ kit
Bold: epitopes previously identified in healthy individuals

Figure S1



FACS-analyses of T-cell responses specific for Aspf1, Aspf2, Aspf3 and Aspf4 in patients with progressive invasive aspergillosis.

FACS-analyses were performed on PBMC of patient MST (A and C) and patient ESF (B and D) after restimulation with Aspf1- Aspf2- Aspf3- or Aspf4-pulsed PBMC or with unpulsed PBMC (no pep). T-cells were analyzed 5 hours after restimulation and FACS-plots are gated on CD4+ T-cells. CD154-expression and IFNγ-production are depicted in figure A and B, CD154-expression and IL-5 production are depicted in figure C and D. In the top of the graphs the timepoints are indicated by Roman numerals and the corresponding timeperiod in months after invasive aspergillosis (Mo after IA) is given for every timepoint. The timepoints in figure A and C correspond to the timepoints in the graph of figure 3B and the timepoints in figure B and D correspond to the timepoints in the graph of figure 3E.



Determination of epitope-specificity, HLA-restriction and *Aspergillus* reactivity.

(A) IFNγ production by T-cell clone 3 from individual ACD after ON stimulation with the subpools of Catalase1 loaded on autologous PBMC. Every peptide is only present in 2 subpools, to be able to identify the target peptide directly from the subpool analysis. The target peptide for clone ACD3 is peptide 1G11 (aa 333-347, DPTKIVPEEFVPITK). (B) The T-cell epitope recognized by the clone was confirmed by stimulating the clone with newly synthesized peptide. (C) HLA-restriction of clone ACD3 was confirmed by testing with peptide-loaded HLA class II transduced Hela cells. The HLA-restriction molecules used for presentation of the epitope are underlined. T-cell clone ACD3 was isolated from an individual with HLA type DRB1*01, DRB1*1101, DRB3*02, DQB1*0301, DQB1*0501, DPB1*0401 and DPB1*0402. (D) Crf1-specific T-cell clones were stimulated with autologous monocyte-derived dendritic cells that were preloaded with *Aspergillus* protein extract for 4 hours and then matured for 3 days. (E) Catalase1-specific T-cell clones were stimulated with unloaded autologous dendritic cells or with autologous dendritic cells preloaded with *Aspergillus* protein extract, dendritic cells loaded with Catalase1 recombinant protein or dendritic cells loaded with Catalase1 overlapping peptide pool. IFNγ production was measured after ON stimulation.