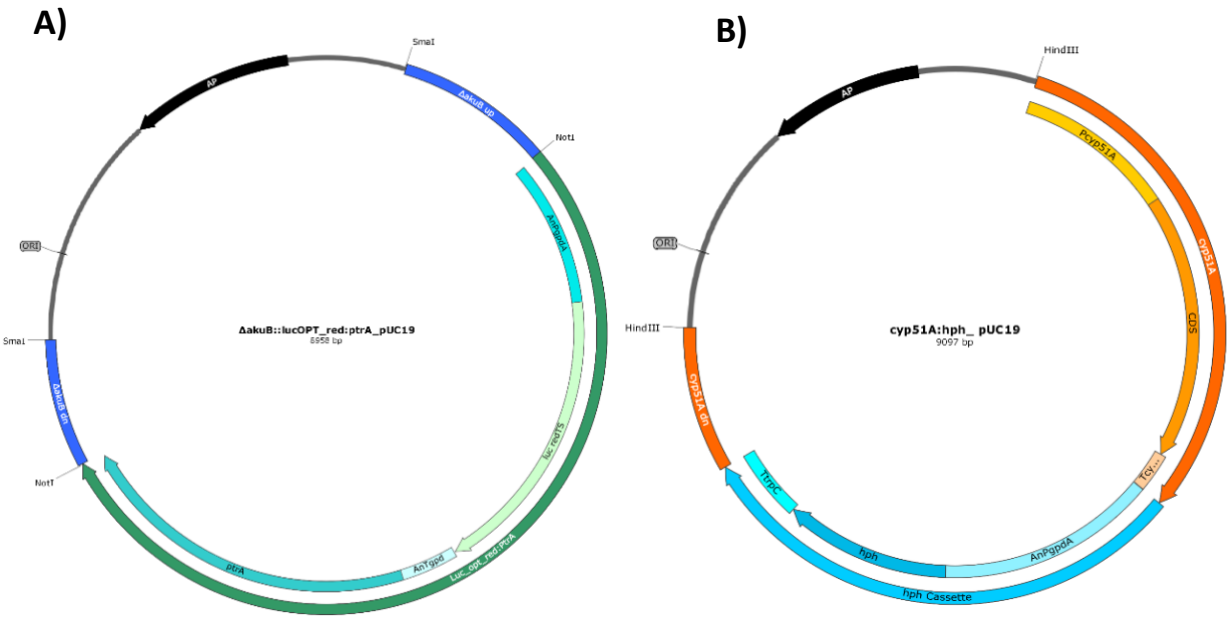

Fig. S1. Maps of plasmids generated in this study. A) Plasmid map of the *akuB* deletion cassette for expression of the red-shifted thermostable luciferase (*luc*_redTS) from the *akuB* locus in *A. fumigatus*. The cassette was excised by *Sma*I restriction and used for transformation of the *A. fumigatus* wild-type strain CBS144.89. **B)** Plasmid map of the cyp51A and hygromycin complementation cassette for the transformation of the genetically modified triazole-resistant *A. fumigatus* strains and reversion to the wild-type *cyp51A* gene. To perform the transformation of Af_Δ*akuB*::*luc*^{OPT_red}_ptrA_4003-new7 and Af_Δ*akuB*:: *luc*$_{OPT_red}$_ptrA_3216-1, the complementation construct was released from the vector backbone by *Hind*III restriction and gel-purified. **Abbreviations***:* Upstream region of the *akuB gene (ΔakuB up),* glyceraldehyde-3-phosphate dehydrogenase gene *(gpdA)* promoter *(AnPgpdA)* and terminator *(AnTgpd)* region*, pyrithiamine resistance gene* (*ptrA*), downstream region *of the akuB* gene (*ΔakuB* dn), *cyp51A* gene (*cyp51A)* promoter (*Pcyp51A*), coding region (CDS) and terminator (T*cy*) regions, hygromycin B resistance gene (*hph*), tryptophan terminator (T*trpC*), downstream region of the *cyp51A* gene (*cyp51A* dn).

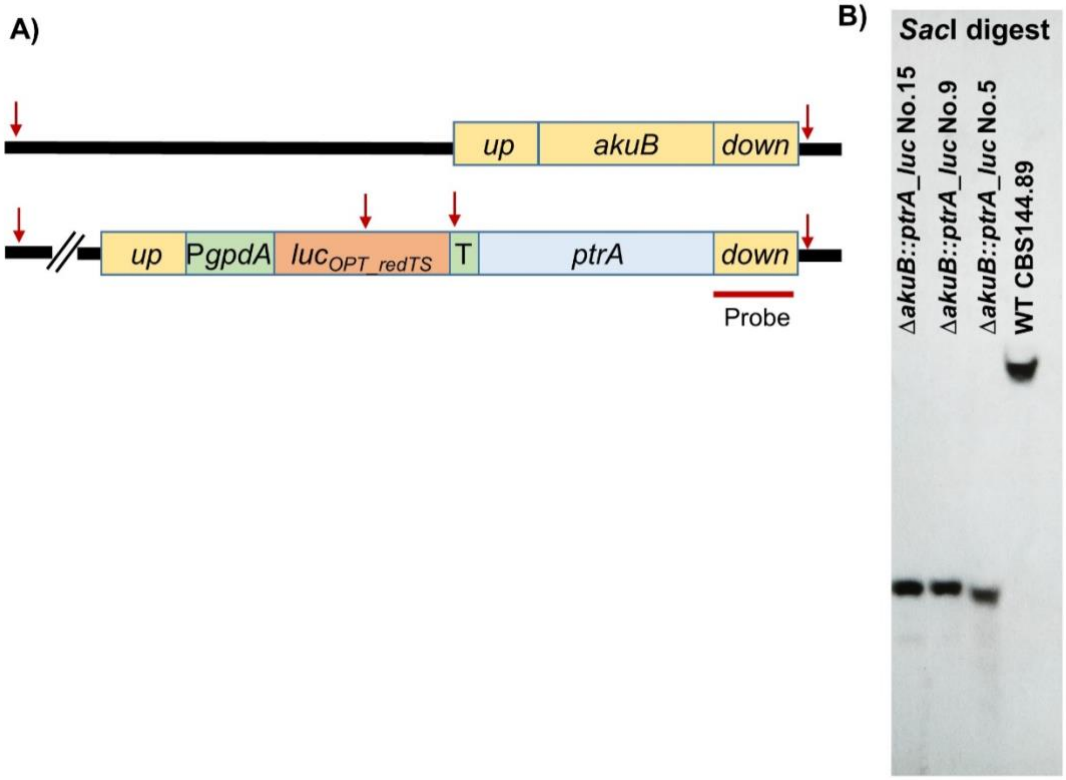

Fig. S2A. Southern blot analysis for confirmation of the replacement of the *akuB* locus in *A. fumigatus* by the luciferase expression construct. (A) Scheme of the genomic *akuB* locus in wild type and deletion mutants. The *akuB* locus was replaced by a codon-optimised and red-shifted thermostable firefly luciferase under control of the *gpdA* promoter (P*gpdA*) and its terminator (T). The pyrithiamine resistance gene (*ptrA*) served as marker in the transformation. *Sac*I restriction sites are indicated by arrows. A probe against the downstream region was generated. **(B)** Southern blot analysis of *Sac*I digested genomic DNA of wild type and deletion mutants showing the expected shift of the wild-type signal from 6193 bp to 3670 bp in the luciferase-expressing *akuB* deletion mutants. Strain No. 5 was used in subsequent experiments as parental strain.

