This is the peer reviewd version of the followng article:

Polymorphisms in host immunity-modulating genes and risk of invasive aspergillosis: Results from the AspBIOmics Consortium / Lupiañez, C. B.; Canet, L. M.; Carvalho, A.; Alcazar Fuoli, L.; Springer, J.; Lackner, M.; Segura Catena, J.; Comino, A.; Olmedo, C.; Ríos, R.; Fernández Montoya, A.; Cuenca Estrella, M.; Solano, C.; López Nevot, M. Á.; Cunha, C.; Oliveira Coelho, A.; Villaescusa, T.; Fianchi, L.; Aguado, J. M.; Pagano, L.; López Fernández, E.; Potenza, Leonardo; Luppi, Mario; Lass Flörl, C.; Loeffler, J.; Einsele, H.; Vazquez, L.; Jurado, M.; Sainz, J.. - In: INFECTION AND IMMUNITY. - ISSN 0019-9567. - STAMPA. - 84:3(2016), pp. 643-657. [10.1128/IAI.01359-15]

Terms of use:

The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

12/10/2024 09:56

(Article begins on next page)

12/10/2024 09:56

IAI Accepted Manuscript Posted Online 14 December 2015
Infect. Immun. doi:10.1128/IAI.01359-15
Copyright © 2015, American Society for Microbiology. All Rights Reserved.

Accepted Manuscript Posted Online
Infection and Immunity
IAI
Infection and Immunity

Polymorphisms in host immunity modulating genes and risk of invasive aspergillosis: Results from the aspBIOmics consortium

Lupiañez CB[1,2], Canet LM[1,2], Carvalho A[3,4], Alcazar-Fuoli L[5], Springer J[6], Lackner M[7], Segura-Catena J[1,2], Comino A[8], Olmedo C[8], Ríos R[1,2], Fernández-Montoya A[9], Cuenca-Estrella M[5], Solano C[10], Miguel Ángel López-Nevot[11], Cunha C[3], Oliveira-Coelho A[3], Villaescusa T[12, 13], Fianchi L[14], Aguado JM[15], Pagano L[14], López-Fernández E[2], Potenza L[16], Luppi M[16], Lass-Flörl C[7], Loeffler J[6], Einsele H[6], Vazquez L[12], PCRAGA Study Group[17], Jurado M[1, 2], Sainz J[1,2]

[1] Genomic Oncology department, GENYO, Centre for Genomics and Oncological Research: Pfizer / University of Granada / Andalusian Regional Government, PTS Granada, Granada, Spain
[2] Hematology department, Virgen de las Nieves University Hospital, Granada, Spain
[3] Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal
[4] ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal
[5] Mycology Reference Laboratory, Centro Nacional de Microbiologia, Instituto de Salud Carlos III, Madrid, Spain
[6] Universitätsklinikum Würzburg, Medizinische Klinik II, Würzburg, Germany
[7] Division of Hygiene and Medical Microbiology, Medical University of Innsbruck, Innsbruck, Austria
[8] Experimental Research Unit, Virgen de las Nieves University Hospital, Granada, Spain
[9] Blood Transfusion Regional Centre and Sectorial Tissue Bank, Granada, Spain
[10] Hematology department, Clinic University Hospital of Valencia, Valencia, Spain
[11] Immunology department, Virgen de las Nieves University Hospital, Granada, Spain
[12] Hematology department, University Hospital of Salamanca, Salamanca, Spain
[13] Hematology department, Jiménez Díaz Foundation, Madrid, Spain
[14] Istituto di Ematologia, Università Cattolica del S. Cuore, Rome, Italy.
[15] Unit of Infectious Diseases, Hospital Universitario "12 de Octubre", Instituto de Investigación Hospital "12 de Octubre" (i+12), Madrid, Spain
[16] Department of Medical and Surgical Sciences, University of Modena and Reggio Emilia, AOU Policlinico, Modena, Italy
[17] PCRAGA study group

Running title: Immune-modulating gene polymorphisms and IA

FUNDING
This study was supported by grants PI12/02688 from Fondo de Investigaciones Sanitarias (Madrid, Spain), PIM2010EPA-00756 from the ERA-NET PathoGenoMics (0315900A) and by the Collaborative Research Center / Transregio 124 FungiNet. Cristina Cunha is supported by the Fundação para a Ciência e Tecnologia, Portugal (SFRH/BPD/96176/2013). This study was also supported by a donation of Consuelo González Moreno, an acute myeloid leukemia survivor.

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

AUTHOR'S CONTRIBUTIONS

MJ and JS conceived the study and participated in its design and coordination. CBL performed the genetic analyses. CBL, LMC, JSC, AO-C, AC and JS performed in vitro analyses. LA-F, AC, JSp, ML, ACo, CO, RR, MC-E, CS, MAL-N, AF-M, CC, TV, LF, JMA, LP, EL-F, LPo, MLu, CL-F, JL, HE, LV, PCRAGA Study Group coordinated patient's recruitment and provided the clinical data. JS analysed the data. MJ and JS drafted the manuscript. All authors read and approved the final version of the manuscript.

Corresponding author:
Juan Sainz, PhD
Genomic Oncology department
GENYO. Centre for Genomics and Oncological Research: Pfizer / University of Granada / Andalusian Regional Government
PTS Granada
Avda. de la Ilustración, 114
18016 Granada, Spain
Phone: +34-958-715500
Fax: +34-958-637071
E-mail: juan.sainz@genyo.es

Word Count: Abstract 286, Text 4648, 4 Figures (+1 Supplementary Figure), 5 Tables (+3 supplementary tables)

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

ABSTRACT

Recent studies suggest that immune-modulating single nucleotide polymorphisms (SNPs) influence the risk of developing cancer-related infections. Here, we evaluated whether 36 SNPs within 14 immune-related genes are associated with the risk of Invasive Aspergillosis (IA) and whether genotyping of these variants might improve disease risk prediction. We conducted a case-control association study of 781 immunocompromised patients, 149 of whom were diagnosed with IA. Association analysis showed that the $IL4R_{rs2107356}$ and $IL8_{rs2227307}$ SNPs were associated with an increased risk of IA (OR=1.92, 95%CI: 1.20-3.09 and OR=1.73, 1.06-2.81) whereas the $IL12B_{rs3212227}$ and $IFN\gamma_{rs2069705}$ variants were significantly associated with a decreased risk of developing the infection (OR=0.60, 0.38-0.96 and OR=0.63, 0.41-0.97). An allogeneic hematopoietic stem cell transplantation (allo-HSCT)-stratified analysis revealed that the effect observed for the $IL4R_{rs2107356}$ and $IFN\gamma_{rs2069705}$ SNPs was stronger in allo-HSCT (OR=5.63, 1.20-3.09 and OR=0.24, 0.10-0.59) than in non-HSCT patients, suggesting that the presence of these SNPs may render patients more vulnerable to infection especially under severe and prolonged immunosuppressive conditions. Importantly, *in vitro* studies revealed that carriers of the $IFN\gamma_{rs2069705C}$ allele showed a significantly increased macrophage-mediated neutralisation of fungal conidia (P=0.0003) and, under stimulation conditions, produced higher levels of *IFNγ*mRNA (P=0.049) and IFNγ and TNFα cytokines ($P_{LPS\text{-}96h}$=0.057, $P_{PHA\text{-}96h}$=0.036 and $P_{LPS+PHA\text{-}96h}$=0.030 and $P_{PHA\text{-}72h}$=0.045, $P_{LPS+PHA\text{-}72h}$=0.018, $P_{LPS\text{-}96h}$=0.058 and $P_{LPS+PHA\text{-}96h}$=0.0058, respectively). Finally, we also observed that the addition of SNPs significantly associated with IA to a model including clinical variables led to a substantial improvement in the discriminatory ability to predict the disease (AUC=0.659 vs. AUC=0.564, $P_{LR}=5.2\cdot10^{-4}$ and $P_{50.000Perm}=9.34\cdot10^{-5}$). These findings suggest that the $IFN\gamma_{rs2069705}$ SNP influences the risk of IA and that predictive models built with *IFNγ, IL8*, *IL12p70* and *VEGFα* variants might be used to predict disease risk and to implement risk-adapted prophylaxis or diagnostic strategies.

Keywords: Invasive Aspergillosis, infection, genetic susceptibility, immuno-modulating genes.

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

110 **INTRODUCTION**

111 Invasive Aspergillosis (IA) is a life-threatening infection caused by *Aspergillus spp.* that affects
112 acute myelogenous leukemia (AML) and allogeneic hematopoietic stem cell transplantation (allo-
113 HSCT) patients (1-3). Despite recent improvements in the prophylaxis and treatment of IA, its
114 incidence and attributable mortality rates remain unacceptably high even among those
115 individuals who lack established risk factors (4, 5).

116

117 The initial immune response against fungal pathogens such as *Aspergillus fumigatus* the
118 principal pathogenic species, mainly relies on phagocytes, endothelial and epithelial cells that
119 recognize this fungal pathogen through pattern recognition receptors (PRRs) thus leading to
120 phagocytosis, antigen presentation and production of specific cytokines and chemokines (6, 7).
121 There are different families of PRRs including C-type lectin receptors (CLRs), Toll-like receptors
122 (TLRs), RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs) that, in response to
123 *Aspergillus* pathogen-associated molecular patterns (PAMPs), activate Th_1-, Th_2- and Th_{17}-
124 related signalling cascades on phagocytes and non-professional immune cells (8-12). These
125 intracellular molecular pathways culminate in the production of both pro-inflammatory (13-18)
126 and anti-inflammatory cytokines (19, 20) and certain chemokines and their receptors (21-23) as
127 well as in the release of certain proangiogenic factors such as *VEGFA* and *bFGF* (17, 24), which
128 are also key determinants in the immune response against *Aspergillus spp*.

129

130 Although both innate and adaptive immune responses against *A. fumigatus* have been
131 extensively characterized (25, 26), it remains unclear why some immunocompromised subjects
132 develop invasive or disseminated fungal infections while others in similar clinical conditions do
133 not. The remarkable genetic variation of immune genes suggests that the presence of specific
134 genetic variants in these genes may influence their biological function and, consequently, impact
135 on the risk of developing invasive fungal infections, such as IA. In support of this hypothesis,
136 recent studies on genetic susceptibility have successfully identified several genetic variants on
137 PRR genes (*DC-SIGN, Dectin-1*, *TLRs, PTX3 and MBL*) (27-40), cytokines (*IL1 gene cluster,*

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

138 *IL10, IL12* and *IFNγ*) (32, 41-44), chemokines (*CXCL10*) (45) and immune receptors (*TNFR1*
139 and *TNFR2*) (46, 47) as factors influencing the risk of developing IA. With this background, the
140 purpose of this study was to comprehensively assess whether the presence of single nucleotide
141 polymorphisms (SNPs) within 14 immune-modulating genes (*IL4, IL4R, IL8, IL8RA, IL8RB, IL10,*
142 *IL12A, IL12B, IL13, IFNγ, IFNγR2, CCR5, MIF and VEGF*) may influence the risk of developing
143 IA. We also decided to evaluate the functional role of key variants in modulating immune
144 responses and whether selected polymorphisms could be used to predict the disease risk.
145

146 **MATERIAL AND METHODS**

147 *Study design and study population*

148 Here, we analysed whether 36 SNPs within 14 immune-modulating genes were associated with
149 IA. SNPs selection was based on three criteria: (1) SNPs within immunoregulatory genes that
150 may affect immune responses, (2) SNPs having laboratory evidence of a biological function
151 and/or (3) SNPs previously reported as associated with infectious diseases (Table 1). SNPs
152 were genotyped using KASPar® assays (LGC Genomics KBioscience, London, UK) as
153 previously described in detail (28). Patient inclusion criteria were either undergoing allo-HSCT or
154 being diagnosed with acute myeloid leukaemia (AML) or acute lymphoid leukaemia (ALL) and
155 receiving intensive remission-induction chemotherapy. A total of five hundred and ninety-three
156 patients were recruited between February 2010 and March 2014 through the aspBIOmics
157 consortium (www.aspbiomics.eu) and through two Spanish medical institutions (University
158 Hospital of Salamanca and Clinic University Hospital of Valencia) and a Spanish multicentre
159 clinical trial (PCRAGA, EU clinical trial number: 2010-019406-17) (48). Based on microbiological
160 and clinical data, one hundred and thirteen patients were diagnosed with proven or probable IA
161 according to the revised EORTC/MSG criteria (2008) (49). In order to further confirm significant
162 associations identified in our population, we extended the analysis to a second patient group
163 consisting of 188 high-risk patients (36 IA patients and 152 without IA; Table 2) recruited from
164 two Italian medical institutions (Università Cattolica del S. Cuore, Rome; and University of
165 Modena and Reggio Emilia, AOU Policlinico, Modena) and from the Virgen de las Nieves

