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Infection and Immunity
IAI
Infection and Immunity

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

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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.

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Infection and Immunity
IAI
Infection and Immunity

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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 *IFNγ*$_{rs2069705}$ 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 *IFNγ*$_{rs2069705}$ 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 *IFNγ*$_{rs2069705C}$ 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, $P_{LR}=5.2\bullet10^{-4}$ and $P_{50.000Perm}=9.34\bullet10^{-5}$). These findings suggest that the *IFNγ*$_{rs2069705}$ 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.

Infection and Immunity
IAI
Infection and Immunity

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Infection and Immunity

IAI

Infection and Immunity

INTRODUCTION

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

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

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

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Infection and Immunity

IAI

Infection and Immunity

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

MATERIAL AND METHODS

Study design and study population

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

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University hospital (Granada, Spain) between January 2013 and January 2015. This ambitious study design provided a population of 781 high-risk patients, 149 of whom were diagnosed with proven and probable IA (19 proven and 130 probable IA). To our knowledge, this is one of the largest population recruited so far exploring genetic susceptibility to IA. The study was approved by the ethical review boards of each participating institution.

Statistical analysis

The Hardy-Weinberg Equilibrium (HWE) tests were performed in the uninfected control group by a standard observed-expected chi-square (χ^2) test. Logistic regression analyses adjusted for age, gender, country of origin, allo-HSCT and receipt or non-receipt of anti-fungal prophylaxis were performed to determine significant associations with IA risk. SNPtool (50) and Haploview were used for linkage disequilibrium (LD) blocks reconstruction and haplotype association statistics. Block structures were determined according to the method of Gabriel *et al.* (51). In order to account for multiple testing, we calculated an adjusted significance level using the Meff method (52), which consider the number of independent marker loci (M_{effLi}=31), and the number of models of inheritance tested (co-dominant, dominant, recessive and log-additive). Detailed information about this method of multiple testing correction is freely available online at http://neurogenetics.qimrberghofer.edu.au/SNPSpDlite. Thus, the resulting threshold for the main effect analysis was 0.0004 ([0.05/31]/4).

Cell isolation and differentiation

Peripheral blood mononuclear cells (PBMCs) and monocytes were isolated from whole blood collected from healthy donors after obtaining written informed consent (PI12/02688 and SECVS 014/2015 protocols). PBMCs were isolated by gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare Bio-Sciences) and monocytes were isolated by immunomagnetic selection of CD_{14}^+ cells (Miltenyi Biotec). Purity of the obtained CD_{14}^+ population was assessed by fluorescence-activated cell sorting analysis. Monocytes were then plated at a density of 5×10^5 cells/mL in 24-well plates, cultivated for 7 days in complete RPMI 1640 medium supplemented

Infection and Immunity
IAI
Infection and Immunity

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with human serum and 20 ng/mL of GM-CSF to allow differentiation into macrophages. The culture medium was replaced every 3 days. Genotyping of significant SNPs was performed and either PBMCs or monocytes were grouped according to the genotype of interest.

Assessment of fungicidal activity

Human monocyte-derived macrophages were infected with conidia from *Aspergillus fumigatus* at an effector-to-target ratio of 1:10. To measure the fungicidal ability, macrophages were allowed to kill the ingested conidia for 2 h. Serial dilutions of macrophage lysates were plated on solid growth media and following a 2-day incubation, the number of colony-forming units (CFU) was enumerated and the percentage of CFU inhibition was calculated. In order to avoid a bias due to differences in internalization rates, the supernatants collected after the co-culture were plated and compared among different donors.

IL12p70 and IFNγ stimulation assays.