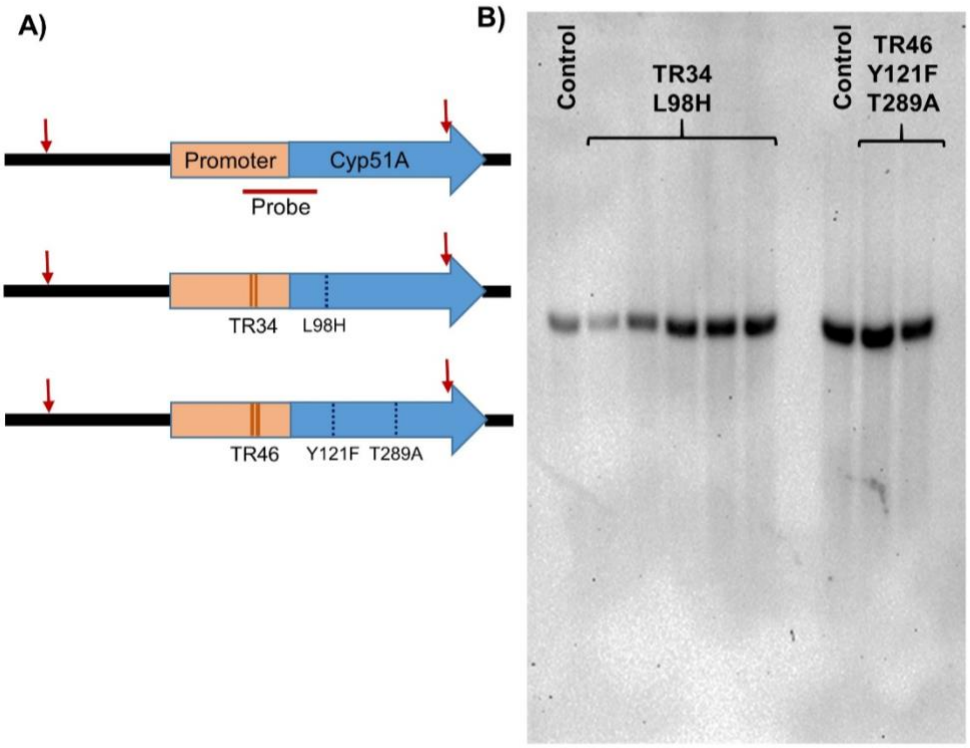

Fig. S2B. Southern blot analysis of itraconazole resistant *A. fumigatus* transformants. The parental Δ*akuB::luc* strain was transformed with the respective promoter and partial *cyp51A* gene fragment to achieve the desired mutations of the *cyp51A* gene. Transformants were regenerated on an itraconazole containing medium. **(A)** Scheme of the genomic situation of the *cyp51A* gene in the parental control strain and the *cyp51A* mutants. *Bam*HI restriction sites are indicated by arrows and result in a 3596 bp fragment for the *cyp51A* wild-type situation, 3630 bp for TR34/L98H mutants and 3642 bp for TR46/Y121/T289A mutants. **(B)** Southern blot analysis of *Bam*HI restricted genomic DNA of parental control strain and itraconazole resistant transformants. Only a single copy of the *cyp51A* gene is detected. Mutations in the *cyp51A* promoter and coding region were confirmed from selected strains by gene sequence analysis.