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

166 University hospital (Granada, Spain) between January 2013 and January 2015. This ambitious
167 study design provided a population of 781 high-risk patients, 149 of whom were diagnosed with
168 proven and probable IA (19 proven and 130 probable IA). To our knowledge, this is one of the
169 largest population recruited so far exploring genetic susceptibility to IA. The study was approved
170 by the ethical review boards of each participating institution.
171
172 *Statistical analysis*
173 The Hardy-Weinberg Equilibrium (HWE) tests were performed in the uninfected control group by
174 a standard observed-expected chi-square (χ^2) test. Logistic regression analyses adjusted for
175 age, gender, country of origin, allo-HSCT and receipt or non-receipt of anti-fungal prophylaxis
176 were performed to determine significant associations with IA risk. SNPtool (50) and Haploview
177 were used for linkage disequilibrium (LD) blocks reconstruction and haplotype association
178 statistics. Block structures were determined according to the method of Gabriel *et al.* (51). In
179 order to account for multiple testing, we calculated an adjusted significance level using the Meff
180 method (52), which consider the number of independent marker loci (M_{effLi}=31), and the number
181 of models of inheritance tested (co-dominant, dominant, recessive and log-additive). Detailed
182 information about this method of multiple testing correction is freely available online at
183 http://neurogenetics.qimrberghofer.edu.au/SNPSpDlite. Thus, the resulting threshold for the
184 main effect analysis was 0.0004 ([0.05/31]/4).
185
186 *Cell isolation and differentiation*
187 Peripheral blood mononuclear cells (PBMCs) and monocytes were isolated from whole blood
188 collected from healthy donors after obtaining written informed consent (PI12/02688 and SECVS
189 014/2015 protocols). PBMCs were isolated by gradient centrifugation using Ficoll-Paque PLUS
190 (GE Healthcare Bio-Sciences) and monocytes were isolated by immunomagnetic selection of
191 CD_{14}^+ cells (Miltenyi Biotec). Purity of the obtained CD_{14}^+ population was assessed by
192 fluorescence-activated cell sorting analysis. Monocytes were then plated at a density of 5×10^5
193 cells/mL in 24-well plates, cultivated for 7 days in complete RPMI 1640 medium supplemented

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

194 with human serum and 20 ng/mL of GM-CSF to allow differentiation into macrophages. The
195 culture medium was replaced every 3 days. Genotyping of significant SNPs was performed and
196 either PBMCs or monocytes were grouped according to the genotype of interest.
197
198 *Assessment of fungicidal activity*
199 Human monocyte-derived macrophages were infected with conidia from *Aspergillus fumigatus* at
200 an effector-to-target ratio of 1:10. To measure the fungicidal ability, macrophages were allowed
201 to kill the ingested conidia for 2 h. Serial dilutions of macrophage lysates were plated on solid
202 growth media and following a 2-day incubation, the number of colony-forming units (CFU) was
203 enumerated and the percentage of CFU inhibition was calculated. In order to avoid a bias due to
204 differences in internalization rates, the supernatants collected after the co-culture were plated
205 and compared among different donors.
206
207 *IL12p70 and IFNγ stimulation assays.*
208 *IL12p70* and *IFNγ* stimulation assays were performed in PBMCs from healthy donors according
209 to a previously reported protocol (53). PBMCs were selected according to the $IL12B_{rs3212227}$ and
210 $IFN\gamma_{rs2069705}$ genotypes and were cultured in 2 ml of culture medium RPMI-1640 supplemented
211 with 10% sterile heat-inactivated fetal bovine serum (FBS) and an antibiotic mixture containing
212 penicillin, streptomycin and neomycin (Gibco®/Life Technologies) at 37ºC in 5% CO_2. PBMCs
213 from healthy subjects harbouring the $IFN\gamma_{rs2069705T/T}$ (n=8), $IFN\gamma_{rs2069705C/T}$ (n=8) and $IFN\gamma_{rs2069705C/C}$
214 (n=3) genotypes were incubated for 72h and 96h with phytohaemagglutinin (PHA, 2μg/ml) alone
215 or in combination with lypopolysaccharide (LPS, 100ng/ml) and IFNγ, IL12p70, TNF and IL8
216 levels were determined in triplicate using the Procartaplex, Multiplex Immunoassay
217 (Affymetrix/eBioscience) according to manufacturer's recommendations. In parallel, PBMCs
218 bearing the $IL12B_{rs3212227A/A}$ (n=13), $IL12B_{rs3212227A/C}$ (n=3) and $IL12B_{rs3212227C/C}$ (n=1) genotypes
219 were treated for 24h and 48h with zymosan (5μg/ml) alone or in combination with LPS
220 (100ng/ml) and correlation of cytokine levels with the $IL12B_{rs3212227}$ or $IL8_{rs321227}$ SNPs was also

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

221 analysed. After the incubation period, supernatants were collected and stored at -80ºC until
222 cytokine measurement.
223
224 *Analysis of IL4R and IFNγ mRNA expression*
225 We measured IL4R and *IFNγ* mRNA gene expression in blood samples collected from healthy
226 blood donors but also in monocyte-derived macrophages at baseline and after the stimulation
227 with conidia from *A. fumigatus* at an effector-to-target ratio of 1:2 for 8 h. Total RNA from blood or
228 monocyte-derived macrophages was extracted using RNeasy Mini Kit (QIAGEN) and reverse
229 transcribed with the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's
230 instructions. Real-time RT-PCR was performed in an Applied Biosystems 7500HT Fast using
231 Taqman probe-based gene expression technology (Life Technologies) according to the
232 manufacturer's instructions. Statistical significance in gene expression changes was determined
233 by unpaired t test with Whelch's correction (assuming unequal variance between groups).
234
235 *Analysis of IL4R protein expression on T- and B-lymphocytes and monocytes by flow cytometry*
236 *IL4R* protein levels were determined in PBMCs carrying the wild-type $IL4R_{rs2107356G/G}$ (n=13),
237 heterozygote $IL4R_{rs2107356A/G}$ (n=24) or mutant $IL4R_{rs2107356A/A}$ genotypes (n=6) by flow cytometry
238 following a slightly modified version of a previously reported protocol (54). Briefly, PBMCs (1 x
239 10^6) were pre-incubated with PBS supplemented with 5% FBS + 2 mM EDTA for 10 minutes to
240 block Fc receptors. Subsequently, cells were stained for 45 minutes at ambient temperature with
241 mouse anti-human antibodies to determine the levels of *IL4R* protein expression on CD_3^+T-cells,
242 CD_{19}^+B-cells and CD_{14}^+ monocytes. The negative control consisted of cells incubated with a
243 mouse PE-IgG1 Kappa (BD Pharmingen). The analysis was performed according to the flow
244 cytometric cell surface staining method and the following antibodies were used: phycoerythrin
245 (PE)-conjugated CD_{124}^+, Peridin chlorophyll protein (PerCP)-conjugated CD_{14}^+, allophycocyanin
246 (APC)-eFluor®780-conjugated CD_3^+ and fluorescein isothiocyanate (FITC)–conjugated CD_{19}^+
247 antibodies (BD Pharmingen). Cells were acquired on a BD FACSVerse flow cytometer (BD
248 Biosciences) and the data were analysed using the FlowJo software (TreeStar Inc.). Median

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

249 fluorescence intensity (MFI) of the positive population was recorded for each cell type and
250 statistical differences were evaluated using an unpaired t-test with Welch's correction (two-tailed
251 P value).
252
253 *Predictive models and discriminative accuracy*
254 The value of immune-modulating polymorphisms for prediction of IA was examined using
255 stepwise logistic regression analysis. A prediction model was built that included age, gender,
256 allo-HSCT and anti-fungal prophylactic status, and those genetic variants that showed significant
257 associations with IA in the single-SNP analysis (P<0.05; phases 1+2). Then, using p-values as a
258 selection criterion, variables with the highest p-value were dropped and analyses were finalized
259 when all variables reached statistical significance (P<0.05). A predictive model with a similar
260 number of "non-significant SNPs" (P>0.10) was also built. The area under the curve (AUC) of a
261 receiver operating characteristic (ROC) curve analysis was used to assess the discriminative
262 accuracy of each particular model compared with a reference model including only demographic
263 and clinical variables as covariates (age, gender, allo-HSCT and anti-fungal prophylaxis status).
264 A -2 log likelihood ratio (LR) test was used to determine whether predictive models including
265 genetic information were statistically different when compared with the reference model. Finally,
266 we run a randomization test to confirm whether the improved predictive ability of the model
267 including genetic variants significantly associated with IA was consistent after 50,000 iterations.
268 Further details are included in supplementary material. All analyses were performed using R
269 (http://www.r-project.org/).
270
271 **RESULTS**
272 A total of 781 patients were enrolled in this case-control study and, among them, one hundred
273 and forty-nine were diagnosed with proven or probable IA according to the revised EORTC/MSG
274 definitions. The remaining 632 patients showed no evidence for proven or probable IA. Baseline
275 and clinical characteristics of IA and non-IA patient groups are summarized in Table 2. Overall, IA
276 and non-IA patients had a similar mean age (52.67 vs. 52.79, *P*=0.935) but IA patients showed a

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

277 significantly higher male to female gender ratio when compared with those patients with no
278 evidence of IA (1.81 vs. 1.19, *P*=0.031). In addition, the percentage of patients diagnosed with
279 ALL was significantly higher in IA compared with non-IA patients (16.11% vs. 8.23%, *P*=0.006)
280 whereas the percentage of patients with AML was significantly lower in IA compared to non-IA
281 cases (67.11% vs. 76.74%, P=0.020). Interestingly, we also observed a significantly lower
282 proportion of IA cases among those receiving posaconazole prophylaxis (*P*=0.017).
283
284 Thirty-six genetic variants within 14 immune-modulating genes were initially genotyped in 593
285 high-risk patients (113 IA and 480 non-IA patients). Logistic regression analysis revealed that
286 patients carrying the $IL4R_{rs2107356A/A}$ and the $VEGFA_{rs2146323A}$ and $VEGFA_{rs6900017T}$ alleles had a
287 significantly increased risk of IA (OR=2.05, 95%CI 1.24-3.40; OR=1.63, 95%CI 1.02-2.61 and
288 OR=1.76, 95%CI 1.02-3.03, respectively) whereas patients carrying the $IL12B_{rs3212227C}$ and
289 $IFN\gamma_{rs2069705C}$ alleles showed a significantly decreased risk of developing the disease (OR=0.57,
290 95%CI 0.35-0.93 and OR=0.56, 95%CI 0.36-0.88, respectively; Table 3). When a log-additive
291 model was assumed, we also found a significant association between the $VEGFA_{rs2146323}$ and
292 $VEGFA_{rs6900017}$ SNPs and an increased risk of IA (per-allele OR=1.45, 95%CI 1.04-2.03 and per-
293 allele OR=1.73, 95%CI 1.08-2.77) and a statistically significant association of the $IFN\gamma_{rs2069705}$
294 SNP with a decreased risk of developing IA (per-allele OR=0.69, 95%CI 0.49-0.97).
295
296 In order to further confirm these significant associations, the study cohort was extended by
297 recruiting 188 additional patients, 36 of whom were diagnosed with proven or probable IA. Given
298 the low number of proven and probable IA cases, we could not consider this second population
299 as an independent population for replication. An overall association analysis including 781
300 patients (149 IA and 632 non-IA patients) confirmed that carriers of the $IL4R_{rs2107356A/A}$ and
301 $IL8_{rs2227307G/G}$ genotypes had a significantly increased risk of IA when compared with those
302 carrying the wild type allele (OR=1.92, 95%CI 1.20-3.09 and OR=1.73, 95%CI 1.06-2.81)
303 whereas those subjects harbouring the $IL12B_{rs3212227C}$ and $IFN\gamma_{rs2069705C}$ alleles showed a
304 decreased risk of developing the infection (OR=0.60, 95%CI 0.38-0.96 and OR=0.63, 95%CI

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

305 0.41-0.97, respectively). When we tested the allele-dose effect of significant SNPs, we found
306 that the *IL12B*$_{rs3212227}$ polymorphism was significantly associated with a reduced risk of getting
307 the infection (per-allele OR=0.67, 95%CI 0.45-0.99; Table 3). As part of these association
308 analyses, we also performed haplotype analysis that confirmed that none of these
309 polymorphisms were part of risk haplotypes. We only observed a significant association with IA
310 for a relatively rare *IFNγ*$_{TC}$ haplotype whose effect was likely due to the *IFNγ*$_{rs2069705}$ SNP
311 (OR=0.34, 95%CI 0.13-0.88; Supplementary Table 1).
312
313 Interestingly, a logistic regression analysis restricted to allo-HSCT patients and considering only
314 donor genotypes and episodes of IA that occurred after transplantation (n=171) also showed that
315 the effect of the *IL4R*$_{2107356}$ and *IFNγ*$_{rs2069705}$ SNPs on the risk of IA was considerably stronger in
316 allo-HSCT patients compared to those patients who did not undergo transplantation (OR=5.63,
317 95%CI 1.98-16.05 vs. OR=1.48, 95%CI 0.81-2.71 and OR=0.24, 95%CI 0.10-0.59 vs. OR=0.86,
318 95%CI 0.52-1.45; respectively; Table 4). In this allo-HSCT-stratified analysis, we also found that
319 allo-HSCT patients carrying the *VEGFA*$_{rs3024994T}$ allele showed an increased risk of IA when
320 compared with those allo-HSCT patients carrying the wild type genotype/allele (OR=4.48, 95%CI
321 1.25-16.08; Table 4).
322
323 Although none of the reported overall, haplotype and allo-HSCT-stratified associations remained
324 significant after correction for multiple testing ($P_{Meff_correction}$=0.0004), the association of
325 *IL4R*$_{rs2107356}$ and *IFNγ*$_{rs2069705}$ polymorphisms showed a marginal level of significance in allo-
326 HSCT patients when a recessive and dominant model were respectively assumed (P_{REC}=0.0009
327 and P_{DOM}=0.0011, respectively). Considering these results and those showing a suggestive
328 association between *IL12B*$_{rs3212227}$ SNP and risk of IA, we decided to evaluate whether the
329 *IL4R*$_{rs2107356}$, *IL8*$_{rs2227307,}$ *IL12B*$_{rs3212227}$ and *IFNγ*$_{rs2069705}$ variants could have a functional effect in
330 modulating the strength of immune responses against specific *Aspergillus* antigens and/or
331 stimulatory molecules. For that purpose, we first investigated whether *IFNγ*$_{rs2069705}$ and
332 *IL4R*$_{rs2107356}$ but also *IL12B*$_{rs3212227}$ and *IL8*$_{rs2227307}$ variants correlated with the ability of monocyte-