IL12p70 and *IFNγ* stimulation assays were performed in PBMCs from healthy donors according to a previously reported protocol (53). PBMCs were selected according to the $IL12B_{rs3212227}$ and $IFN\gamma_{rs2069705}$ genotypes and were cultured in 2 ml of culture medium RPMI-1640 supplemented with 10% sterile heat-inactivated fetal bovine serum (FBS) and an antibiotic mixture containing penicillin, streptomycin and neomycin (Gibco®/Life Technologies) at 37ºC in 5% CO_2. PBMCs from healthy subjects harbouring the $IFN\gamma_{rs2069705T/T}$ (n=8), $IFN\gamma_{rs2069705C/T}$ (n=8) and $IFN\gamma_{rs2069705C/C}$ (n=3) genotypes were incubated for 72h and 96h with phytohaemagglutinin (PHA, 2μg/ml) alone or in combination with lypopolysaccharide (LPS, 100ng/ml) and IFNγ, IL12p70, TNF and IL8 levels were determined in triplicate using the Procartaplex, Multiplex Immunoassay (Affymetrix/eBioscience) according to manufacturer's recommendations. In parallel, PBMCs bearing the $IL12B_{rs3212227A/A}$ (n=13), $IL12B_{rs3212227A/C}$ (n=3) and $IL12B_{rs3212227C/C}$ (n=1) genotypes were treated for 24h and 48h with zymosan (5μg/ml) alone or in combination with LPS (100ng/ml) and correlation of cytokine levels with the $IL12B_{rs3212227}$ or $IL8_{rs321227}$ SNPs was also

Infection and Immunity
IAI
Infection and Immunity

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analysed. After the incubation period, supernatants were collected and stored at -80ºC until cytokine measurement.

Analysis of IL4R and IFNγ mRNA expression

We measured IL4R and *IFNγ* mRNA gene expression in blood samples collected from healthy blood donors but also in monocyte-derived macrophages at baseline and after the stimulation with conidia from *A. fumigatus* at an effector-to-target ratio of 1:2 for 8 h. Total RNA from blood or monocyte-derived macrophages was extracted using RNeasy Mini Kit (QIAGEN) and reverse transcribed with the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. Real-time RT-PCR was performed in an Applied Biosystems 7500HT Fast using Taqman probe-based gene expression technology (Life Technologies) according to the manufacturer's instructions. Statistical significance in gene expression changes was determined by unpaired t test with Whelch's correction (assuming unequal variance between groups).

Analysis of IL4R protein expression on T- and B-lymphocytes and monocytes by flow cytometry

IL4R protein levels were determined in PBMCs carrying the wild-type $IL4R_{rs2107356G/G}$ (n=13), heterozygote $IL4R_{rs2107356A/G}$ (n=24) or mutant $IL4R_{rs2107356A/A}$ genotypes (n=6) by flow cytometry following a slightly modified version of a previously reported protocol (54). Briefly, PBMCs (1 x 10^6) were pre-incubated with PBS supplemented with 5% FBS + 2 mM EDTA for 10 minutes to block Fc receptors. Subsequently, cells were stained for 45 minutes at ambient temperature with mouse anti-human antibodies to determine the levels of *IL4R* protein expression on CD_3^+T-cells, CD_{19}^+B-cells and CD_{14}^+ monocytes. The negative control consisted of cells incubated with a mouse PE-IgG1 Kappa (BD Pharmingen). The analysis was performed according to the flow cytometric cell surface staining method and the following antibodies were used: phycoerythrin (PE)-conjugated CD_{124}^+, Peridin chlorophyll protein (PerCP)-conjugated CD_{14}^+, allophycocyanin (APC)-eFluor®780-conjugated CD_3^+ and fluorescein isothiocyanate (FITC)–conjugated CD_{19}^+ antibodies (BD Pharmingen). Cells were acquired on a BD FACSVerse flow cytometer (BD Biosciences) and the data were analysed using the FlowJo software (TreeStar Inc.). Median

Infection and Immunity
IAI
Infection and Immunity

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fluorescence intensity (MFI) of the positive population was recorded for each cell type and statistical differences were evaluated using an unpaired t-test with Welch's correction (two-tailed P value).