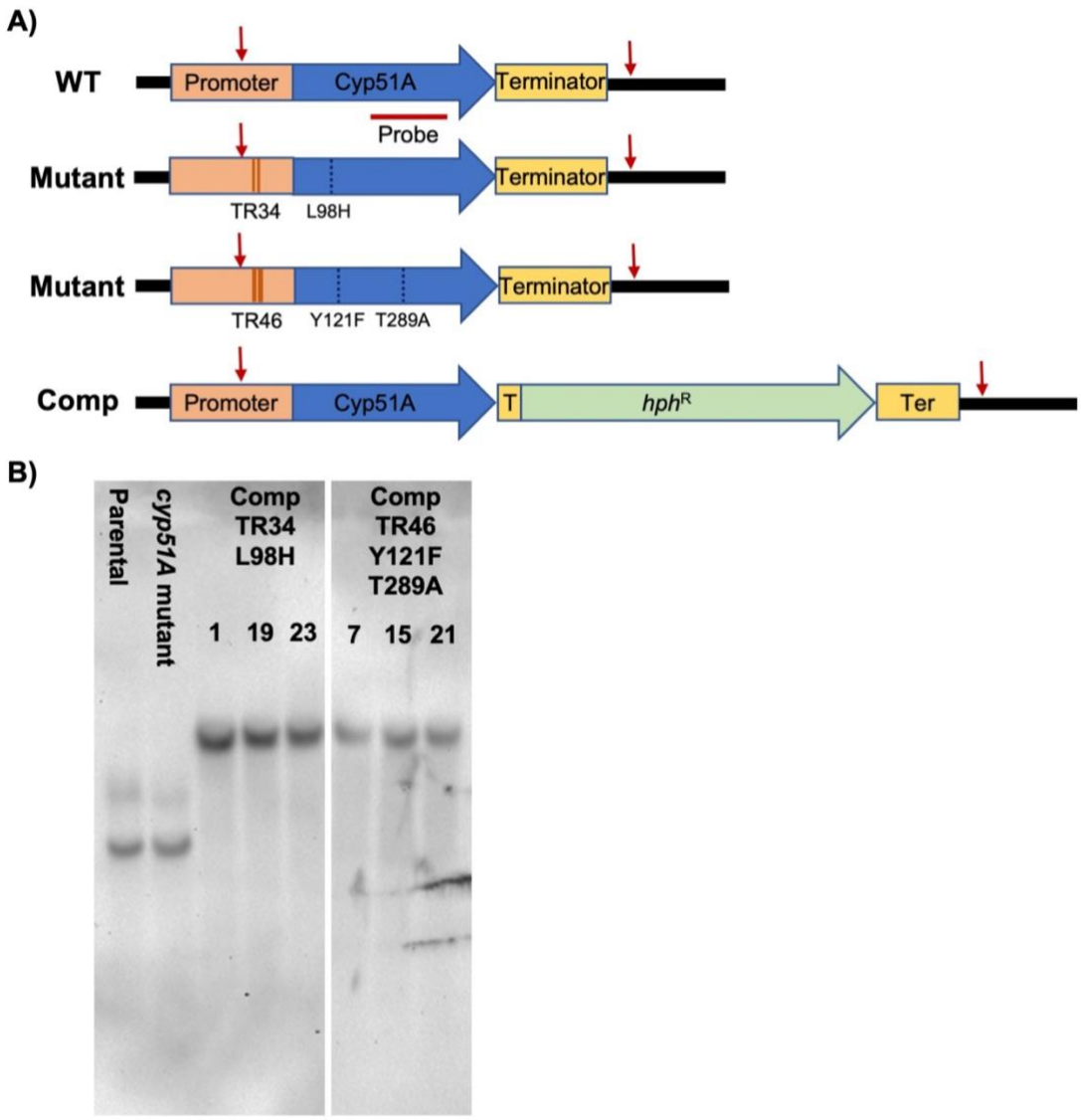

Fig. S2C. Southern blot analysis confirming the integration of the complementation construct into the *cyp51A* locus of triazole resistant mutants. The mutated *cyp51A* gene copy was replaced by the wild-type *cyp51A* gene using the hygromycin B resistance marker for selection of transformants. (A) Scheme of the genomic *cyp51A* gene situation in the in the parental control strain (WT), the *cyp51A* mutants and complemented mutants (Comp). *Xba*I restriction sites are indicated by arrows and result in a 3 kb fragment before and a 6 kb fragment after complementation. (B) Southern blot of *Xba*I restricted genomic DNA of parental control strain a *cyp51A* mutant strain and complemented mutants (Comp). A single signal with the expected size shift is observed in the complemented strains.

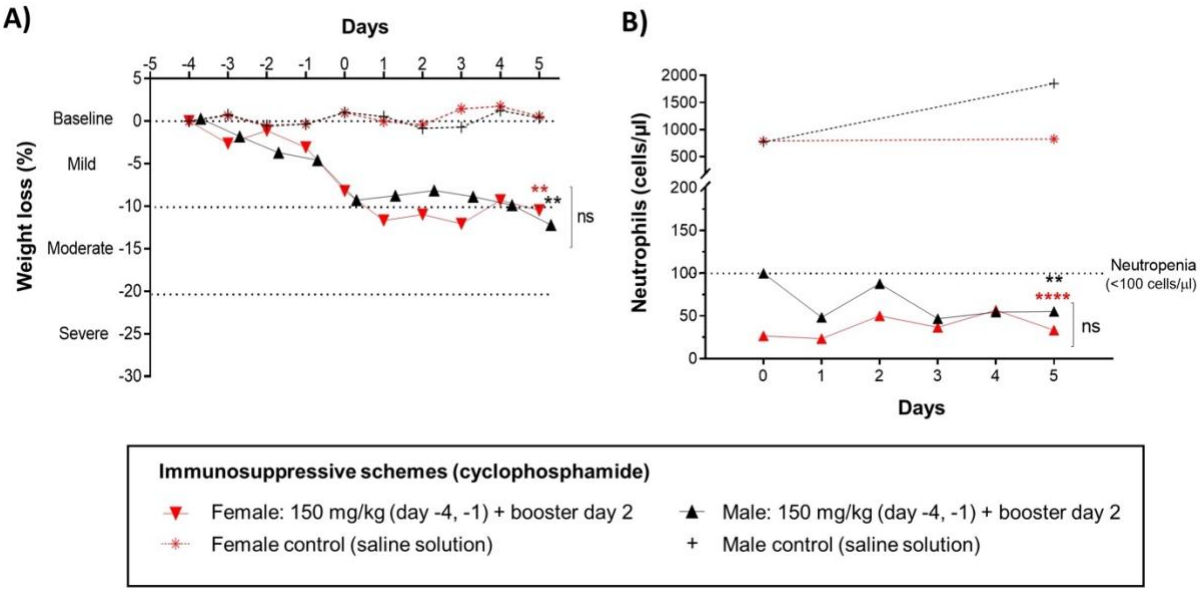

Fig. S3. Scheme of immunosuppressive effects on weight loss and neutropenia induction by cyclophosphamide injection with 150 mg/kg (day -4, -1) + booster at day 2 amongst male and female mice. A) Evolution of weight loss (% compared to baseline; mild 0-10%, moderate 10-20% and severe >20%) of tested cyclophosphamide immunosuppressive scheme between male and female mice and corresponding control group (saline solution) from day -4 (initiation of immunosuppressive therapy/baseline) until day 5. Graph represents mean value of weight loss percentage. **B)** Neutrophil count (cells/µl) kinetics after cyclophosphamide-induced immunosuppression between the female and male group and controls. Graph represents mean value (n = 3, per group per time point). *Mixed model ANOVA (multiple comparison analysis, Tukey correction)* to control group (above) and between gender *(lateral); * p = <0.05, ** p = <0.005, *** p = <0.0005, ns: non-significant.*

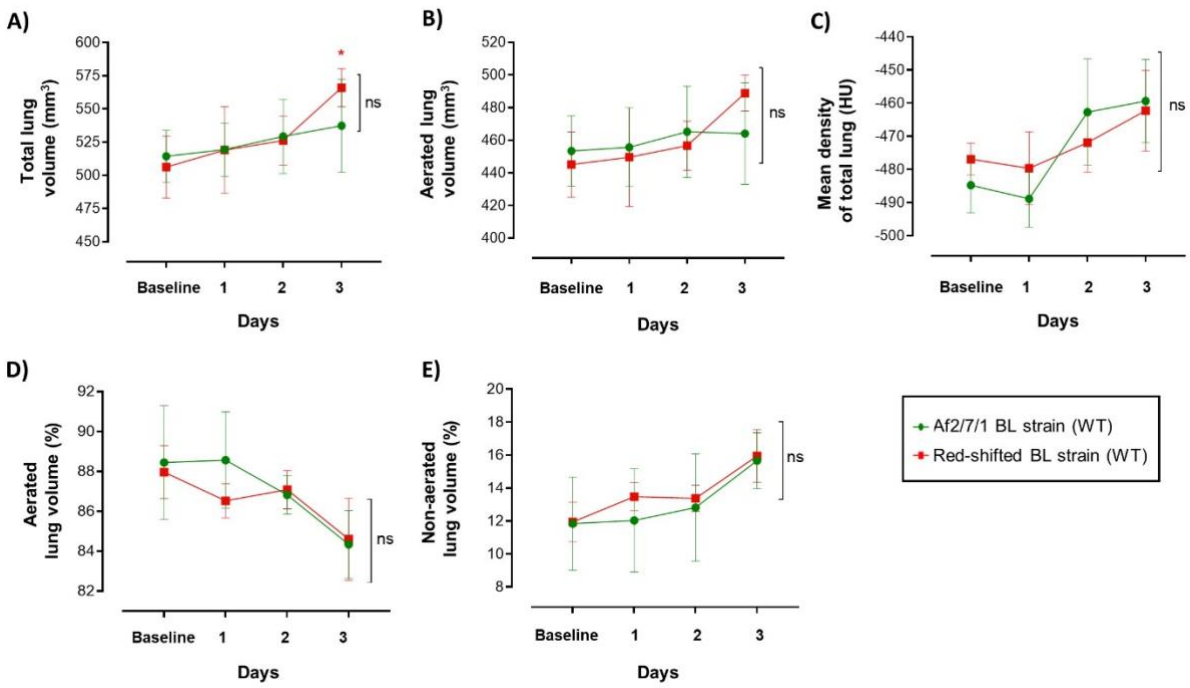

Fig. S4. Micro-CT-derived lung biomarkers of mice infected with the wild-type Af2/7/1 and Af_luc$_{OPT_red}$_WT ***Aspergillus fumigatus*** **strains.**