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

333 derived macrophages to efficiently kill fungal conidia. Interestingly, we found that macrophages
334 from donors carrying the *IFNγ*$_{rs2069705C}$ allele showed a significantly increased capability to kill
335 fungal spores that those from subjects carrying the wild type genotype (TT vs. TC, *P*=0.0043; TT
336 vs CC, *P*=0.0012; and TT vs. TC+CC, P=0.0003; Figure 1A). No differences in killing ability were
337 observed in macrophages from donors carrying the *IL4R*$_{rs2107356A/A}$ and *IL8*$_{rs2227307G/G}$ genotypes
338 or *IL12B*$_{rs3212227C}$ allele in comparison with their respective wild type allele/genotype (Figure 1B-
339 D).
340
341 Motivated by these results, we also decided to investigate whether the presence of the above-
342 mentioned SNPs correlated with cytokine levels after stimulation of PBMCs from healthy donors
343 with fungal antigens (zymosan) or stimulatory molecules (LPS and PHA). These *in vitro*
344 stimulation experiments revealed that carriers of the *IFNγ*$_{rs2069705C}$ allele showed an increased
345 production of IFNγ after 4 days of incubation with LPS or PHA and when both stimulating
346 reagents were used in combination (P_{LPS}=0.057, P_{PHA}=0.036 and $P_{LPS+PHA}$=0.030; Figure 2A and
347 Supplementary Table 2). We also observed that donors carrying the *IFNγ*$_{rs2069705C}$ allele showed
348 a drastic increase in the production of TNF at almost all time points when compared with those
349 bearing the wild type genotype ($P_{PHA-72h}$=0.045, $P_{LPS+PHA-72h}$=0.018, $P_{LPS-96h}$=0.058, and $P_{LPS+PHA-}$
350 $_{96h}$=0.0058; Figure 2B and Supplementary Table 2). In addition, we observed that subjects
351 carrying the *IFNγ*$_{rs2069705C}$ allele tended to have an increased production of IL12p70 when
352 compared with those carrying the wild type genotype (Figure 2C and Supplementary Table 2).
353 No correlation between *IL12p70 and IL8* levels and *IL12B*$_{rs3212227}$ and *IL8*$_{rs2227307}$ genotypes was
354 found. These findings suggest that the *IFNγ*$_{rs2069705}$ SNP might contribute to modulate the risk of
355 IA likely through the regulation of IFNγ mRNA levels.
356
357 In order to test this hypothesis, we measured *IFNγ*mRNA expression in PBMCs from healthy
358 donors (n=21) that were grouped according to the *IFNγ*$_{rs2069705}$ genotype. Importantly, we found
359 that carriers of the *IFNγ*$_{rs2069705C}$ allele (C/T+CC) showed a significantly increased level of *IFNγ*

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

360 mRNA when compared with those carrying the wild type genotype (40.85±11.65 vs. 13.87±5.43,
361 *P*=0.049; Figure 3A and 3B). Although this result pointed toward a role of this SNP in modulating
362 *IFNγ* gene expression in PBMCs, we decided to further confirm this result by looking at the
363 publicly available blood expression quantitative trait loci (eQTL) browser
364 (http://genenetwork.nl/bloodeqtlbrowser/). Of note, we found that, in agreement with our gene
365 expression data, this variant located in the promoter region of the gene (but also those
366 neighboring SNPs within the same linkage disequilibrium block) showed a positive correlation
367 with *IFNγ* mRNA expression level that ranged between $P=1.01 \cdot 10^{-3}$ and $P=1.70 \cdot 10^{-3}$
368 (Supplementary Figure 1).
369
370 Next, we analysed the correlation between the $IL4R_{rs2107356}$ SNP and *IL4R* mRNA expression
371 levels in whole blood samples collected from healthy donors (n=43) and in monocyte-derived
372 macrophages at baseline and after *in vitro* stimulation with *A. fumigatus* conidia (n=12). Gene
373 expression data in blood samples did not show differences in IL4R mRNA expression levels
374 between homozygotes (GG vs. AA, *P*=0.304) but they showed a significantly increased level of
375 expression in heterozygotes when were compared either with wild type or mutant homozygotes
376 (AG vs. GG, *P*=0.0045 and AG vs. AA, *P*=0.031, respectively; Figure 3C). When carriers of the
377 $IL4R_{rs2107356AA}$ genotype were compared with those carrying the wild type allele (according to the
378 genetic model used in our genetic analysis), we failed to find statistical differences in IL4R
379 mRNA expression levels ($P_{AAvs.GG+AG}$=0.2937; Figure 3D). These findings did not support our
380 hypothesis suggesting a functional effect for this promoter polymorphism on mRNA expression
381 but were in agreement with those data from the blood eQTL browser that reported no
382 association of this promoter variant with IL4R mRNA expression levels in PBMCs. In line with
383 these results, we neither found any correlation between the $IL4R_{rs2107356}$ variant and IL4R mRNA
384 expression levels in monocytes-derived macrophages at baseline and after stimulation with *A.*
385 *fumigatus* conidia (Figure 3E and 3F). The lack of correlation between this promoter
386 polymorphism and IL4R protein level was also confirmed by flow cytometry analysis in different
387 immune cell types (CD_{19}^{+}, CD_{14}^{+} and CD_{3}^{+}, cells; Figure 3G). We only found a significantly

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

388 increased level of IL4R in CD_{19}^{+} cells in heterozygotes when compared with those subjects
389 carrying the AA genotype (*P*=0.032; Figure 3G), which was in line with our gene expression data
390 in blood samples from healthy donors and confirmed that the effect attributed to this promoter
391 variant cannot be explained by changes in mRNA or protein expression levels. Given that *IL4R*
392 is internalized in a time-dependent manner after stimulation with *IL4*, we could not confidently
393 measure the correlation of the $IL4R_{2107356}$ SNP and *IL4R* protein levels under stimulation
394 conditions (data not shown).
395
396 Finally, considering the association of some of the immune-modulating SNPs with the risk of
397 developing IA and given the correlation of some of these genetic markers with cytokine levels,
398 we tested the capacity of these variants to predict with confidence the disease risk. We
399 assessed the predictive capacity of a model built with "significant SNPs" and demographic and
400 clinical variables in comparison with a reference model including only demographic and clinical
401 variables. Despite the modest population size, we found that a predictive model including 4
402 variants associated with IA, age, gender, allo-HSCT and antifungal prophylaxis status showed
403 an improvement in discriminatory ability to predict the disease when compared with the
404 reference model (AUC=0.659, 95%IC 0.596-0.722, *P*=0.000005 vs. AUC=0.564, 95%IC 0.499-
405 0.630, *P*=0.064; $P_{-2_log_likehood_ratio_test}$=0.00052; Table 5 and Figure 4). Importantly, we also
406 observed that a model built with a similar number of "non-significant SNPs", together with
407 demographic and clinical variables, did not show any significant change in predictive capacity
408 when compared with the reference model (Supplementary Table 3), which confirmed the utility of
409 significant SNPs in predicting IA. The consistency of the predictive results was supported
410 through a 50.000 permutation test that showed that none of the 50.000 permuted models
411 showed a better prediction capacity than our genetic model built with SNPs significantly
412 associated with IA ($AUC_{sort\text{-}average}$=0.6001, $SD_{sort\text{-}AUC}$=0.0158, Z score=3.7361 and $P_{Z_score\text{-}}$
413 $_{value_(50.000perm)}$=$9.34 \cdot 10^{-05}$; Table 5 and Supplementary material).
414
415 **DISCUSSION**

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

416 In this study, we report an association between genetic variants within *IL4R*, *IL8*, *IL12B* and *IFNγ*
417 genes and the risk of developing IA. Carriers of the *IL4R*$_{rs2107356A/A}$ and *IL8*$_{rs2227307G/G}$ genotypes
418 had a significantly increased risk of developing the infection whereas patients carrying the
419 *IL12B*$_{rs3212227C}$ and *IFNγ*$_{rs2069705C}$ alleles showed a substantially decreased risk of IA when
420 compared with those harbouring the wild type allele/genotype. Although none of these
421 associations persisted after a restrictive correction for multiple testing, we found that the
422 association of the *IL4R*$_{rs2107356}$ and *IFNγ*$_{rs2069705}$ polymorphisms reached marginal significance in
423 the allo-HSCT patient population, which pointed towards an impact of these variants in
424 modulating the risk of developing IA, particularly in high-risk populations. Based on these results
425 but also those suggesting overall associations of polymorphisms within the *IL4R*, *IL8*, *IL12B* and
426 *IFNγ* genes at the conventional significance threshold of $P \leq 0.05$, it seems plausible to suggest
427 that polymorphisms within these genes may affect gene function and therefore contribute to the
428 pathogenesis of IA. We hypothesized that both *IL4R*$_{rs2107356}$ and *IFNγ*$_{rs2069705}$ polymorphisms,
429 located in the promoter region of their respective genes, may affect gene expression and,
430 consequently, have an effect in modulating *IL4*- and *IFNγ*-mediated immune responses against
431 *Aspergillus*. Likewise, we hypothesized that *IL8*$_{rs2227307}$ and *IL12B*$_{rs3212227}$ intronic polymorphisms
432 might affect alternative splicing of *IL8* and *IL12B* mRNA and even alter mRNA expression
433 thereby dysregulating *IL8*- and *IL12*-mediated Th1 immune responses against fungal pathogens.
434 In line with these arguments, a number of previous studies have reported associations of these
435 polymorphisms with immune-related diseases (55, 56) and infections (57-60) including IA (45).
436 In particular, we confirmed an association previously reported by Mezger *et al.* (2008) between
437 the *IFNγ*$_{rs2069705}$ SNP and the risk of IA (45). This supports our hypothesis that this association is
438 likely to be true and that this variant may play a role in modulating the immune response against
439 *Aspergillus*.
440
441 Recent studies in humans and animal models have demonstrated that *IL4/IL4R*, *IL8*, *IL12* and
442 *IFNγ* have a central role in IA (19, 61-65). In particular, *IFNγ* seems to be a key factor as its

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

443 enhanced production (14, 66) or its therapeutic administration (63), boosts the production of free
444 oxygen radicals and the neutrophil-mediated damage of fungal hyphae and promotes resistance
445 to the infection (19, 67). It is also well documented that the production of *IL8 and IL12p70* by
446 epithelial and dendritic cells in response to conidia enhances Th_1-mediated immune responses
447 (61, 68, 69) and increases resistance to IA (62) whereas its neutralization produces a marked
448 increase in susceptibility to IA (15, 19). IL12p70 also mediates enhanced cytotoxic activity of NK
449 cells and CD_8^+-T cells and promotes the secretion of *IFNγ* by CD_4^+-T cells, which is an essential
450 process for an efficient clearance of inhaled *Aspergillus fumigatus* spores. Conversely, *IL4*
451 secretion activates Th_2-CD_4^+-T cell immune responses and leads to a significant decrease in Th_1
452 immune responses and, consequently, increases susceptibility to *Aspergillus* infection (19, 70).
453 Similarly, it has also been demonstrated that the lack of *IL4* cytokine increases Th_1 immune
454 responses characterized by high production of *IL12* and an enhanced *IL12*-mediated production
455 of *IFNγ* by T lymphocytes thereby leading to an increase in resistance to developing the infection
456 (19).
457
458 In light of these results and in order to better characterize the role of *IFNγ*, *IL4R*, *IL8* and *IL12B*
459 polymorphisms in modulating immune responses against fungal antigens and/or specific
460 stimulatory molecules, we proceeded to perform functional assays in PBMCs and monocyte-
461 derived macrophages from healthy donors. Importantly, we found that subjects carrying the
462 $IFN\gamma_{rs2069705C}$ allele showed an increased ability to kill *A. fumigatus* conidia than those carrying
463 the wild type allele. This important finding support our genetic findings but also those from a
464 previous study (45) suggesting that $IFN\gamma_{rs2069705}$ promoter variant might play a key role in
465 modulating the strength of immune responses against *Aspergillus* likely through the modulation
466 on IFNγ mRNA expression. Importantly, we also observed that, under stimulating conditions,
467 PBMCs from carriers of the $IFN\gamma_{rs2069705C}$ allele showed an increased production of IFNγ and TNF
468 cytokines than those individuals carrying the wild type allele. In addition to this, we found that the
469 $IFN\gamma_{rs2069705}$ SNP correlated with *IFNγ* mRNA levels in PBMCs from healthy donors, which again