Predictive models and discriminative accuracy

The value of immune-modulating polymorphisms for prediction of IA was examined using stepwise logistic regression analysis. A prediction model was built that included age, gender, allo-HSCT and anti-fungal prophylactic status, and those genetic variants that showed significant associations with IA in the single-SNP analysis (P<0.05; phases 1+2). Then, using p-values as a selection criterion, variables with the highest p-value were dropped and analyses were finalized when all variables reached statistical significance (P<0.05). A predictive model with a similar number of "non-significant SNPs" (P>0.10) was also built. The area under the curve (AUC) of a receiver operating characteristic (ROC) curve analysis was used to assess the discriminative accuracy of each particular model compared with a reference model including only demographic and clinical variables as covariates (age, gender, allo-HSCT and anti-fungal prophylaxis status). A -2 log likelihood ratio (LR) test was used to determine whether predictive models including genetic information were statistically different when compared with the reference model. Finally, we run a randomization test to confirm whether the improved predictive ability of the model including genetic variants significantly associated with IA was consistent after 50,000 iterations. Further details are included in supplementary material. All analyses were performed using R (http://www.r-project.org/).

RESULTS

A total of 781 patients were enrolled in this case-control study and, among them, one hundred and forty-nine were diagnosed with proven or probable IA according to the revised EORTC/MSG definitions. The remaining 632 patients showed no evidence for proven or probable IA. Baseline and clinical characteristics of IA and non-IA patient groups are summarized in Table 2. Overall, IA and non-IA patients had a similar mean age (52.67 vs. 52.79, *P*=0.935) but IA patients showed a

Infection and Immunity
IAI
Infection and Immunity

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significantly higher male to female gender ratio when compared with those patients with no evidence of IA (1.81 vs. 1.19, *P*=0.031). In addition, the percentage of patients diagnosed with ALL was significantly higher in IA compared with non-IA patients (16.11% vs. 8.23%, *P*=0.006) whereas the percentage of patients with AML was significantly lower in IA compared to non-IA cases (67.11% vs. 76.74%, P=0.020). Interestingly, we also observed a significantly lower proportion of IA cases among those receiving posaconazole prophylaxis (*P*=0.017).

Thirty-six genetic variants within 14 immune-modulating genes were initially genotyped in 593 high-risk patients (113 IA and 480 non-IA patients). Logistic regression analysis revealed that patients carrying the *IL4R*$_{rs2107356A/A}$ and the *VEGFA*$_{rs2146323A}$ and *VEGFA*$_{rs6900017T}$ alleles had a significantly increased risk of IA (OR=2.05, 95%CI 1.24-3.40; OR=1.63, 95%CI 1.02-2.61 and OR=1.76, 95%CI 1.02-3.03, respectively) whereas patients carrying the *IL12B*$_{rs3212227C}$ and *IFN*$\gamma_{rs2069705C}$ alleles showed a significantly decreased risk of developing the disease (OR=0.57, 95%CI 0.35-0.93 and OR=0.56, 95%CI 0.36-0.88, respectively; Table 3). When a log-additive model was assumed, we also found a significant association between the *VEGFA*$_{rs2146323}$ and *VEGFA*$_{rs6900017}$ SNPs and an increased risk of IA (per-allele OR=1.45, 95%CI 1.04-2.03 and per-allele OR=1.73, 95%CI 1.08-2.77) and a statistically significant association of the *IFN*$\gamma_{rs2069705}$ SNP with a decreased risk of developing IA (per-allele OR=0.69, 95%CI 0.49-0.97).

In order to further confirm these significant associations, the study cohort was extended by recruiting 188 additional patients, 36 of whom were diagnosed with proven or probable IA. Given the low number of proven and probable IA cases, we could not consider this second population as an independent population for replication. An overall association analysis including 781 patients (149 IA and 632 non-IA patients) confirmed that carriers of the *IL4R*$_{rs2107356A/A}$ and *IL8*$_{rs2227307G/G}$ genotypes had a significantly increased risk of IA when compared with those carrying the wild type allele (OR=1.92, 95%CI 1.20-3.09 and OR=1.73, 95%CI 1.06-2.81) whereas those subjects harbouring the *IL12B*$_{rs3212227C}$ and *IFN*$\gamma_{rs2069705C}$ alleles showed a decreased risk of developing the infection (OR=0.60, 95%CI 0.38-0.96 and OR=0.63, 95%CI

Infection and Immunity
IAI
Infection and Immunity

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0.41-0.97, respectively). When we tested the allele-dose effect of significant SNPs, we found that the *IL12B*$_{rs3212227}$ polymorphism was significantly associated with a reduced risk of getting the infection (per-allele OR=0.67, 95%CI 0.45-0.99; Table 3). As part of these association analyses, we also performed haplotype analysis that confirmed that none of these polymorphisms were part of risk haplotypes. We only observed a significant association with IA for a relatively rare *IFNγ*$_{TC}$ haplotype whose effect was likely due to the *IFNγ*$_{rs2069705}$ SNP (OR=0.34, 95%CI 0.13-0.88; Supplementary Table 1).