A) Micro-CT derived biomarkers quantifying the total lung volume and **B)** aerated lung volume of intranasally infected mice (5×10^5 spores) with the wild-type BL Af2/7/1/2 and Af_luc$_{OPT_red}$_WT strains. **C)** Mean density of total lung (HU) evolution over time. **D)** Aerated, and **E)** non-aerated lung volume percentages from total lung volume variances over time for infected mice. *Error bars represent SD of the results (mean) from multiple mice (n = 5 male). Two-way repeated measures ANOVA (multiple comparison analysis, Tukey correction); * p = 0.05, to baseline (above) and between groups (lateral).* ***Abbreviations:*** *Wild type (WT), Bioluminescent (BL), micro-computed tomography (micro-CT), Hounsfield Unit (HU), non-significant (ns).*

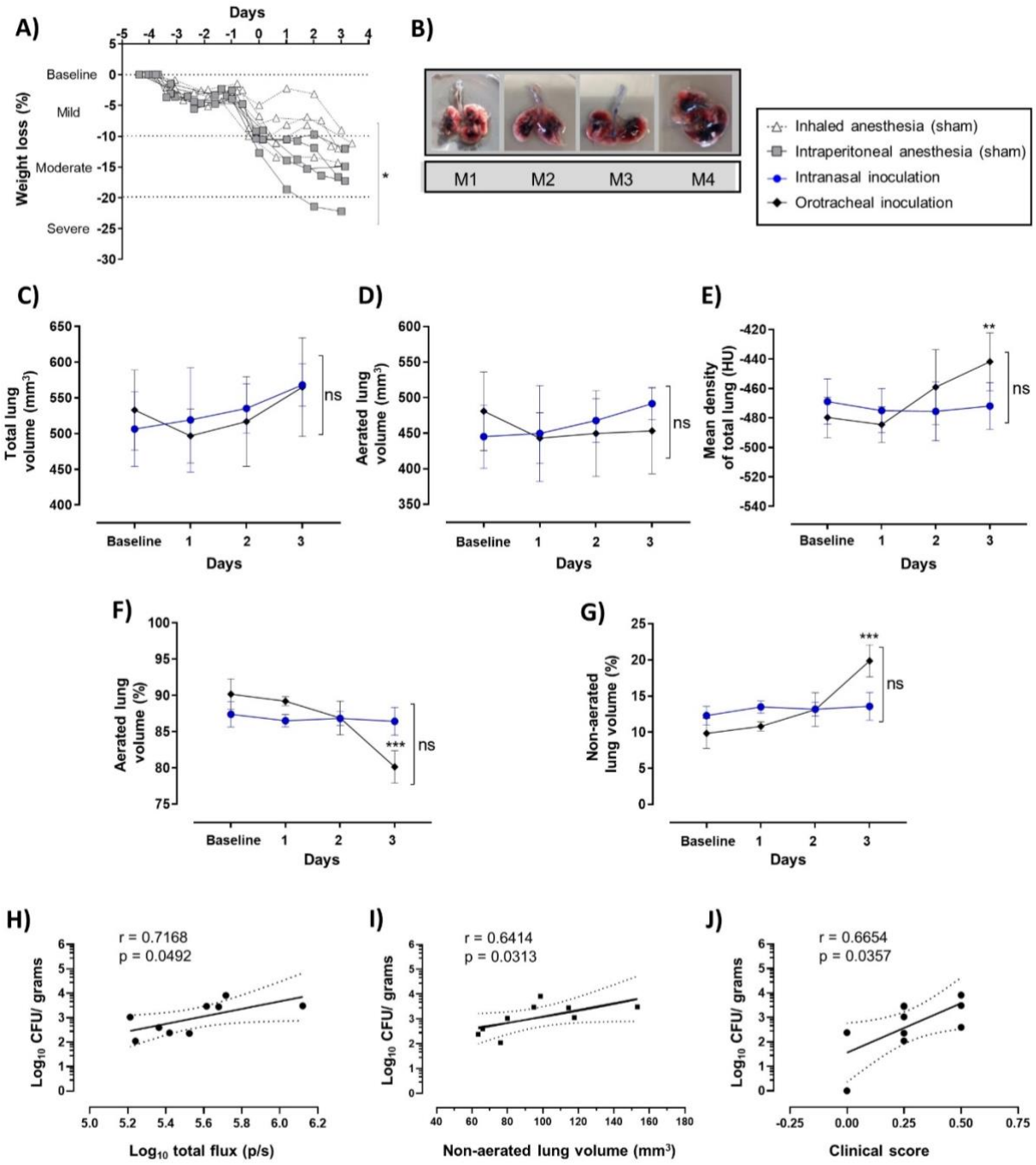

Fig. S5. Orotracheal route of inoculation characterization of non-infected mice, micro-CT derived biomarkers and correlation of imaging biomarkers from orotracheally and intranasally infected mice. A) Relative weight loss evolution in non-infected mice orotracheally sham-infected (25 µl saline-solution) immunocompromised mice after inhaled or peritoneally injected anaesthesia. **B)** Non-infectious *ex vivo* orotracheal route technique-assessment in mice (n = 4 male) after instillation with trypan blue (25 µl). **C)** Quantitative total and **D)** aerated lung tissue volume measurements of intranasally and orotracheally infected mice (5×10^5 Af_luc$_{OPT_red}$_WT spores) over time. **E)** Mean total lung density volume per mouse group over the time course of infection (HU). Percentage variances of **F)** aerated and **G)** non-aerated lung volumes from total lung volume before (baseline) and after infection.

Correlation and linear regression analysis of infected mice of quantified imaging biomarker readouts: **H)** bioluminescent signal (log_{10} total flux total), **I)** lung lesion development (non-aerated lung volume; mm^3) and **J)** cumulative visual clinical score of lung lesion based on imaging scans to lung CFU counts (log_{10}) on day 3. *Error bars represent SD of the results (mean) from multiple mice (n = 5 male), dashed lines represent the 95% confidence band. Two-way ANOVA (multiple comparison analysis, Tukey correction), Pearson correlation; * p = <0.05, ** p = <0.005, *** p = <0.0005, to baseline (above) and between groups (lateral).* ***Abbreviations:*** *Wild type (WT), micro-computed tomography (micro-CT), non-significant (ns), Hounsfield Unit (HU), colony forming units (CFU).*