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

470 suggested that this polymorphism or another causative polymorphism in strong linkage
471 disequilibrium with it may be involved not only in the control of *IFNγ* production but also in the
472 subsequent induction of *TNF* production. Taking all these findings together, we propose a central
473 role for this variant in determining the risk of IA in allo-HSCT and in leukaemia patients
474 undergoing intensive chemotherapy.
475
476 Although genetic data suggested that polymorphisms within IL4R, IL8 and IL12B influence the
477 risk of developing IA, functional experiments did not show any correlation between these SNPs
478 and their respective mRNA and/or protein levels. Therefore, we suggest that these variants
479 might exert their biological function by modulating other biological processes such as mRNA
480 processing (splicing or turnover) or mRNA stability or even act at post-transcriptional level.
481 Further studies are now warranted to replicate our findings and to experimentally identify the
482 functional role of these SNPs in determining the risk of IA.
483
484 Finally, given the genetic and/or functional effect observed for variants within *IFNγ*, *IL4R*, *IL8*,
485 *IL12B* and *VEGFA* genes, we found also interesting to determine the impact of these variants in
486 predicting the disease risk. We found that a model built with $IL8_{rs2227307}$, $IL12B_{rs3212227}$, $IFN\gamma_{rs2069705}$
487 and $VEGFA_{rs6900017}$ SNPs showed a significantly improved discriminatory ability to predict the
488 disease when it was compared with a model that included demographic and clinical variables.
489 Importantly, when a similar number of "non-significant SNPs" were added to the reference
490 model, we did not observe any significant change in predictive capacity, which confirmed that
491 only a model built with these significant SNPs could have capacity to predict the infection. In
492 support of this finding, we also observed that the AUC of this model was systematically higher
493 than the AUC observed for 50.000 iterative models, which emphasizes the importance of
494 considering predictive models to assist in the clinical decision-making process and to improve in
495 novel strategies to prevent IA occurrence.
496

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

497 This study has both strengths and limitations. Study strengths include a multicentre population-
498 based design, a relatively large sample size, and the high number of genetic polymorphisms
499 analysed. This allowed us for the first time to perform predictive analyses to assess the potential
500 utility of genetic variants in predicting with confidence the risk of developing IA. Potential
501 weaknesses include limited antifungal prophylaxis data availability for a subset of patients and a
502 relatively low number of proven or probable IA cases that limited the study's statistical power to
503 rule out spurious associations. To minimize this limitation, the most relevant associations were
504 functionally validated.
505
506 In conclusion, our data suggest that immune-modulating polymorphisms have an impact on the
507 risk of IA and that genotyping of these variants could help to predict the risk of IA and therefore
508 be useful to establish a risk-adapted anti-fungal prophylaxis strategy.
509

510 **ACKNOWLEDGMENTS**

511 We thank Thomas Rogers (St James's Hospital, Dublin, Ireland) for reviewing and editing the
512 English language and Dr. António Marques (Hospital de Braga, Portugal) who provided the buffy
513 coats. We also thank Astella Pharma Inc. for supporting this work.
514

515 **REFERENCES**

516 1. **Neofytos D, Lu K, Hatfield-Seung A, Blackford A, Marr KA, Treadway S, Ostrander D,**
517 **Nussenblatt V, Karp J.** 2013. Epidemiology, outcomes, and risk factors of invasive fungal
518 infections in adult patients with acute myelogenous leukemia after induction chemotherapy.
519 Diagn Microbiol Infect Dis **75:**144-149.
520 2. **Neofytos D, Treadway S, Ostrander D, Alonso CD, Dierberg KL, Nussenblatt V, Durand**
521 **CM, Thompson CB, Marr KA.** 2013. Epidemiology, outcomes, and mortality predictors of
522 invasive mold infections among transplant recipients: a 10-year, single-center experience.
523 Transpl Infect Dis doi:10.1111/tid.12060.
524 3. **Steinbach WJ, Marr KA, Anaissie EJ, Azie N, Quan SP, Meier-Kriesche HU, Apewokin**

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

525 **S, Horn DL.** 2012. Clinical epidemiology of 960 patients with invasive aspergillosis from the
526 PATH Alliance registry. J Infect **65:**453-464.
527 4. **Hammond SP, Marty FM, Bryar JM, DeAngelo DJ, Baden LR.** 2010. Invasive fungal
528 disease in patients treated for newly diagnosed acute leukemia. Am J Hematol **85:**695-699.
529 5. **Baddley JW, Stephens JM, Ji X, Gao X, Schlamm HT, Tarallo M.** 2013. Aspergillosis in
530 Intensive Care Unit (ICU) patients: epidemiology and economic outcomes. BMC Infect Dis
531 **13:**29.
532 6. **Gresnigt MS, Netea MG, van de Veerdonk FL.** 2012. Pattern recognition receptors and
533 their role in invasive aspergillosis. Ann N Y Acad Sci **1273:**60-67.
534 7. **Balloy V, Chignard M.** 2009. The innate immune response to Aspergillus fumigatus.
535 Microbes Infect **11:**919-927.
536 8. **Serrano-Gomez D, Leal JA, Corbi AL.** 2005. DC-SIGN mediates the binding of Aspergillus
537 fumigatus and keratinophylic fungi by human dendritic cells. Immunobiology **210:**175-183.
538 9. **Sun WK, Lu X, Li X, Sun QY, Su X, Song Y, Sun HM, Shi Y.** 2012. Dectin-1 is inducible
539 and plays a crucial role in Aspergillus-induced innate immune responses in human bronchial
540 epithelial cells. Eur J Clin Microbiol Infect Dis **31:**2755-2764.
541 10. **Mambula SS, Sau K, Henneke P, Golenbock DT, Levitz SM.** 2002. Toll-like receptor (TLR)
542 signaling in response to Aspergillus fumigatus. J Biol Chem **277:**39320-39326.
543 11. **Gersuk GM, Underhill DM, Zhu L, Marr KA.** 2006. Dectin-1 and TLRs permit macrophages
544 to distinguish between different Aspergillus fumigatus cellular states. J Immunol **176:**3717-
545 3724.
546 12. **Said-Sadier N, Padilla E, Langsley G, Ojcius DM.** 2010. Aspergillus fumigatus stimulates
547 the NLRP3 inflammasome through a pathway requiring ROS production and the Syk
548 tyrosine kinase. PLoS One **5:**e10008.
549 13. **Mehrad B, Strieter RM, Standiford TJ.** 1999. Role of TNF-alpha in pulmonary host
550 defense in murine invasive aspergillosis. J Immunol **162:**1633-1640.
551 14. **Cenci E, Mencacci A, Fe d'Ostiani C, Del Sero G, Mosci P, Montagnoli C, Bacci A,**
552 **Romani L.** 1998. Cytokine- and T helper-dependent lung mucosal immunity in mice with

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

553 invasive pulmonary aspergillosis. J Infect Dis **178:**1750-1760.

554 15. **Brieland JK, Jackson C, Menzel F, Loebenberg D, Cacciapuoti A, Halpern J, Hurst S,**
555 **Muchamuel T, Debets R, Kastelein R, Churakova T, Abrams J, Hare R, O'Garra A.** 2001.
556 Cytokine networking in lungs of immunocompetent mice in response to inhaled Aspergillus
557 fumigatus. Infect Immun **69:**1554-1560.

558 16. **Gringhuis SI, den Dunnen J, Litjens M, van der Vlist M, Wevers B, Bruijns SC,**
559 **Geijtenbeek TB.** 2009. Dectin-1 directs T helper cell differentiation by controlling
560 noncanonical NF-kappaB activation through Raf-1 and Syk. Nat Immunol **10:**203-213.

561 17. **Sha Q, Truong-Tran AQ, Plitt JR, Beck LA, Schleimer RP.** 2004. Activation of airway
562 epithelial cells by toll-like receptor agonists. Am J Respir Cell Mol Biol **31:**358-364.

563 18. **Balloy V, Sallenave JM, Wu Y, Touqui L, Latge JP, Si-Tahar M, Chignard M.** 2008.
564 Aspergillus fumigatus-induced interleukin-8 synthesis by respiratory epithelial cells is
565 controlled by the phosphatidylinositol 3-kinase, p38 MAPK, and ERK1/2 pathways and not
566 by the toll-like receptor-MyD88 pathway. J Biol Chem **283:**30513-30521.

567 19. **Cenci E, Mencacci A, Del Sero G, Bacci A, Montagnoli C, d'Ostiani CF, Mosci P,**
568 **Bachmann M, Bistoni F, Kopf M, Romani L.** 1999. Interleukin-4 causes susceptibility to
569 invasive pulmonary aspergillosis through suppression of protective type I responses. J Infect
570 Dis **180:**1957-1968.

571 20. **Clemons KV, Grunig G, Sobel RA, Mirels LF, Rennick DM, Stevens DA.** 2000. Role of
572 IL-10 in invasive aspergillosis: increased resistance of IL-10 gene knockout mice to lethal
573 systemic aspergillosis. Clin Exp Immunol **122:**186-191.

574 21. **Mehrad B, Strieter RM, Moore TA, Tsai WC, Lira SA, Standiford TJ.** 1999. CXC
575 chemokine receptor-2 ligands are necessary components of neutrophil-mediated host
576 defense in invasive pulmonary aspergillosis. J Immunol **163:**6086-6094.

577 22. **Mehrad B, Moore TA, Standiford TJ.** 2000. Macrophage inflammatory protein-1 alpha is a
578 critical mediator of host defense against invasive pulmonary aspergillosis in neutropenic
579 hosts. J Immunol **165:**962-968.

580 23. **Morrison BE, Park SJ, Mooney JM, Mehrad B.** 2003. Chemokine-mediated recruitment of

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

581 NK cells is a critical host defense mechanism in invasive aspergillosis. J Clin Invest
582 **112:**1862-1870.
583 24. **Yoshida S, Ono M, Shono T, Izumi H, Ishibashi T, Suzuki H, Kuwano M.** 1997.
584 Involvement of interleukin-8, vascular endothelial growth factor, and basic fibroblast growth
585 factor in tumor necrosis factor alpha-dependent angiogenesis. Mol Cell Biol **17:**4015-4023.
586 25. **Park SJ, Mehrad B.** 2009. Innate immunity to Aspergillus species. Clin Microbiol Rev
587 **22:**535-551.
588 26. **Romani L.** 2011. Immunity to fungal infections. Nat Rev Immunol **11:**275-288.
589 27. **Crosdale DJ, Poulton KV, Ollier WE, Thomson W, Denning DW.** 2001. Mannose-binding
590 lectin gene polymorphisms as a susceptibility factor for chronic necrotizing pulmonary
591 aspergillosis. J Infect Dis **184:**653-656.
592 28. **Sainz J, Lupianez CB, Segura-Catena J, Vazquez L, Rios R, Oyonarte S, Hemminki K,**
593 **Forsti A, Jurado M.** 2012. Dectin-1 and DC-SIGN polymorphisms associated with invasive
594 pulmonary Aspergillosis infection. PLoS One **7:**e32273.
595 29. **Kesh S, Mensah NY, Peterlongo P, Jaffe D, Hsu K, M VDB, O'Reilly R, Pamer E,**
596 **Satagopan J, Papanicolaou GA.** 2005. TLR1 and TLR6 polymorphisms are associated
597 with susceptibility to invasive aspergillosis after allogeneic stem cell transplantation. Ann N Y
598 Acad Sci **1062:**95-103.
599 30. **Bochud PY, Chien JW, Marr KA, Leisenring WM, Upton A, Janer M, Rodrigues SD, Li**
600 **S, Hansen JA, Zhao LP, Aderem A, Boeckh M.** 2008. Toll-like receptor 4 polymorphisms
601 and aspergillosis in stem-cell transplantation. N Engl J Med **359:**1766-1777.
602 31. **Carvalho A, Cunha C, Carotti A, Aloisi T, Guarrera O, Di Ianni M, Falzetti F, Bistoni F,**
603 **Aversa F, Pitzurra L, Rodrigues F, Romani L.** 2009. Polymorphisms in Toll-like receptor
604 genes and susceptibility to infections in allogeneic stem cell transplantation. Exp Hematol
605 **37:**1022-1029.
606 32. **de Boer MG, Jolink H, Halkes CJ, van der Heiden PL, Kremer D, Falkenburg JH, van**
607 **de Vosse E, van Dissel JT.** 2011. Influence of polymorphisms in innate immunity genes on
608 susceptibility to invasive aspergillosis after stem cell transplantation. PLoS One **6:**e18403.