Interestingly, a logistic regression analysis restricted to allo-HSCT patients and considering only donor genotypes and episodes of IA that occurred after transplantation (n=171) also showed that the effect of the *IL4R*$_{2107356}$ and *IFNγ*$_{rs2069705}$ SNPs on the risk of IA was considerably stronger in allo-HSCT patients compared to those patients who did not undergo transplantation (OR=5.63, 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, 95%CI 0.52-1.45; respectively; Table 4). In this allo-HSCT-stratified analysis, we also found that allo-HSCT patients carrying the *VEGFA*$_{rs3024994T}$ allele showed an increased risk of IA when compared with those allo-HSCT patients carrying the wild type genotype/allele (OR=4.48, 95%CI 1.25-16.08; Table 4).

Although none of the reported overall, haplotype and allo-HSCT-stratified associations remained significant after correction for multiple testing ($P_{Meff_correction}$=0.0004), the association of *IL4R*$_{rs2107356}$ and *IFNγ*$_{rs2069705}$ polymorphisms showed a marginal level of significance in allo-HSCT patients when a recessive and dominant model were respectively assumed (P_{REC}=0.0009 and P_{DOM}=0.0011, respectively). Considering these results and those showing a suggestive association between *IL12B*$_{rs3212227}$ SNP and risk of IA, we decided to evaluate whether the *IL4R*$_{rs2107356}$, *IL8*$_{rs2227307}$, *IL12B*$_{rs3212227}$ and *IFNγ*$_{rs2069705}$ variants could have a functional effect in modulating the strength of immune responses against specific *Aspergillus* antigens and/or stimulatory molecules. For that purpose, we first investigated whether *IFNγ*$_{rs2069705}$ and *IL4R*$_{rs2107356}$ but also *IL12B*$_{rs3212227}$ and *IL8*$_{rs2227307}$ variants correlated with the ability of monocyte-

Infection and Immunity
IAI
Infection and Immunity

Accepted Manuscript Posted Online

derived macrophages to efficiently kill fungal conidia. Interestingly, we found that macrophages from donors carrying the *IFNγ*$_{rs2069705C}$ allele showed a significantly increased capability to kill fungal spores that those from subjects carrying the wild type genotype (TT vs. TC, *P*=0.0043; TT vs CC, *P*=0.0012; and TT vs. TC+CC, P=0.0003; Figure 1A). No differences in killing ability were observed in macrophages from donors carrying the *IL4R*$_{rs2107356A/A}$ and *IL8*$_{rs2227307G/G}$ genotypes or *IL12B*$_{rs3212227C}$ allele in comparison with their respective wild type allele/genotype (Figure 1B-D).

Motivated by these results, we also decided to investigate whether the presence of the above-mentioned SNPs correlated with cytokine levels after stimulation of PBMCs from healthy donors with fungal antigens (zymosan) or stimulatory molecules (LPS and PHA). These *in vitro* stimulation experiments revealed that carriers of the *IFNγ*$_{rs2069705C}$ allele showed an increased production of IFNγ after 4 days of incubation with LPS or PHA and when both stimulating reagents were used in combination (P_{LPS}=0.057, P_{PHA}=0.036 and $P_{LPS+PHA}$=0.030; Figure 2A and Supplementary Table 2). We also observed that donors carrying the *IFNγ*$_{rs2069705C}$ allele showed a drastic increase in the production of TNF at almost all time points when compared with those bearing the wild type genotype ($P_{PHA-72h}$=0.045, $P_{LPS+PHA-72h}$=0.018, $P_{LPS-96h}$=0.058, and $P_{LPS+PHA-96h}$=0.0058; Figure 2B and Supplementary Table 2). In addition, we observed that subjects carrying the *IFNγ*$_{rs2069705C}$ allele tended to have an increased production of IL12p70 when compared with those carrying the wild type genotype (Figure 2C and Supplementary Table 2). No correlation between *IL12p70 and IL8* levels and *IL12B*$_{rs3212227}$ and *IL8*$_{rs2227307}$ genotypes was found. These findings suggest that the *IFNγ*$_{rs2069705}$ SNP might contribute to modulate the risk of IA likely through the regulation of IFNγ mRNA levels.