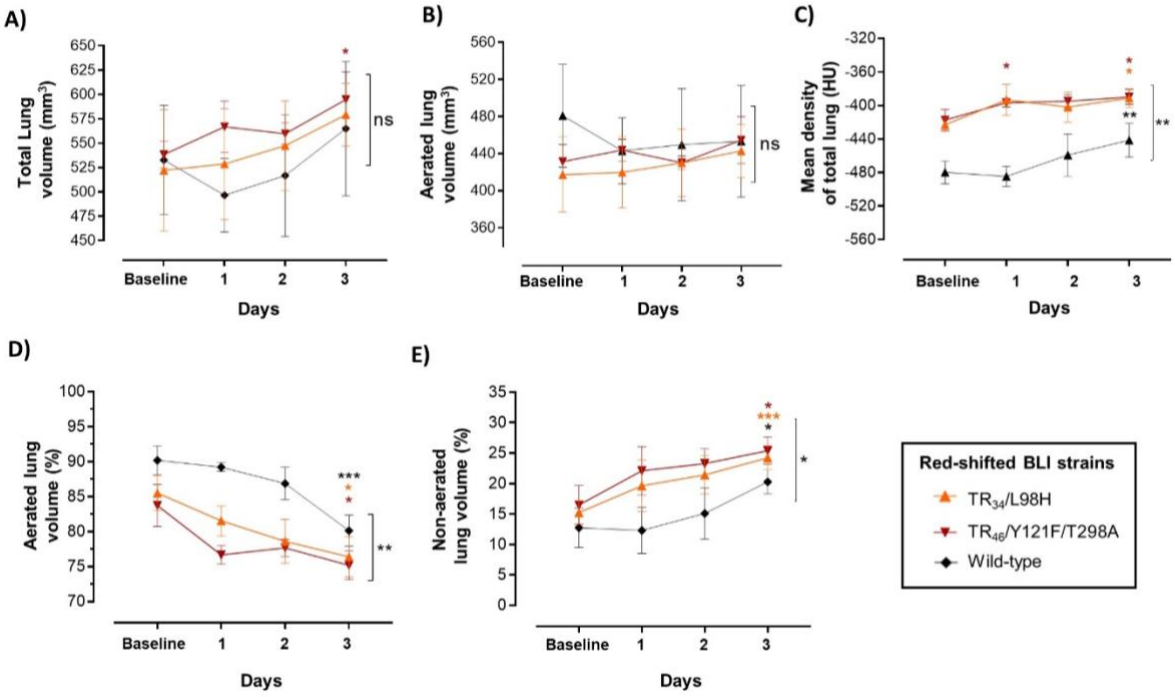

Fig. S6. Micro-CT derived lung biomarkers upon IPA development after infection with red-shifted luciferase expressing wild-type and triazole-resistant *Aspergillus fumigatus* strains.

A) Quantification of micro-CT derived total lung volume and **B)** aerated lung volume of mice orotracheally infected with red-shifted luciferase expressing and triazole-resistant (Af_luc$_{OPT_red}$_TR34, _TR46) and WT (Af_luc$_{OPT_red}$_WT) *A. fumigatus* strains (5 × 10^5 spores) before and after infection. **C)** Mean density of total lung volume over time (HU). **D)** Longitudinal aerated and **E)** non-aerated lung volumes percentages relative to total lung volume of infected animals upon infection per day. *Error bars represent SD of the results (mean) from multiple mice (n = 5 male). Two-way repeated measures ANOVA (multiple comparison analysis, Tukey correction); * p = <0.05, ** p = <0.005, *** p = <0.0005, to baseline (above) and WT (lateral).* ***Abbreviations:*** *IPA (invasive pulmonary aspergillosis), wild type (WT), micro-computed tomography (micro-CT), non-significant (ns).*