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

609 33. **Carvalho A, Pasqualotto AC, Pitzurra L, Romani L, Denning DW, Rodrigues F.** 2008.
610 Polymorphisms in toll-like receptor genes and susceptibility to pulmonary aspergillosis. J
611 Infect Dis **197:**618-621.
612 34. **Chai LY, de Boer MG, van der Velden WJ, Plantinga TS, van Spriel AB, Jacobs C,**
613 **Halkes CJ, Vonk AG, Blijlevens NM, van Dissel JT, Donnelly PJ, Kullberg BJ, Maertens**
614 **J, Netea MG.** 2011. The Y238X stop codon polymorphism in the human beta-glucan
615 receptor dectin-1 and susceptibility to invasive aspergillosis. J Infect Dis **203:**736-743.
616 35. **Cunha C, Di Ianni M, Bozza S, Giovannini G, Zagarella S, Zelante T, D'Angelo C, Pierini**
617 **A, Pitzurra L, Falzetti F, Carotti A, Perruccio K, Latge JP, Rodrigues F, Velardi A,**
618 **Aversa F, Romani L, Carvalho A.** 2010. Dectin-1 Y238X polymorphism associates with
619 susceptibility to invasive aspergillosis in hematopoietic transplantation through impairment
620 of both recipient- and donor-dependent mechanisms of antifungal immunity. Blood
621 **116:**5394-5402.
622 36. **Granell M, Urbano-Ispizua A, Suarez B, Rovira M, Fernandez-Aviles F, Martinez C,**
623 **Ortega M, Uriburu C, Gaya A, Roncero JM, Navarro A, Carreras E, Mensa J, Vives J,**
624 **Rozman C, Montserrat E, Lozano F.** 2006. Mannan-binding lectin pathway deficiencies
625 and invasive fungal infections following allogeneic stem cell transplantation. Exp Hematol
626 **34:**1435-1441.
627 37. **Grube M, Loeffler J, Mezger M, Kruger B, Echtenacher B, Hoffmann P, Edinger M,**
628 **Einsele H, Andreesen R, Holler E.** 2013. TLR5 stop codon polymorphism is associated
629 with invasive aspergillosis after allogeneic stem cell transplantation. Med Mycol
630 doi:10.3109/13693786.2013.809630.
631 38. **Cunha C, Giovannini G, Pierini A, Bell AS, Sorci G, Riuzzi F, Donato R, Rodrigues F,**
632 **Velardi A, Aversa F, Romani L, Carvalho A.** 2011. Genetically-determined hyperfunction of
633 the S100B/RAGE axis is a risk factor for aspergillosis in stem cell transplant recipients.
634 PLoS One **6:**e27962.
635 39. **Cunha C, Aversa F, Lacerda JF, Busca A, Kurzai O, Grube M, Loffler J, Maertens JA,**
636 **Bell AS, Inforzato A, Barbati E, Almeida B, Santos e Sousa P, Barbui A, Potenza L,**

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

637 **Caira M, Rodrigues F, Salvatori G, Pagano L, Luppi M, Mantovani A, Velardi A, Romani**
638 **L, Carvalho A.** 2014. Genetic PTX3 deficiency and aspergillosis in stem-cell
639 transplantation. N Engl J Med **370:**421-432.

640 40. **Koldehoff M, Beelen DW, Elmaagacli AH.** 2013. Increased susceptibility for aspergillosis
641 and post-transplant immune deficiency in patients with gene variants of TLR4 after stem cell
642 transplantation. Transpl Infect Dis **15:**533-539.

643 41. **Sainz J, Perez E, Gomez-Lopera S, Jurado M.** 2008. IL1 gene cluster polymorphisms and
644 its haplotypes may predict the risk to develop invasive pulmonary aspergillosis and
645 modulate C-reactive protein level. J Clin Immunol **28:**473-485.

646 42. **Sainz J, Hassan L, Perez E, Romero A, Moratalla A, Lopez-Fernandez E, Oyonarte S,**
647 **Jurado M.** 2007. Interleukin-10 promoter polymorphism as risk factor to develop invasive
648 pulmonary aspergillosis. Immunol Lett **109:**76-82.

649 43. **Seo KW, Kim DH, Sohn SK, Lee NY, Chang HH, Kim SW, Jeon SB, Baek JH, Kim JG,**
650 **Suh JS, Lee KB.** 2005. Protective role of interleukin-10 promoter gene polymorphism in the
651 pathogenesis of invasive pulmonary aspergillosis after allogeneic stem cell transplantation.
652 Bone Marrow Transplant **36:**1089-1095.

653 44. **Johnson MD, Plantinga TS, van de Vosse E, Velez Edwards DR, Smith PB, Alexander**
654 **BD, Yang JC, Kremer D, Laird GM, Oosting M, Joosten LA, van der Meer JW, van**
655 **Dissel JT, Walsh TJ, Perfect JR, Kullberg BJ, Scott WK, Netea MG.** 2012. Cytokine gene
656 polymorphisms and the outcome of invasive candidiasis: a prospective cohort study. Clin
657 Infect Dis **54:**502-510.

658 45. **Mezger M, Steffens M, Beyer M, Manger C, Eberle J, Toliat MR, Wienker TF, Ljungman**
659 **P, Hebart H, Dornbusch HJ, Einsele H, Loeffler J.** 2008. Polymorphisms in the chemokine
660 (C-X-C motif) ligand 10 are associated with invasive aspergillosis after allogeneic stem-cell
661 transplantation and influence CXCL10 expression in monocyte-derived dendritic cells. Blood
662 **111:**534-536.

663 46. **Sainz J, Salas-Alvarado I, Lopez-Fernandez E, Olmedo C, Comino A, Garcia F, Blanco**
664 **A, Gomez-Lopera S, Oyonarte S, Bueno P, Jurado M.** 2010. TNFR1 mRNA expression

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

665 level and TNFR1 gene polymorphisms are predictive markers for susceptibility to develop
666 invasive pulmonary aspergillosis. Int J Immunopathol Pharmacol **23:**423-436.
667 47. **Sainz J, Perez E, Hassan L, Moratalla A, Romero A, Collado MD, Jurado M.** 2007.
668 Variable number of tandem repeats of TNF receptor type 2 promoter as genetic biomarker of
669 susceptibility to develop invasive pulmonary aspergillosis. Hum Immunol **68:**41-50.
670 48. **Aguado JM, Vazquez L, Fernandez-Ruiz M, Villaescusa T, Ruiz-Camps I, Barba P, Silva**
671 **JT, Batlle M, Solano C, Gallardo D, Heras I, Polo M, Varela R, Vallejo C, Olave T, Lopez-**
672 **Jimenez J, Rovira M, Parody R, Cuenca-Estrella M, Group PS, Spanish Stem Cell**
673 **Transplantation Group tSGoMMotSSoCM, Infectious D, the Spanish Network for**
674 **Research in Infectious D.** 2015. Serum galactomannan versus a combination of
675 galactomannan and polymerase chain reaction-based Aspergillus DNA detection for early
676 therapy of invasive aspergillosis in high-risk hematological patients: a randomized controlled
677 trial. Clin Infect Dis **60:**405-414.
678 49. **De Pauw B, Walsh TJ, Donnelly JP, Stevens DA, Edwards JE, Calandra T, Pappas PG,**
679 **Maertens J, Lortholary O, Kauffman CA, Denning DW, Patterson TF, Maschmeyer G,**
680 **Bille J, Dismukes WE, Herbrecht R, Hope WW, Kibbler CC, Kullberg BJ, Marr KA,**
681 **Munoz P, Odds FC, Perfect JR, Restrepo A, Ruhnke M, Segal BH, Sobel JD, Sorrell TC,**
682 **Viscoli C, Wingard JR, Zaoutis T, Bennett JE, European Organization for R, Treatment**
683 **of Cancer/Invasive Fungal Infections Cooperative G, National Institute of A, Infectious**
684 **Diseases Mycoses Study Group Consensus G.** 2008. Revised definitions of invasive
685 fungal disease from the European Organization for Research and Treatment of
686 Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy
687 and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. Clin
688 Infect Dis **46:**1813-1821.
689 50. **Chen B, Wilkening S, Drechsel M, Hemminki K.** 2009. SNP_tools: A compact tool
690 package for analysis and conversion of genotype data for MS-Excel. BMC Res Notes **2:**214.
691 51. **Gabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, Blumenstiel B, Higgins J,**
692 **DeFelice M, Lochner A, Faggart M, Liu-Cordero SN, Rotimi C, Adeyemo A, Cooper R,**

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

693 **Ward R, Lander ES, Daly MJ, Altshuler D.** 2002. The structure of haplotype blocks in the
694 human genome. Science **296:**2225-2229.
695 52. **Nyholt DR.** 2004. A simple correction for multiple testing for single-nucleotide
696 polymorphisms in linkage disequilibrium with each other. Am J Hum Genet **74:**765-769.
697 53. **Byrnes HD, Kaminski H, Mirza A, Deno G, Lundell D, Fine JS.** 1999. Macrophage
698 inflammatory protein-3 beta enhances IL-10 production by activated human peripheral blood
699 monocytes and T cells. J Immunol **163:**4715-4720.
700 54. **Mozo L, Gayo A, Suarez A, Rivas D, Zamorano J, Gutierrez C.** 1998. Glucocorticoids
701 inhibit IL-4 and mitogen-induced IL-4R alpha chain expression by different
702 posttranscriptional mechanisms. J Allergy Clin Immunol **102:**968-976.
703 55. **Hesselmar B, Bergin AM, Park H, Hahn-Zoric M, Eriksson B, Hanson LA, Padyukov L.**
704 2010. Interleukin-4 receptor polymorphisms in asthma and allergy: relation to different
705 disease phenotypes. Acta Paediatr **99:**399-403.
706 56. **Kim K, Cho SK, Sestak A, Namjou B, Kang C, Bae SC.** 2010. Interferon-gamma gene
707 polymorphisms associated with susceptibility to systemic lupus erythematosus. Ann Rheum
708 Dis **69:**1247-1250.
709 57. **Stappers MH, Thys Y, Oosting M, Plantinga TS, Ioana M, Reimnitz P, Mouton JW,**
710 **Netea MG, Joosten LA, Gyssens IC.** 2014. Polymorphisms in cytokine genes IL6, TNF,
711 IL10, IL17A and IFNG influence susceptibility to complicated skin and skin structure
712 infections. Eur J Clin Microbiol Infect Dis **33:**2267-2274.
713 58. **Wang D, Zhong X, Huang D, Chen R, Bai G, Li Q, Yu B, Fan Y, Sun X.** 2014. Functional
714 polymorphisms of interferon-gamma affect pneumonia-induced sepsis. PLoS One **9:**e87049.
715 59. **Morris GA, Edwards DR, Hill PC, Wejse C, Bisseye C, Olesen R, Edwards TL, Gilbert**
716 **JR, Myers JL, Stryjewski ME, Abbate E, Estevan R, Hamilton CD, Tacconelli A, Novelli**
717 **G, Brunetti E, Aaby P, Sodemann M, Ostergaard L, Adegbola R, Williams SM, Scott**
718 **WK, Sirugo G.** 2011. Interleukin 12B (IL12B) genetic variation and pulmonary tuberculosis:
719 a study of cohorts from The Gambia, Guinea-Bissau, United States and Argentina. PLoS
720 One **6:**e16656.

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

721 60. **Zhang N, Xu Y, Zhang B, Zhang T, Yang H, Zhang B, Feng Z, Zhong D.** 2014. Analysis of
722 interleukin-8 gene variants reveals their relative importance as genetic susceptibility factors
723 for chronic periodontitis in the Han population. PLoS One **9:**e104436.
724 61. **Bozza S, Gaziano R, Spreca A, Bacci A, Montagnoli C, di Francesco P, Romani L.**
725 2002. Dendritic cells transport conidia and hyphae of Aspergillus fumigatus from the airways
726 to the draining lymph nodes and initiate disparate Th responses to the fungus. J Immunol
727 **168:**1362-1371.
728 62. **Cenci E, Perito S, Enssle KH, Mosci P, Latge JP, Romani L, Bistoni F.** 1997. Th1 and
729 Th2 cytokines in mice with invasive aspergillosis. Infect Immun **65:**564-570.
730 63. **Nagai H, Guo J, Choi H, Kurup V.** 1995. Interferon-gamma and tumor necrosis factor-
731 alpha protect mice from invasive aspergillosis. J Infect Dis **172:**1554-1560.
732 64. **Bellocchio S, Bozza S, Montagnoli C, Perruccio K, Gaziano R, Pitzurra L, Romani L.**
733 2005. Immunity to Aspergillus fumigatus: the basis for immunotherapy and vaccination. Med
734 Mycol **43 Suppl 1:**S181-188.
735 65. **Phadke AP, Mehrad B.** 2005. Cytokines in host defense against Aspergillus: recent
736 advances. Med Mycol **43 Suppl 1:**S173-176.
737 66. **Park SJ, Hughes MA, Burdick M, Strieter RM, Mehrad B.** 2009. Early NK cell-derived
738 IFN-{gamma} is essential to host defense in neutropenic invasive aspergillosis. J Immunol
739 **182:**4306-4312.
740 67. **Roilides E, Uhlig K, Venzon D, Pizzo PA, Walsh TJ.** 1993. Enhancement of oxidative
741 response and damage caused by human neutrophils to Aspergillus fumigatus hyphae by
742 granulocyte colony-stimulating factor and gamma interferon. Infect Immun **61:**1185-1193.
743 68. **Bellanger AP, Millon L, Khoufache K, Rivollet D, Bieche I, Laurendeau I, Vidaud M,**
744 **Botterel F, Bretagne S.** 2009. Aspergillus fumigatus germ tube growth and not conidia
745 ingestion induces expression of inflammatory mediator genes in the human lung epithelial
746 cell line A549. J Med Microbiol **58:**174-179.
747 69. **Chiang LY, Sheppard DC, Gravelat FN, Patterson TF, Filler SG.** 2008. Aspergillus
748 fumigatus stimulates leukocyte adhesion molecules and cytokine production by endothelial

Accepted Manuscript Posted Online

749 cells in vitro and during invasive pulmonary disease. Infect Immun **76:**3429-3438.