In order to test this hypothesis, we measured *IFNγ*mRNA expression in PBMCs from healthy donors (n=21) that were grouped according to the *IFNγ*$_{rs2069705}$ genotype. Importantly, we found that carriers of the *IFNγ*$_{rs2069705C}$ allele (C/T+CC) showed a significantly increased level of *IFNγ*

Infection and Immunity
IAI
Infection and Immunity

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mRNA when compared with those carrying the wild type genotype (40.85±11.65 vs. 13.87±5.43, *P*=0.049; Figure 3A and 3B). Although this result pointed toward a role of this SNP in modulating *IFNγ* gene expression in PBMCs, we decided to further confirm this result by looking at the publicly available blood expression quantitative trait loci (eQTL) browser (http://genenetwork.nl/bloodeqtlbrowser/). Of note, we found that, in agreement with our gene expression data, this variant located in the promoter region of the gene (but also those neighboring SNPs within the same linkage disequilibrium block) showed a positive correlation with *IFNγ* mRNA expression level that ranged between $P=1.01\bullet10^{-3}$ and $P=1.70\bullet10^{-3}$ (Supplementary Figure 1).

Next, we analysed the correlation between the $IL4R_{rs2107356}$ SNP and *IL4R* mRNA expression levels in whole blood samples collected from healthy donors (n=43) and in monocyte-derived macrophages at baseline and after *in vitro* stimulation with *A. fumigatus* conidia (n=12). Gene expression data in blood samples did not show differences in IL4R mRNA expression levels between homozygotes (GG vs. AA, *P*=0.304) but they showed a significantly increased level of expression in heterozygotes when were compared either with wild type or mutant homozygotes (AG vs. GG, *P*=0.0045 and AG vs. AA, *P*=0.031, respectively; Figure 3C). When carriers of the $IL4R_{rs2107356AA}$ genotype were compared with those carrying the wild type allele (according to the genetic model used in our genetic analysis), we failed to find statistical differences in IL4R mRNA expression levels ($P_{AAvs.GG+AG}$=0.2937; Figure 3D). These findings did not support our hypothesis suggesting a functional effect for this promoter polymorphism on mRNA expression but were in agreement with those data from the blood eQTL browser that reported no association of this promoter variant with IL4R mRNA expression levels in PBMCs. In line with these results, we neither found any correlation between the $IL4R_{rs2107356}$ variant and IL4R mRNA expression levels in monocytes-derived macrophages at baseline and after stimulation with *A. fumigatus* conidia (Figure 3E and 3F). The lack of correlation between this promoter polymorphism and IL4R protein level was also confirmed by flow cytometry analysis in different immune cell types (CD_{19}^+, CD_{14}^+ and CD_3^+, cells; Figure 3G). We only found a significantly

Infection and Immunity
IAI
Infection and Immunity

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increased level of IL4R in CD_{19}^{+} cells in heterozygotes when compared with those subjects carrying the AA genotype (*P*=0.032; Figure 3G), which was in line with our gene expression data in blood samples from healthy donors and confirmed that the effect attributed to this promoter variant cannot be explained by changes in mRNA or protein expression levels. Given that *IL4R* is internalized in a time-dependent manner after stimulation with *IL4*, we could not confidently measure the correlation of the $IL4R_{2107356}$ SNP and *IL4R* protein levels under stimulation conditions (data not shown).