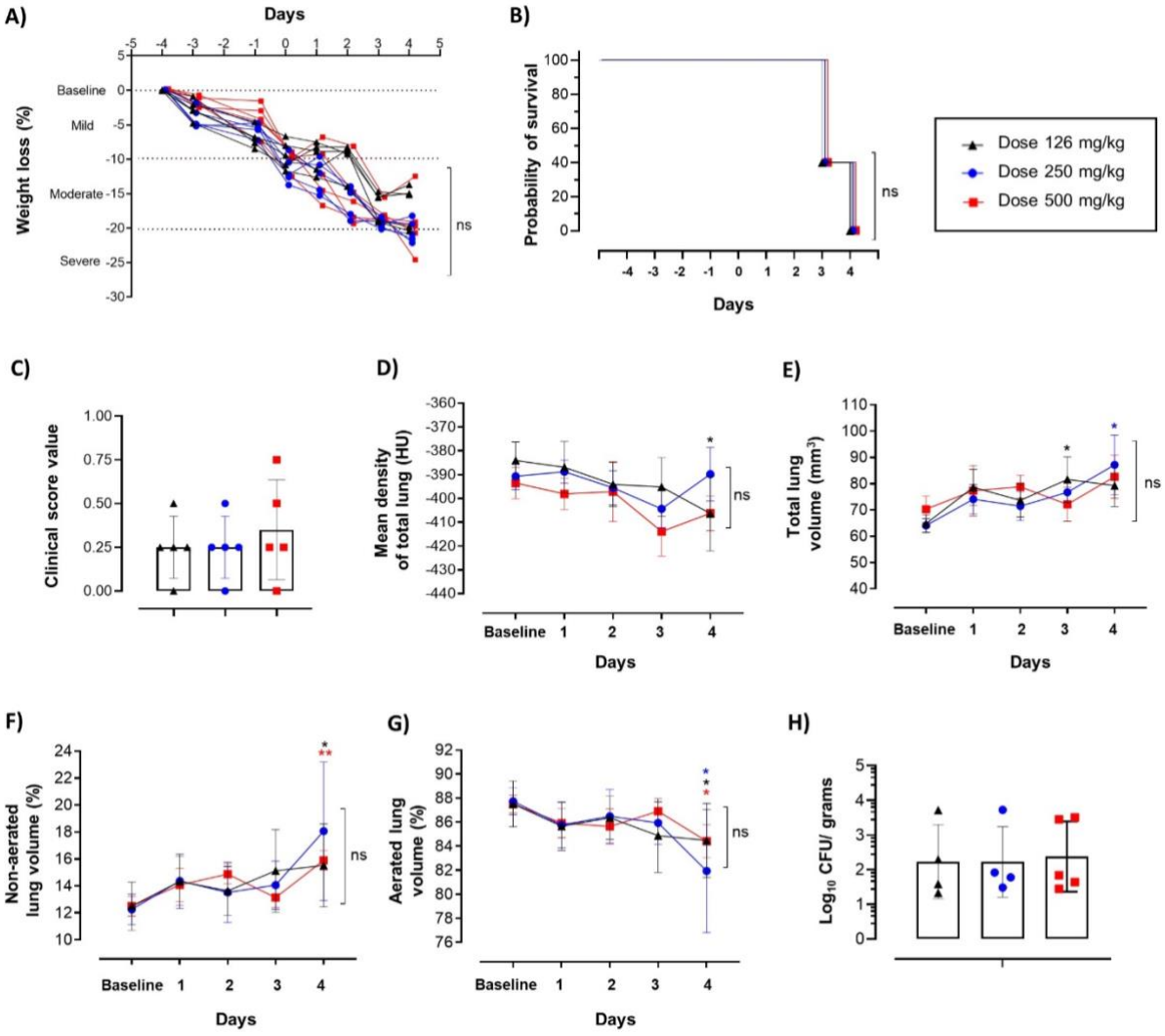

Fig. S7. Multimodal imaging of *in vivo* luciferin dose-dependent effects in *Aspergillus* infected mice A) Longitudinal weight loss percentage (%) evolution and **B)** survival comparison of orotracheally infected mice (Af_luc$_{OPT_red}$_WT 5 × 10^5 spores) according to the administered luciferin dose of 126 mg/kg, 250 mg/kg and 500 mg/kg. **C)** Micro-CT lung scan lesion development visual score (cumulative clinical score) of infected mice, grouped per luciferin dose. **D)** Longitudinal quantification of mouse lung micro-CT scans: mean density of total lung volume (HU) and **E)** non-aerated (lung lesion) lung volume (mm^3), grouped according to administered luciferin dose. **F)** Lung non-aerated and **G)** aerated lung volume percentage variances from total lung volume evolution over time, grouped per administered luciferin dose. **H)** Mean colony forming unit counts (log_{10} CFU/gram counts) of lung homogenates of infected mice per luciferin dose, evaluated at sacrifice (day 3-4 after inoculation). *Error bars represent SD of the results (mean) from multiple mice (n = 5 male). Two-way repeated measures ANOVA (multiple comparison analysis, Tukey correction; A, C-H), Log-rank (Mantel-Cox) test (B); * p = <0.05, ** p = <0.005; to baseline (above) and between groups (lateral).* ***Abbreviations:*** *Wild type (WT), micro-computed tomography (micro-CT), Hounsfield Units (HU), non-significant (ns), colony forming units (CFU).*

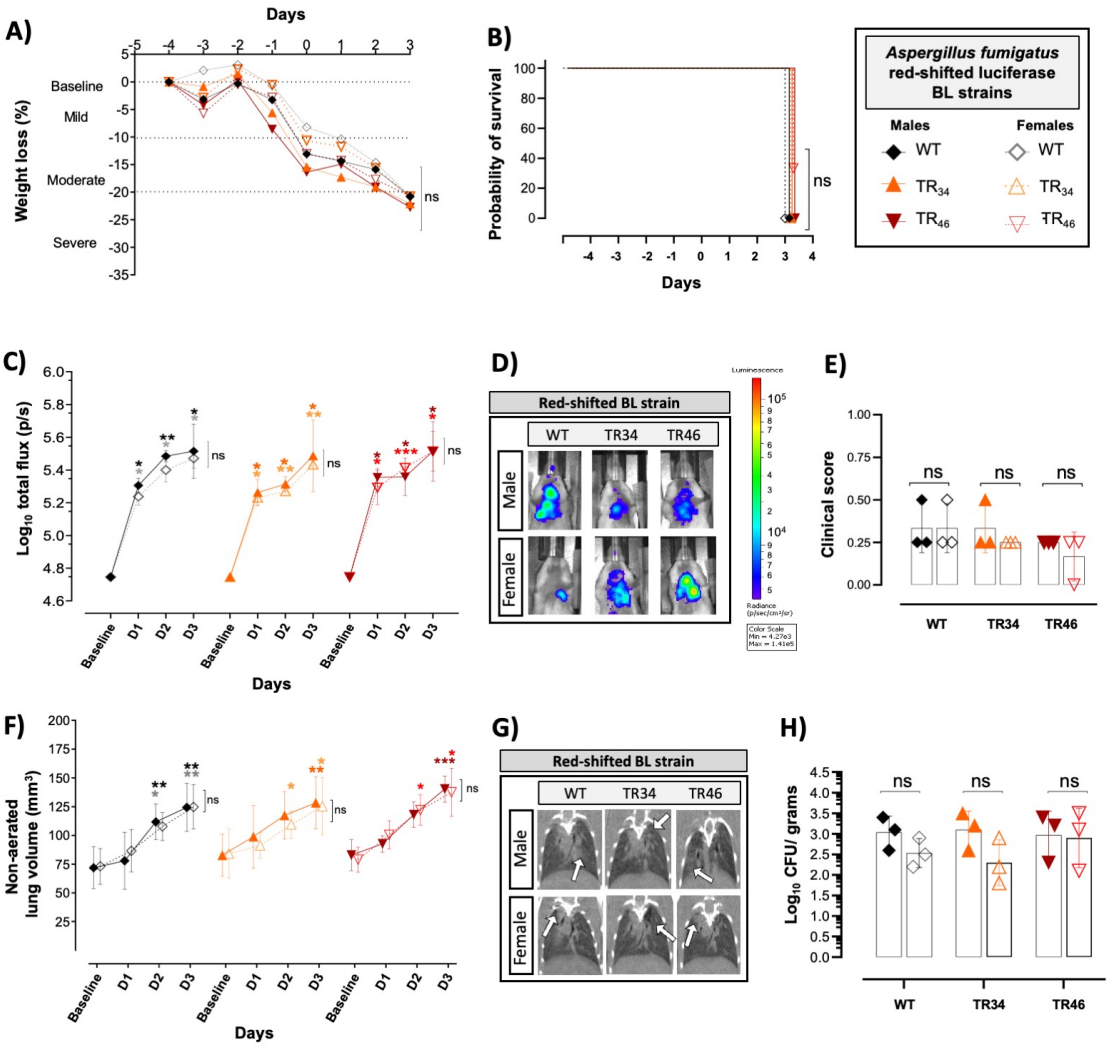

Fig. S8. Multimodal disease development comparison of bioluminescent *Aspergillus fumigatus* strains in male and female mice. A) Longitudinal weight loss percentage (%) and **B)** survival comparison of orotracheally inoculated cyclophosphamide immunosuppressed male and female mice with the Af_luc$_{OPT_red}$_WT, Af_luc$_{OPT_red}$_TR34 and Af_luc$_{OPT_red}$_TR46 strains (5 × 10^5 spores; n = 3 per group). **C)** Lung BL signals (log_{10} total flux) of infected mice before (baseline) and after infection, **D)** and representative BL images on day three after inoculation. **E)** Cumulative clinical scores representing visual assessment of lung lesion development on micro-CT scans and**, F)** quantitative evaluation of lung lesion development (non-aerated lung volume) over time. **G)** Representative coronal pulmonary micro-CT images of infected groups (arrows denote site of lesions). **H)** Colony-forming unit counts (log_{10} CFU/gram) from lung homogenates of infected mice at sacrifice (day 3 after inoculation). *Error bars represent SD of the results (mean) from multiple mice (n = 3), Two-way repeated measures ANOVA (multiple comparison analysis, Tukey correction; A,C,E,F,H), Log-rank (Mantel-Cox) test (B); * p = <0.05, ** p = <0.005, *** p = <0.0005.* ***Abbreviations:*** *Wild type (WT), bioluminescent (BL), micro-computed tomography (micro-CT), colony forming units (CFU), non-significant (ns).*