750 70. **Mencacci A, Perruccio K, Bacci A, Cenci E, Benedetti R, Martelli MF, Bistoni F,**
751 **Coffman R, Velardi A, Romani L.** 2001. Defective antifungal T-helper 1 (TH1) immunity in
752 a murine model of allogeneic T-cell-depleted bone marrow transplantation and its restoration
753 by treatment with TH2 cytokine antagonists. Blood **97:**1483-1490.

754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775
776

Infection and Immunity
IAI
Infection and Immunity

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

TABLE AND FIGURE LEGENDS

Table 1. Selected SNPs within immune-modulating genes.

Abbreviations: SNP, single nucleotide polymorphisms, UTR, untranslated region. References included in this table are listed in the supplementary material.

Table 2. Baseline and clinical characteristic of IA and non-IA patient groups.

Abbreviations: HSCT: Hematopoietic stem cell transplantation, AML: acute myeloid leukemia, ALL: acute lymphoid leukemia; UHS, University hospital of Salamanca (Spain); GHV, General hospital of Valencia (Spain); PCRAGA clinical trial (EU clinical trial number: 2010-019406-17); CSC, Università Cattolica del S. Cuore, Rome (Italy); MO, University of Modena and Reggio Emilia, Modena (Italy). P≤0.05 was considered significant.
* Some patients had several prophylactic drugs.
† Prophylaxis status was only available in 99 subjects (15 IA and 72 non-IA patients).
∂ Percentage calculated according to the number of patients with prophylaxis data available.

Table 3. Association of polymorphisms within immunoregulatory genes and invasive aspergillosis.

Abbreviations: OR, odds ratio; CI, confidence interval. Abbreviations: n/s, not specified; SNP, single nucleotide polymorphism; UTR, untranslated region. Estimates were adjusted for age, sex, country of origin, allo-SCT, and prophylaxis status (ever use of prophylaxis). P<0.05 in bold.

[a]Discovery population: aspBIOmics+PCRAGA+Valencia+Salamanca populations; N=593 hematological patients
[b]Overall: n=781; after extension with 188 high-risk patients [39 HSCT and 149 non-HSCT patients]; 87 of them with prophylaxis data.
[†]Estimates according a recessive model of inheritance.
[§]SNP signifcantly associated with IA according a log-additive model of inheritance.

[§]*IFNG*$_{rs2069705}$ (per-allele OR= 0.69, 95%CI 0.49-0.97; P_{trend}=0.032).
[§]*VEGFA*$_{rs2146323}$ (per-allele OR= 1.45, 95%CI 1.04-2.03; P_{trend} =0.029).
[§]*VEGFA*$_{rs6900017}$ (per-allele OR= 1.73, 95%CI 1.08-2.77; P_{trend}=0.027).
[§]*IL12B*$_{rs3212227}$ (per-allele OR= 0.67, 95%CI 0.45-0.99; P_{trend} =0.040).

Table 4. Association of immunoregulatory SNPs and IA in allo-HSCT patients (n=171).

Abbreviations: OR, odds ratio; CI, confidence interval. Abbreviations: n/s, not specified; SNP, single nucleotide polymorphism; UTR, untranslated region. Estimates were adjusted for age, sex, country of origin, severe neutropenia, and prophylactic status (ever having prophylaxis). P<0.05 in bold.

[a]Overall population (Discovery population+extension).
[†]Estimates according a recessive model of inheritance.
[§]SNP associated with IA infection according to a log-additive model of inheritance.
*Estimates calculated according a co-dominant model (homozygotes for the rare allele were not found).

[§]*IL4R*$_{rs2107356}$ (per-allele OR= 2.17, 95%CI 1.18-3.98; P_{trend}=0.0097).
[§]*IFNG*$_{rs2069705}$ (per-allele OR= 0.50, 95%CI 0.26-0.95; P_{trend}=0.027).
[§]*VEGFA*$_{rs3024994}$ (per-allele OR= 3.19, 95%CI 1.08-9.45; P_{trend}=0.033).

Table 5. Discriminative value *AUC* for models including immune-modulating variants.

[a]Including age, gender, allo-HSCT and prophylactic status as variables never dropped from models.
****IL4R*$_{rs2107356}$ and *VEGFA*$_{rs2146323}$ polymorphisms were not significant and were dropped from the model.
Residual deviance (Reference model): 433.21
Residual deviance (Significant SNPs model): 413.31
After removing missing values, 455 subjects (85 IA and 370 non-IA cases) were available for prediction capacity analysis.

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

840
841 **Figure 1.** Fungicidal activity of monocyte-derived macrophage according to *IFNγ*[A], *IL12B* [B],
842 *IL4R* [C] and *IL8* [D] genotypes.
843
844
845 **Figure 2.** *IFNγ*, *TNFα* and *IL12p70* cytokine levels on stimulated PBMCs according to the
846 $IFN\gamma_{rs2069705}$ genotypes.
847
848 PBMCs from healthy donors were stimulated with Zymosan (5µg/ml) and PHA (2 µg/ml) alone or in combination
849 with LPS (100 ng/ml). Supernatants were harvested for *IFNγ*, *TNFα* and *IL12p70* analysis at 72 and 96h.
850
851
852 **Figure 3.** *IFNγ* [A and B] and *IL4R* mRNA [C-F] and protein [G] expression levels according to
853 the $IL4R_{rs2107356}$ genotypes.
854
855 Correlation between $IFN\gamma_{rs2069705}$ genotypes and *IFNγ* mRNA expression levels were analysed in PBMCs from
856 healthy donors at baseline or stimulated with *Aspergillus* conidia [A and B]. Correlation between $IL4R_{rs2107356}$
857 genotypes and IL4R mRNA expression levels were analysed in blood samples from healthy donors [C and D] and
858 in monocyte-derived macrophages at baseline or stimulated with *Aspergillus* conidia [E and F]. Correlation
859 between this promoter variant and IL4R protein levels were also analysed in different immune cells (CD_{19}^{+}, CD_{14}^{+}
860 and CD_{3}^{+}) by flow cytometry [G].
861
862
863 **Figure 4.** Receiver operating characteristics (ROC) curve analysis.
864
865 ROC curves summarize the accuracy of prediction for each particular model. The model including SNPs
866 significantly associated with IA and demographic and clinical variables (marked in blue) showed a significantly
867 improved predictive capacity comparared with a reference model including only demographic and clinical variables
868 (marked in red).
869 AUC=0.659 vs. AUC=0.564.
870

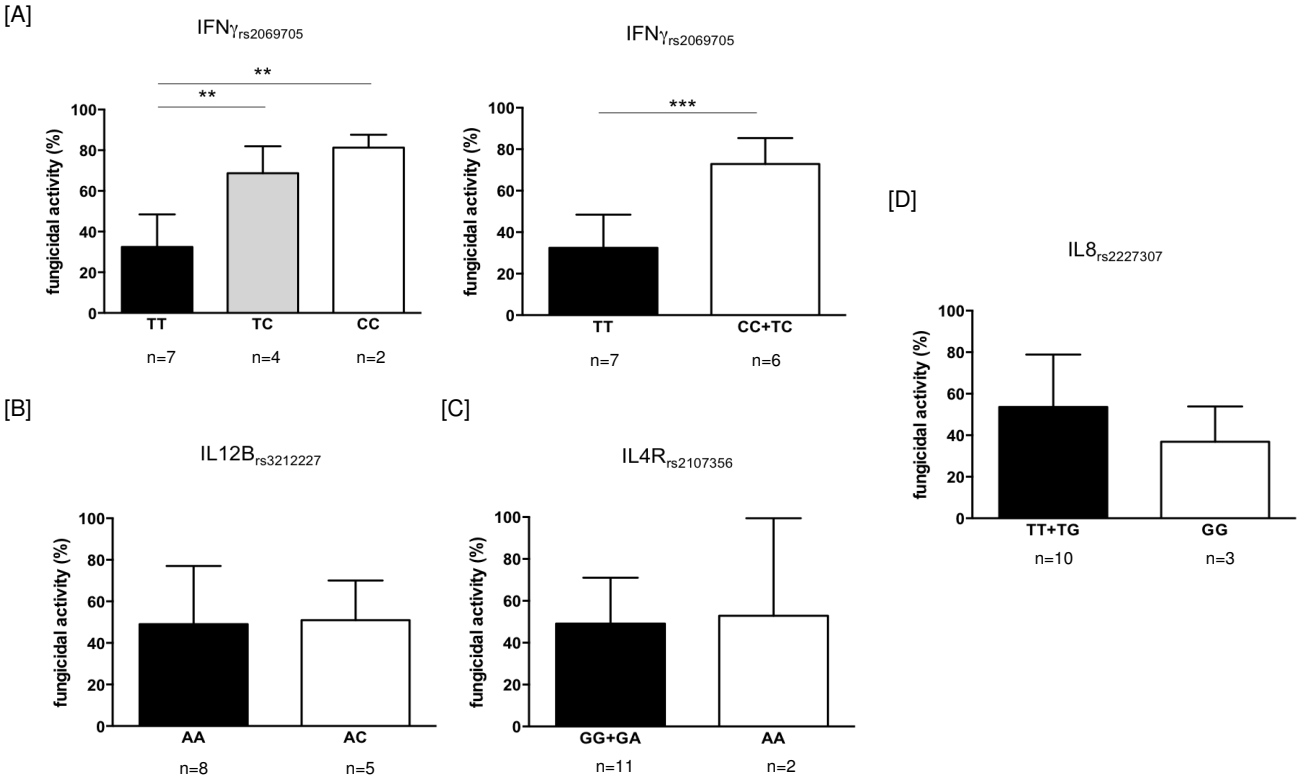
Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

Figure 1



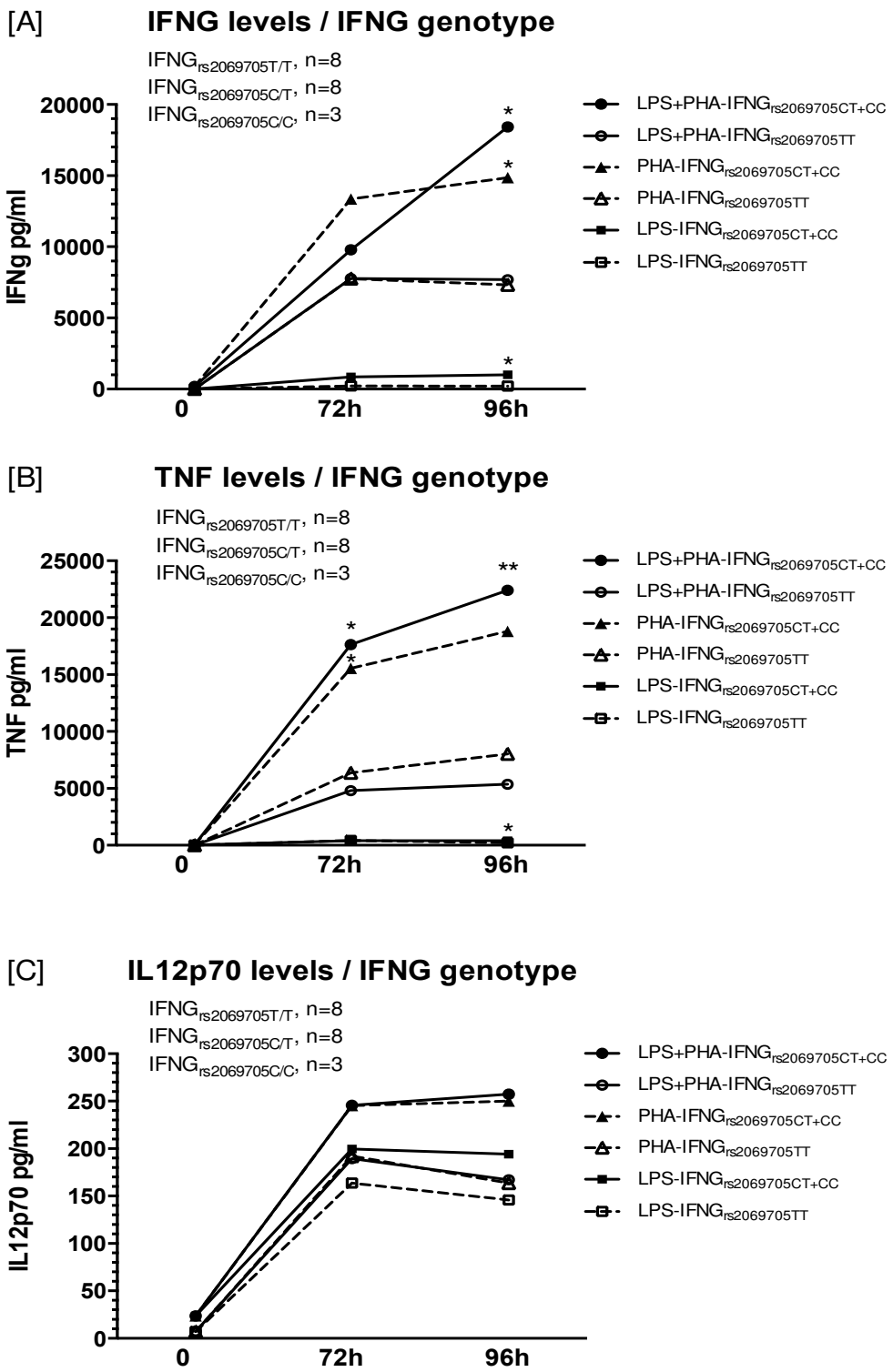
Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