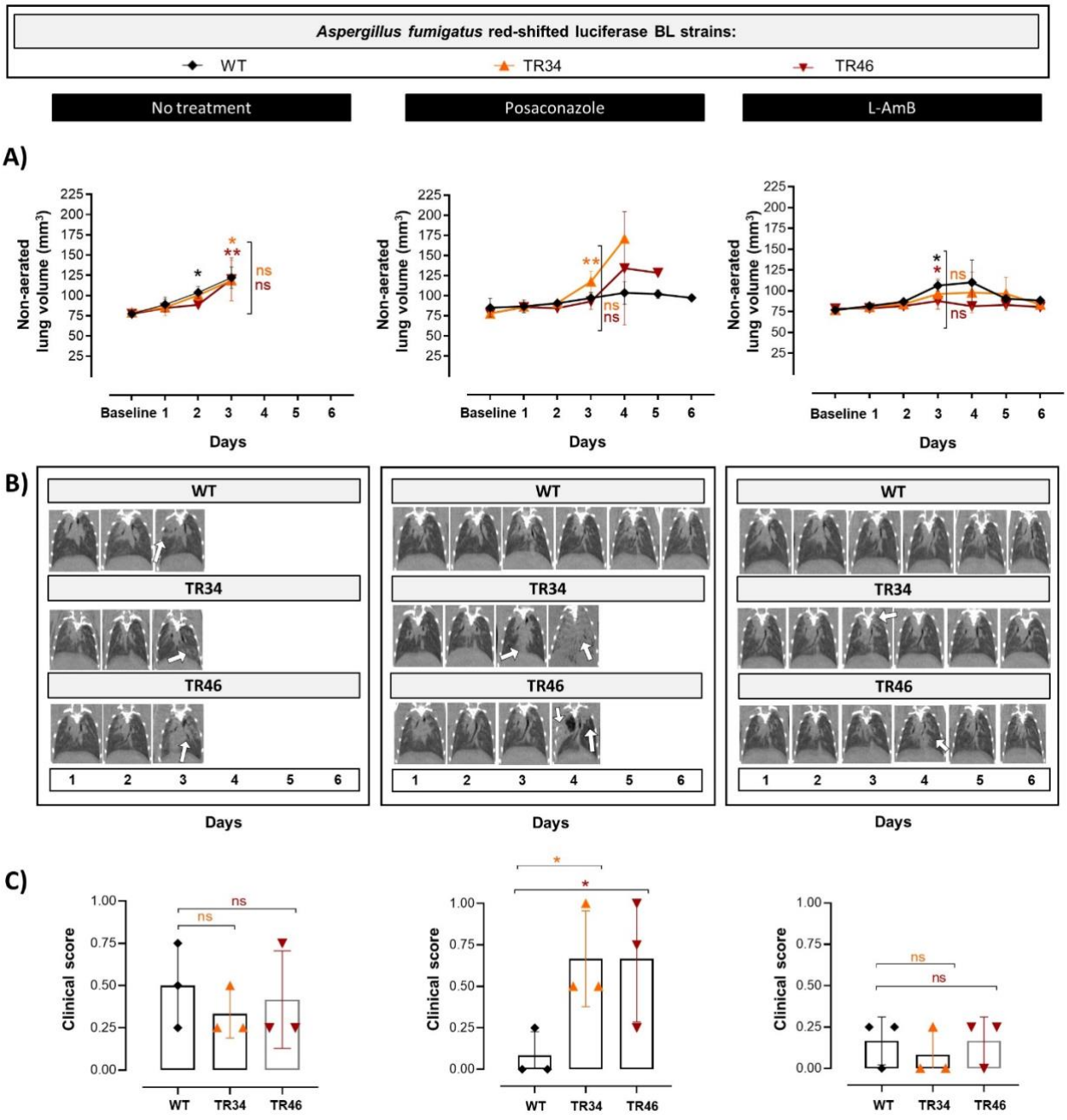

Fig. S9. Micro-CT biomarker comparison on IPA development after *in vivo* antifungal treatment of infected mice with triazole-susceptible and -resistant BL *A. fumigatus* strains. A) Lung lesion development (non-aerated lung volume) before (baseline) and after infection. **B)** Longitudinal representative coronal pulmonary micro-CT images of infected groups and **C)** cumulative clinical scores representing visual assessment of lung lesion development on micro-CT scans (arrows denote site of lesions). *Error bars represent SD of the results (mean) from multiple mice (n = 3 male). Two-way repeated measures ANOVA (multiple comparison analysis, Tukey correction); * p = <0.05, ** p = <0.005.* ***Abbreviations:*** *BL (bioluminescent), wild type (WT), micro-computed tomography (micro-CT), L-AmB (liposomal amphotericin B), non-significant (ns).*

Table S1. Blood cell analysis comparison of cyclophosphamide immunosuppressive schemes *in vivo*

Day	Blood analysis/ weight loss (%)	Cyclophosphamide 100 mg/kg (Day -4, -3, -2, -1, ± booster day 3)	Cyclophosphamide 100 mg/kg (Day -4, -3, -2, -1, + grapefruit juice)	Cyclophosphamide 150 mg/kg (Day -4, -1, ± booster day 2)		Cyclophosphamide 150 mg/kg (Day -4, -1, + grapefruit juice)	Control
0	White blood cells (cells/µl)	283.3	204.5	328.0		226.7	1656.7
	Neutrophils (cells/µl)	135.5	67.3	97.8		78.0	760.0
	Lymphocytes (cells/µl)	131.4	112.5	188.2		94.9	750.3
	Monocytes (cells/µl)	4.6	0.4	5.9		2.0	43.7
	Reticulocytes (cells/µl)	0.1	0.0	0.0		0.0	3.0
	Platelets (cells/µl x 10^3)	147.3	181.5	421.2		292.0	489.7
	Weight loss (%)	*10.1*	*17.3*	*9.6*		*15.8*	*+ 1.1*
1	White blood cells (cells/µl)	233.3	203.3	210.0		229.3	
	Neutrophils (cells/µl)	57.4	82.0	48.3		62.9	
	Lymphocytes (cells/µl)	150.7	65.8	145.6		138.4	
	Monocytes (cells/µl)	3.0	2.4	1.9		6.6	
	Reticulocytes (cells/µl)	0.0	0.0	0.1		0.1	
	Platelets (cells/µl x 10^3)	148.7	175.7	278.5		100.7	
	Weight loss (%)	*9.9*	*22.3*	*9.0*		*19.1*	*+ 0.1*
2	White blood cells (cells/µl)	243.3	583.3	278.3		185.0	
	Neutrophils (cells/µl)	102.8	110.73	87.7		109.9	
	Lymphocytes (cells/µl)	123.6	213.4	155.2		63.1	
	Monocytes (cells/µl)	8.4	11.2	13.6		3.3	
	Reticulocytes (cells/µl)	0.0	0.0	0.0		0.0	
	Platelets (cells/µl x 10^3)	189.7	179.8	282.0		132.3	
	Weight loss (%)	*7.8*	*25.0*	*8.4*		*20.9*	*+ 0.8*
				No booster	***Booster***		
3	White blood cells (cells/µl)	263.3	482.5	376.7	123.3	526.0	
	Neutrophils (cells/µl)	142.5	243	158.9	46.7	95.3	
	Lymphocytes (cells/µl)	109.4	341.1	195.5	35.5	350.2	
	Monocytes (cells/µl)	10.4	11.2	10.6	1.5	17.7	
	Reticulocytes (cells/µl)	0.0	0.0	0.0	0.0	0.2	
	Platelets (cells/µl x 10^3)	252.0	221.8	178.7	476.0	520.0	
	Weight loss (%)	*11.4*	*21.0*	*9.2*	*9.2*	*22.6*	*+ 0.7*