Figure 2



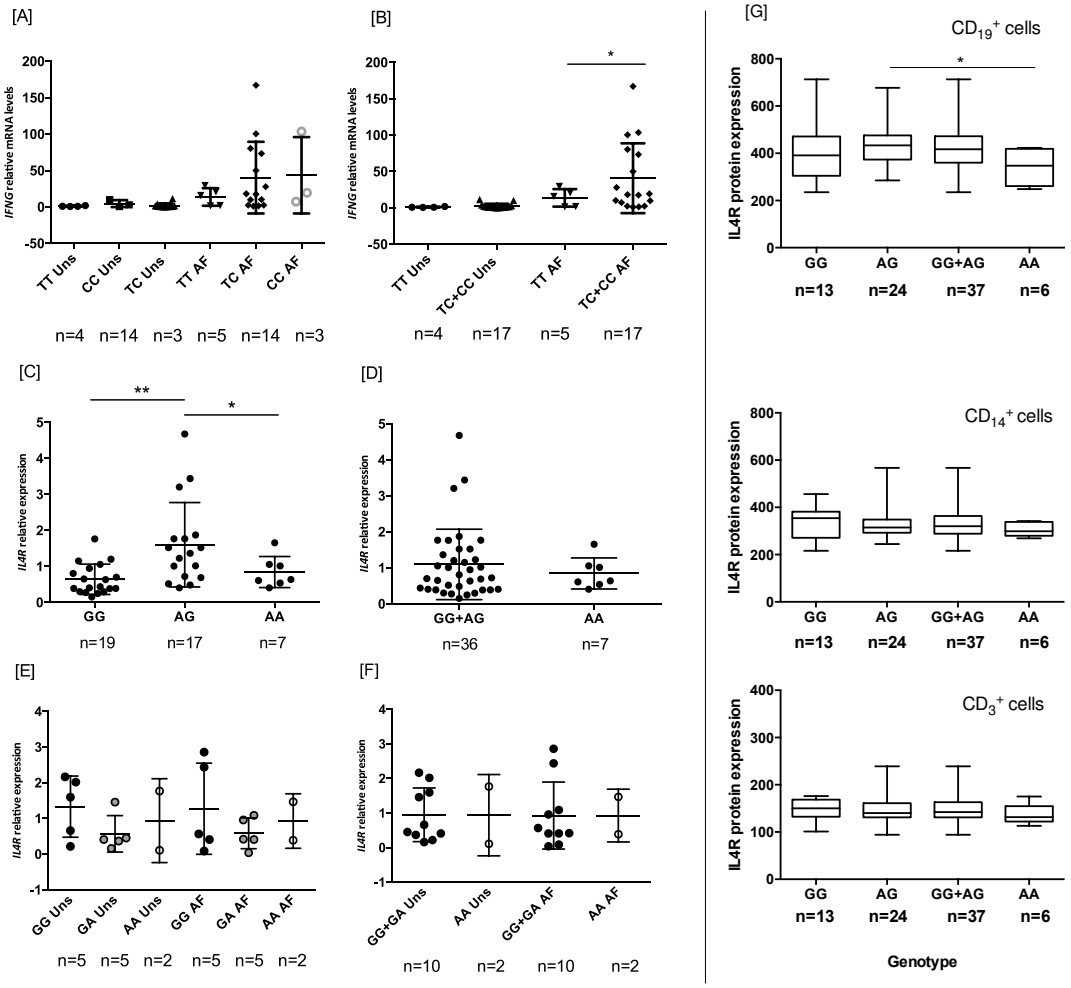
Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

Figure 3



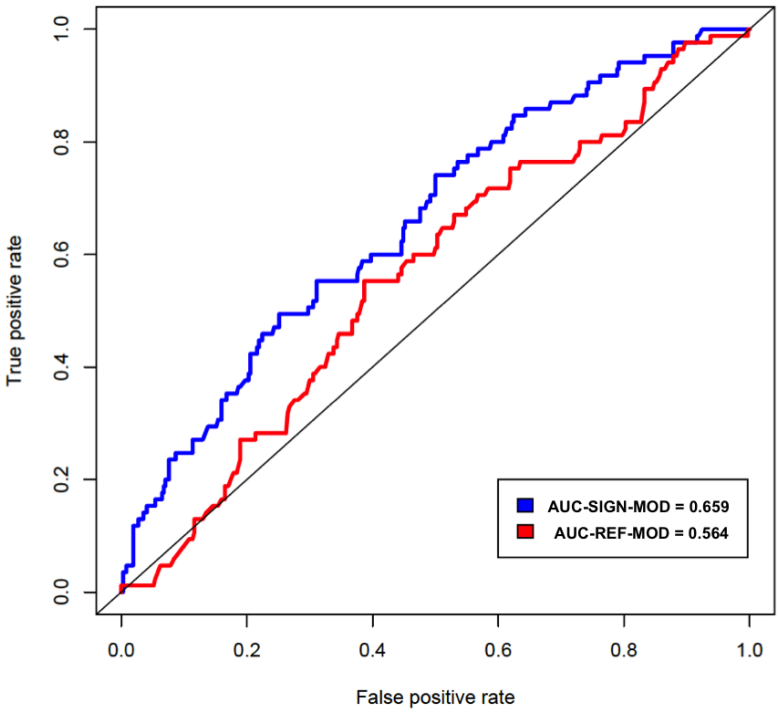
Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

Figure 4



Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

Table 1. Selected SNPs within immune-modulating genes.

Gene name	*Gene symbol_SNP*	*dbSNP rs#*	*Nucleotide substitution*	*Aa change/Location*	*Reported associations with infectious diseases / reported or potential functionality*	*References*
Interleukin 4 (IL4)	*IL4_-1098*	rs2243248	G/T	Promoter	Associated with chronic disseminated candidiasis	(1)
	IL4_Ex1-168	rs2070874	C/T	Intronic	Unknown	
	IL4_IVS2-1443	rs2243268	A/C	Intronic	Unknown	
	IL4_ IVS3-9	rs2243290	A/C	Intronic	Unknown	
Interleukin 4 receptor (IL4R)	*IL4R_-29429 (-3223)*	rs2057768	A/G	Promoter	Associated with soluble IL4R protein levels	(2)
	IL4R_-28120 (-1914)	rs2107356	A/G	Promoter	Unknown	
	IL4R_Ex11+828	rs1801275	A/G	Q576R	Associated with enhanced responsiveness to IL4	(3, 4)
Interleukin 8 (IL8)	*IL8_-251*	rs4073	A/T	Promoter	Associated with increased levels of IL-8 and susceptibility to bacterial urinary tract infection, recurrent Clostridium difficile infection, AIDS, Helicobacter pylori related gastric diseases and mycetoma	(5-8)
	IL8_ IVS1+230 (+396)	rs2227307	G/T	Intronic	Associated with susceptibility to periodontitis	(9)
CXC-Chemokine receptor 1 (IL8RA)	*CXCR1_Ex2+860*	rs2234671	G/C	S276T	Associated with chronic HBV infection. Predicted to affect IL8 signalling (Benign; Polyphen)	(10)
CXC-Chemokine receptor 2 (IL8RB)	*CXCR2_Ex3-1010*	rs1126580	A/G	Intronic	Unknown	
Interleukin 10 (IL10)	*IL10_IVS1-286*	rs3024491	G/T	Intronic	Unknown	
	IL10_Ex5+210	rs3024496	C/T	Intronic	IL10_Ex5+210G alleles is associated with the decreased production of IL-10 by peripheral blood leukocytes in response to helminth infection	(11)
Interleukin 12 alpha (IL12A)	*IL12A_IVS2-798*	rs582054	A/T	Intronic	Unknown	
Interleukin 12 beta (IL12B)	*IL12B_Ex8+159 (+1188)*	rs3212227	A/C	Intronic	IL12B_+1188C allele is associated with an increased risk of lepromatous leprosy	(12)
Interleukin 13 (IL13)	*IL13_-1069*	rs1800925	C/T	Promoter	Alters the expression of *IL13* and the binding of nuclear factors to the *IL13* promoter. Associated with an increased risk of severe respiratory syncytial virus (RSV) infection.	(13, 14)
	IL13_Ex4+98	rs20541	C/T	R144Q	Modifies the *IL13*-mediated Th2 effector functions and correlates with *IL13* activity and levels (114Q carriers have higher levels of *IL13* compared to 144R)	(15)
	IL13_IVS3-24	rs1295686	A/G	Intronic	Unknown	
Interferon gamma (IFNγ)	*IFNG_-1615*	rs2069705	C/T	Promoter	Associated with a reduced risk of IA	(16)
	IFNG_IVS3+284 (+2109)	rs1861494	C/T	Intronic	Unknown	
Interferon gamma receptor 2 (IFNγR2)	*IFNGR2_Ex7-128*	rs1059293	C/T	Intronic	Unknown	
	IFNGR2_Ex2-16	rs9808753	A/G	Q64R	Predicted to affect *IFNγ* signalling (Possibly damaging; Polyphen)	
C-C chemokine receptor type 5 (CCR5)	*CCR5_IVS1+246*	rs1799987	A/G	Intronic	Associated with CCR5 protein levels and HIV-1	(17, 18)
	CCR5_IVS1+151	rs2734648	G/T	Intronic	Part of an haplotype associated with HIV-1	(19)

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

Macrophage migration inhibitory factor (MIF)	*MIF_-173*	rs755622	C/G	Promoter	MIF_-173CC is associated with pulmonary tuberculosis	(20, 21)
Vascular Endothelial Growth Factor alpha (VEGFA)	*VEGFA_-2578*	rs699947	A/C	Promoter	VEGF_-2578CC was associated with higher or lower VEGF expression. Associated with urinary tract infection.	(22-24)
	VEGFA_-7	rs25648	C/T	Promoter	Associated with higher levels of VEGFA mRNA	(25)
	VEGFA_IVS2+1378	rs3024994	C/T	Intronic	Unknown	
	VEGFA_IVS7-919	rs3025035	C/T	Intronic	Unknown	
	VEGFA_6112	rs2146323	A/C	-	Unknown	
	VEGFA_IVS-99	rs3024997	A/G	Intronic	Unknown	
	VEGFA_IVS7+763	rs3025030	C/G	Intronic	Unknown	
	VEGFA_5530	rs998584	G/T	-	Unknown	
	VEGFA_5958bp 3'of STP	rs6899540	A/C	-	Unknown	
	VEGFA_6119bp 3'of STP	rs6900017	C/T	-	Unknown	
	VEGFA_Near gene	rs6905288	A/G	-		

Abbreviations: SNP, single nucleotide polymorphisms, UTR, untranslated region. References included in this table are listed in the supplementary material.

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

Table 2. Baseline and clinical characteristic of patients with or without invasive aspergillosis (IA).

Phases 1	**aspBIOmics consortium + UHS-GHV-PCR-AGA populations**			
	overall (n=593)	IA patients (n=113)	non-IA patients (n=480)	*P* value
Demographic variables				
age (range)	51.46 ± 15.08	51.70 ± 13.20	51.40 ± 15.51	0.834
sex ratio (male/female)	1.27 (332/261)	1.90 (74/39)	1.16 (258/222)	**0.031**
Haematological disease				
AML	412 (69.48)	66 (58.41)	346 (72.08)	**0.006**
ALL	73 (12.31)	23 (20.35)	50 (10.42)	**0.006**
other	108 (18.21)	24 (21.24)	84 (17.50)	0.429
allo-HSCT	299 (50.42)	65 (57.52)	234 (48.75)	0.116
Ever received prophylaxis *				
posaconazole	73 (12.31)	10 (8.85)	63 (13.13)	0.277
itraconazole	49 (8.26)	13 (11.50)	36 (7.50)	0.230
echinocandins	30 (5.06)	2 (1.77)	28 (5.83)	0.125
voriconazole	21 (3.54)	6 (5.31)	15 (3.13)	0.397
amphotericin B	4 (0.67)	0 (0.00)	4 (0.83)	0.738
Never received prophylaxis	450 (75.89)	87 (76.99)	363 (75.63)	0.855
Phase 2	**CSC-MO + VNH population**			
	overall (n=188)	IA patients (n=36)	non-IA patients (n=152)	*P* value
Demographic variables				
age (range)	56.93 ± 18.12	55.75 ± 19.50	57.21 ± 17.84	0.665
sex ratio (male/female)	1.32 (107/81)	1.57 (22/14)	1.27 (85/67)	0.705
Haematological disease				
AML	173 (92.02)	34 (94.44)	139 (91.45)	0.799
ALL	3 (1.60)	1 (2.78)	2 (1.32)	0.912
other	12 (6.38)	1 (2.78)	11 (7.24)	0.545
allo-HSCT	39 (20.74)	3 (8.33)	36 (23.68)	0.069
Ever received prophylaxis†				
posaconazole	40 (40.40)	3 (11.11)	37 (51.39)	**0.0007**
itraconazole	7 (7.07)	3 (11.11)	4 (5.56)	0.603
echinocandins	1 (1.01)	0 (0.00)	1 (1.39)	0.608
voriconazole	2 (2.02)	0 (0.00)	2 (2.78)	0.942
amphotericin B	16 (16.16)	2 (7.41)	14 (19.44)	0.253
Never received prophylaxis∂	69 (69.70)	24 (88.89)	45 (62.50)	**0.022**
Phases 1+2	**aspBIOmics consortium + UHS-GHV-PCR-AGA + CSC-MO population**			
	overall (n=781)	IA patients (n=149)	non-IA patients (n=632)	*P* value
Demographic variables				
age (range)	52.77 ± 16.03	52.67 ± 15.00	52.79 ± 16.27	0.935
sex ratio (male/female)	1.28 (439/342)	1.81 (96/53)	1.19 (343/289)	**0.031**
Haematological disease				
AML	585 (74.90)	100 (67.11)	485 (76.74)	**0.020**
ALL	76 (09.73)	24 (16.11)	52 (08.23)	**0.006**
other	120 (15.36)	25 (16.78)	95 (15.03)	0.685
allo-HSCT	338 (43.28)	68 (45.64)	270 (42.72)	0.579
Ever received prophylaxis				
posaconazole	113 (16.33)	13 (9.29)	100 (18.12)	**0.017**
itraconazole	56 (8.09)	16 (11.43)	40 (7.25)	0.148
echinocandins	31 (4.48)	2 (1.43)	29 (5.25)	0.085
voriconazole	23 (3.32)	6 (4.29)	17 (3.08)	0.655
amphotericin B	20 (2.89)	2 (1.43)	18 (3.26)	0.383
Never received prophylaxis∂	519 (75.00)	111 (79.29)	408 (73.91)	0.229

Abbreviations: HSCT: Haematopoietic stem cell transplantation, AML: acute myeloid leukemia, ALL: acute lymphoid leukemia; UHS, University hospital of Salamanca (Spain); GHV, General hospital of Valencia (Spain); PCRAGA clinical trial (EU clinical trial number: 2010-019406-17); CSC, Università Cattolica del S. Cuore, Rome (Italy); MO, University of Modena and Reggio Emilia, Modena (Italy) and Virgen de las Nieves University hospital (VNH). P≤0.05 was considered significant.

* Some patients had several prophylactic drugs.

† Prophylaxis status was only available in 99 subjects (15 IA and 72 non-IA patients).

∂ Percentage calculated according to the number of patients with prophylaxis data available.

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

Table 3. Associations found between immunoregulatory polymorphisms and invasive aspergillosis.

Variant_dbSNP	Gene	OR (95% CI)[a]	P_{value}	OR (95% CI)[b]	P_{value}
rs2243248	*IL4*	1.19 (0.63-2.26)	0.59		
rs2070874	*IL4*	0.91 (0.53-1.55)	0.72		
rs2243268	*IL4*	0.85 (0.52-1.38)	0.50		
rs2243290	*IL4*	0.67 (0.39-1.16)	0.14		
rs2057768	*IL4R*	1.20 (0.75-1.92)	0.44		
rs2107356	*IL4R*	**2.05 (1.24-3.40)**[†]	**0.0063**	**1.92 (1.20-3.09)**[†]	**0.008**
rs1801275	*IL4R*	1.00 (0.63-1.59)	0.99		
rs4073	*IL8*	1.02 (0.64-1.61)	0.95		
rs2227307	*IL8*	1.72 (1.00-2.94)[†]	**0.049**	**1.73 (1.06-2.81)**[†]	**0.031**
rs2234671	*IL8RA*	1.57 (0.80-3.08)	0.20		
rs1126580	*IL8RB*	1.50 (0.88-2.54)	0.13		
rs3024491	*IL10*	1.09 (0.67-1.78)	0.72		
rs3024496	*IL10*	1.16 (0.71-1.90)	0.55		
rs582054	*IL12A*	1.09 (0.64-1.84)	0.76		
rs3212227	*IL12B*	**0.57 (0.35-0.93)**	**0.021**	**0.60 (0.38-0.96)**[§]	**0.029**
rs20541	*IL13*	0.76 (0.46-1.24)	0.26		
rs1800925	*IL13*	0.85 (0.54-1.36)	0.51		
rs1295686	*IL13*	0.73 (0.45-1.16)	0.18		
rs2069705	*IFNG*	**0.56 (0.36-0.88)**[§]	**0.012**	**0.63 (0.41-0.97)**	**0.035**
rs1861494	*IFNG*	0.74 (0.47-1.17)	0.20		
rs1059293	*IFNGR2*	0.98 (0.57-1.67)	0.93		
rs9808753	*IFNGR2*	1.10 (0.65-1.85)	0.72		
rs1799987	*CCR5*	1.40 (0.83-2.36)	0.20		
rs2734648	*CCR5*	1.07 (0.67-1.71)	0.76		
rs755622	*MIF*	1.38 (0.84-2.25)	0.20		
rs25648	*VEGFA*	1.11 (0.63-1.97)	0.72		
rs699947	*VEGFA*	1.28 (0.75-2.18)	0.35		
rs3024994	*VEGFA*	1.61 (0.86-3.03)	0.15		
rs3025035	*VEGFA*	1.31 (0.78-2.22)	0.31		
rs2146323	*VEGFA*	**1.63 (1.02-2.61)**	**0.040**	1.46 (0.95-2.27)	0.085
rs3024997	*VEGFA*	1.04 (0.67-1.61)	0.87		
rs3025030	*VEGFA*	1.00 (0.58-1.70)	0.99		
rs998584	*VEGFA*	0.66 (0.41-1.06)	0.088		
rs6899540	*VEGFA*	0.84 (0.51-1.40)	0.50		
rs6900017	*VEGFA*	**1.76 (1.02-3.03)**[§]	**0.046**	1.47 (0.87-2.47)	0.16
rs6905288	*VEGFA*	0.83 (0.52-1.31)	0.42		

Abbreviations: OR, odds ratio; CI, confidence interval. Abbreviations: n/s, not specified; SNP, single nucleotide polymorphism; UTR, untranslated region. Estimates were adjusted for age, sex, country of origin, allo-SCT, and prophylaxis status (ever use of prophylaxis). P<0.05 in bold. *P*<0.0004 was defined as multiple testing significance threshold.

[a]Phase 1 (aspBIOmics + PCRAGA+Valencia+Salamanca populations; N=593 hematological patients).
[b]Overall (N=781; after extension with 188 high-risk patients [39 HSCT and 149 non-HSCT patients]; 87 of them with prophylaxis data).
[†]Estimates according a recessive model of inheritance.
[§]SNP signifcantly associated with IA according a log-additive model of inheritance.

[§]$IFNG_{rs2069705}$ (per-allele OR= 0.69, 95%CI 0.49-0.97; P_{trend}=0.032).
[§]$VEGFA_{rs2146323}$ (per-allele OR= 1.45, 95%CI 1.04-2.03; P_{trend} =0.029).
[§]$VEGFA_{rs6900017}$ (per-allele OR= 1.73, 95%CI 1.08-2.77; P_{trend}=0.027).
[§]$IL12B_{rs3212227}$ (per-allele OR= 0.67, 95%CI 0.45-0.99; P_{trend} =0.040).

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

Table 4. Associations found between immunoregulatory SNPs and IA in allo-HSCT patients (n=171).

Variant_dbSNP	Gene	OR (95%CI)	P_{value}
rs2243248	*IL4*	0.93 (0.25-3.44)	0.91
rs2070874	*IL4*	1.40 (0.56-3.52)	0.47
rs2243268	*IL4*	1.15 (0.49-2.73)	0.75
rs2243290	*IL4*	0.93 (0.35-2.45)	0.88
rs2057768	*IL4R*	1.30 (0.56-3.03)	0.54
rs2107356	*IL4R*	**5.63 (1.98-16.05)**[†§]	**0.0009**
rs1801275	*IL4R*	0.50 (0.20-1.24)	0.12
rs4073	*IL8*	0.76 (0.33-1.73)	0.51
rs2227307	*IL8*	1.21 (0.51-2.86)	0.67
rs2234671	*IL8RA*	1.48 (0.42-5.15)	0.55
rs1126580	*IL8RB*	2.39 (0.92-6.20)[†]	0.072
rs3024491	*IL10*	1.08 (0.44-2.70)	0.86
rs3024496	*IL10*	0.74 (0.30-1.83)	0.52
rs582054	*IL12A*	1.73 (0.61-4.91)	0.29
rs3212227	*IL12B*	0.64 (0.26-1.57)	0.32
rs20541	*IL13*	0.80 (0.32-1.99)	0.63
rs1800925	*IL13*	1.96 (0.84-4.58)	0.12
rs1295686	*IL13*	0.53 (0.22-1.29)	0.16
rs2069705	*IFNG*	**0.24 (0.10-0.59)**[§]	**0.0011**
rs1861494	*IFNG*	0.63 (0.27-1.49)	0.29
rs1059293	*IFNGR2*	1.53 (0.59-3.97)	0.37
rs9808753	*IFNGR2*	0.78 (0.29-2.09)	0.62
rs1799987	*CCR5*	1.75 (0.65-4.69)	0.26
rs2734648	*CCR5*	1.04 (0.44-2.48)	0.93
rs755622	*MIF*	0.63 (0.25-1.61)*	0.33
rs25648	*VEGFA*	1.39 (0.53-3.66)	0.51
rs699947	*VEGFA*	0.51 (0.19-1.36)	0.18
rs3024994	*VEGFA*	**4.48 (1.25-16.08)**[§]	**0.022**
rs3025035	*VEGFA*	1.96 (0.77-4.99)	0.16
rs2146323	*VEGFA*	0.86 (0.38-1.97)	0.72
rs3024997	*VEGFA*	0.71 (0.31-1.62)	0.41
rs3025030	*VEGFA*	1.11 (0.41-3.01)*	0.84
rs998584	*VEGFA*	0.84 (0.32-2.22)	0.72
rs6899540	*VEGFA*	0.62 (0.23-1.69)	0.34
rs6900017	*VEGFA*	2.68 (0.97-7.42)	0.061
rs6905288	*VEGFA*	1.04 (0.43-2.53)	0.92

Abbreviations: OR, odds ratio; CI, confidence interval. Abbreviations: n/s, not specified; SNP, single nucleotide polymorphism; UTR, untranslated region. Estimates were adjusted for age, sex, country of origin, severe neutropenia, and prophylactic status (ever having prophylaxis). P<0.05 in bold. *P*<0.0004 was defined as corrected significance threshold.

[†]Estimates according a recessive model of inheritance.
[§]SNP associated with IA infection according to a log-additive model of inheritance.
*Estimates calculated according a co-dominant model (homozygotes for the rare allele were not found).

[§] $IL4R_{rs2107356}$ (per-allele OR= 2.17, 95%CI 1.18-3.98; P_{trend}=0.0097).
[§] $IFNG_{rs2069705}$ (per-allele OR= 0.50, 95%CI 0.26-0.95; P_{trend}=0.027).
[§] $VEGFA_{rs3024994}$ (per-allele OR= 3.19, 95%CI 1.08-9.45; P_{trend}=0.033).

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

Table 5. Discriminative value *AUC* for models with or without immune-modulating variants.

Reference model				
SNPs	P-value	OR 95%CI	AUC 95%CI[a]	*P*-value
Age	0.898	1.001 (0.985-1.017)		
Gender	0.033	1.721 (1.045-2.835)		
Allo-SCT	0.785	0.934 (0.570-1.529)		
Prophylactic status	0.790	1.080 (0.612-1.906)	0.564 (0.499-0.630)[∂]	0.064
Predictive model built with 4 significant SNPs*				
SNPs	P-value	OR 95%CI	AUC 95%CI[a]	*P*-value
IL8$_{rs2227307}$	0.024	1.952 (1.093-3.489)		
IL12B$_{rs3212227}$	0.016	0.508 (0.292-0.884)		
IFNG$_{rs2069705}$	0.031	0.583 (0.358-0.952)		
VEGFA$_{rs6900017}$	0.040	1.814 (1.026-3.207)		
Age	0.951	1.001 (0.984-1.018)		
Gender	0.064	1.626 (0.972-2.719)		
Allo-SCT	0.757	0.923 (0.557-1.532)		
Prophylactic status	0.525	1.210 (0.672-2.179)	0.659 (0.596-0.722)[∂]	0.000005

[a]Including age, gender, allo-SCT and prophylactic status as variables never dropped from models.

*$IL4R_{rs2107356}$ and $VEGFA_{rs2146323}$ polymorphisms were not significant and were dropped from the model.

∂ These models showed a statistically different prediction capacity (-2log likehood ratio test, df=4, *P*=0.00052).

Residual deviance (Reference model): 433.21

Residual deviance (Significant SNPs model): 413.31

After removing missing values, 455 subjects (85 IA and 370 non-IA cases) were available for prediction capacity analysis.

Permutation analysis: Average AUC of null distribution (50.000 models)=0.6001; $SD_{50.000AUC}$=0.0158;

Z-score_value$_{50.000perm}$=3.7361 and $P_{50.000perm}$=$9.34 \cdot 10^{-05}$.