		No booster	***Booster***					
4	White blood cells (cells/µl)	350.0	540.0	2514.0	880.0	243.3	1804.0	1838.0
	Neutrophils (cells/µl)	101.1	132.1	900.3	118.4	54.4	650.9	413.8
	Lymphocytes (cells/µl)	206.2	344.0	1268.8	640.2	137.6	840.8	1275.7
	Monocytes (cells/µl)	30.1	16.4	93.2	32.5	0.5	55.2	34.9
	Reticulocytes (cells/µl)	0.0	0.1	1.1	0.1	0.0	0.7	2.6
	Platelets (cells/µl x 10^3)	228.3	329.0	472.6	571.7	502.3	329.2	501.0
	Weight loss (%)	*10.1*	*11.1*	*18.6*	*4.6*	*10.1*	*27.1*	*+ 1.23*
5	White blood cells (cells/µl)		1881.7			128.3		
	Neutrophil (cells/µl)		544.1			55.2		
	Lymphocytes (cells/µl)		1127.3			57.8		
	Monocytes (cells/µl)		65.8			0.9		
	Reticulocytes (cells/µl)		1.5			0.0		
	Platelets (cells/µl x 10^3)		637.5			403.0		
	Weight loss (%)		*9.03*			*12.49*		

Grouped mean cell counts of 3 mice per analyzed regimen group per time point

Table S2. Primer sequences for the development of red-shifted luciferase expressing *Aspergillus fumigatus* bioluminescent strains

Name	Sequence (5' → 3')	Function (Amplification)
IF_Luc_PgpdA_f	ATG GAG GAC GCC AAG AAC ATC	Amplification of Af_*luc*$_{OPT\_red}$
IF_Luc_TgpdA_r	CTA GAC GGC GAT CTT GCCG	Amplification of Af_*luc*$_{OPT\_red}$
IF_PgpdAAn_f	CTG CCA ATT GGA TCC CGG GCT GAG TAA TAA GCG CAC	Amplification of *gpdA gene* promoter from *A. nidulans*
IF_PgpdAAn_Luc_r	CTT GGC GTC CTC CAT TGT GAT GTC TGC TCA AGCG	Fusion of *gpdA* gene promoter of *A. nidulans* with Af_*luc*$_{OPT\_red}$
IF_TgpdA_Luc_f	AAG ATC GCC GTC TAG GAA ACA GGT CGG AAG CC	Amplification of the terminator of *gpdA* gene of *A. nidulans*
IF_PgpdAter_r	GCG CTG CAG GGG CCC GGG TGG TAG CTC GTT GTC GAC	Amplification of the terminator of *gpdA* gene of *A. nidulans*
KU80upIFpUC_f	CGA GCT CGG TAC CCG GGA CTC AAT TTC TAT TCT AGA GCA TC	Amplification of upstream region of *akuB* and fusion with pUC19
KU80upIFPgpdA_r	GCG GCC GCC TCA GGG TCG TCA AAG TCA GTAC	Amplification of upstream region of *akuB* and fusion with *gpdA* gene promoter
PgpdAIFKu80up_f	CCC TGA GGC GGC CGC GGG CTG AGT AAT AAG	Amplification of P*gpdA*:Af_*luc*$_{OPT_red}$T*gpdA_ptrA* and fusion with upstream region of *akuB* gene
ptrAIFKU80do_r	AAC TTT GGG CGG CCG CGT ATT ATA CTG TC	Amplification of P*gpdA*:Af_*luc*$_{OPT_red}$T*gpdA_ptrA* and fusion with downstream region of *akuB* gene
KU80doIFptrA_f	GCG GCC GCC CAA AGT TAA AGG GCG CAA GC	Amplification of downstream region of *akuB* gene and fusion with *ptrA*
KU80doIFpUC_r	CTC TAG AGG ATC CCC GGG CTA TCA CTT TGC CCA GTC	Amplification of downstream region of *akuB* gene and fusion with pUC19
pCYP51A_f	CCT ATA AGT CGA GTG CAG GAC	Amplification of azole resistance gene (*cyp51A*)
tCYP51A_r	GAA GTC CTC GAT GGT TAC AAC	Amplification of azole resistance gene (*cyp51A*)
CypAfHind_up_f	CTG CAG GCA TGC AAG CTT CTT CAA TGC TCA GGC ATG	Amplification of the complementation *cyp51A* construct and fusion with pUC19
CypAfNotDown_f	*GCA CAG CGG CCG CAC GCA AAG ACG AGA AGG*	Amplification of the complementation *cyp51A* construct and fusion with pUC19
CypAfNotTer_r	GCG TGC GGC CGC TGT GCA CTG TTC TGG TTCC	Amplification of the complementation *cyp51A* construct and fusion with pUC19
CypAfHindDown_r	CAT GAT TAC GCC AAG CTT GAA CAT CGA ACC TCT CGT GTG	Amplification of the complementation *cyp51A* construct and fusion with pUC19

Table S3. *In vitro* mycological characteristics of genetically modified *Aspergillus fumigatus* strains

	Parental Strain	**Complemented strain**	**Parental Strain**	**Complemented strain**
	Af_luc$_{OPT_red}$_TR34	Af_luc$_{OPT_red}$_TR34 + *WTcyp51A -TR$_{34}$/L98H* (comp 3216-1, Δ*akuB*::*luc* No. 23 hygR)	Af_luc$_{OPT_red}$_TR46	Af_luc$_{OPT_red}$_TR46 +*WTcyp51A -TR$_{46}$/Y121F/T289A* (comp4003new7, Δ*akuB*:: *luc* No. 15 hygR)
Characteristics				
Susceptibility (MIC; mg/L) [1]				
Voriconazole	8	1	>16	0.5
Posaconazole	0.5	0.125	0.5	0.125
Itraconazole	16	0.5	16	0.25
Phenotype [2]	Resistant	WT	Resistant	WT
***Cyp51A* gene mutation**				
	TR$_{34}$/L98H	-	TR$_{46}$/Y121F/T289A	-

[1] EUCAST method for susceptibility testing of molds (version 9.3.2). [2] EUCAST clinical breakpoints version 10.0 (Resistance MIC values: itraconazole > 1, voriconazole > 1, posaconazole > 0.25 mg/L). ***Abbreviations:*** *MIC (minimum inhibitory concentration), WT (Wild type= susceptible to triazole antifungals).*