Finally, considering the association of some of the immune-modulating SNPs with the risk of developing IA and given the correlation of some of these genetic markers with cytokine levels, we tested the capacity of these variants to predict with confidence the disease risk. We assessed the predictive capacity of a model built with "significant SNPs" and demographic and clinical variables in comparison with a reference model including only demographic and clinical variables. Despite the modest population size, we found that a predictive model including 4 variants associated with IA, age, gender, allo-HSCT and antifungal prophylaxis status showed an improvement in discriminatory ability to predict the disease when compared with the reference model (AUC=0.659, 95%IC 0.596-0.722, *P*=0.000005 vs. AUC=0.564, 95%IC 0.499-0.630, *P*=0.064; $P_{-2_log_likehood_ratio_test}$=0.00052; Table 5 and Figure 4). Importantly, we also observed that a model built with a similar number of "non-significant SNPs", together with demographic and clinical variables, did not show any significant change in predictive capacity when compared with the reference model (Supplementary Table 3), which confirmed the utility of significant SNPs in predicting IA. The consistency of the predictive results was supported through a 50.000 permutation test that showed that none of the 50.000 permuted models showed a better prediction capacity than our genetic model built with SNPs significantly associated with IA ($AUC_{sort\text{-}average}$=0.6001, $SD_{sort\text{-}AUC}$=0.0158, Z score=3.7361 and $P_{Z_score\text{-}value_(50.000perm)}$=$9.34 \bullet 10^{-05}$; Table 5 and Supplementary material).

DISCUSSION

Infection and Immunity
IAI
Infection and Immunity

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Infection and Immunity

IAI

Infection and Immunity

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

Recent studies in humans and animal models have demonstrated that *IL4/IL4R*, *IL8*, *IL12* and *IFNγ* have a central role in IA (19, 61-65). In particular, *IFNγ* seems to be a key factor as its

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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

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suggested that this polymorphism or another causative polymorphism in strong linkage disequilibrium with it may be involved not only in the control of *IFNγ* production but also in the subsequent induction of *TNF* production. Taking all these findings together, we propose a central role for this variant in determining the risk of IA in allo-HSCT and in leukaemia patients undergoing intensive chemotherapy.

Although genetic data suggested that polymorphisms within IL4R, IL8 and IL12B influence the risk of developing IA, functional experiments did not show any correlation between these SNPs and their respective mRNA and/or protein levels. Therefore, we suggest that these variants might exert their biological function by modulating other biological processes such as mRNA processing (splicing or turnover) or mRNA stability or even act at post-transcriptional level. Further studies are now warranted to replicate our findings and to experimentally identify the functional role of these SNPs in determining the risk of IA.

Finally, given the genetic and/or functional effect observed for variants within *IFNγ*, *IL4R*, *IL8*, *IL12B* and *VEGFA* genes, we found also interesting to determine the impact of these variants in predicting the disease risk. We found that a model built with $IL8_{rs2227307}$, $IL12B_{rs3212227}$, $IFN\gamma_{rs2069705}$ and $VEGFA_{rs6900017}$ SNPs showed a significantly improved discriminatory ability to predict the disease when it was compared with a model that included demographic and clinical variables. Importantly, when a similar number of “non-significant SNPs” were added to the reference model, we did not observe any significant change in predictive capacity, which confirmed that only a model built with these significant SNPs could have capacity to predict the infection. In support of this finding, we also observed that the AUC of this model was systematically higher than the AUC observed for 50.000 iterative models, which emphasizes the importance of considering predictive models to assist in the clinical decision-making process and to improve in novel strategies to prevent IA occurrence.

Infection and Immunity
IAI
Infection and Immunity

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This study has both strengths and limitations. Study strengths include a multicentre population-based design, a relatively large sample size, and the high number of genetic polymorphisms analysed. This allowed us for the first time to perform predictive analyses to assess the potential utility of genetic variants in predicting with confidence the risk of developing IA. Potential weaknesses include limited antifungal prophylaxis data availability for a subset of patients and a relatively low number of proven or probable IA cases that limited the study's statistical power to rule out spurious associations. To minimize this limitation, the most relevant associations were functionally validated.

In conclusion, our data suggest that immune-modulating polymorphisms have an impact on the risk of IA and that genotyping of these variants could help to predict the risk of IA and therefore be useful to establish a risk-adapted anti-fungal prophylaxis strategy.

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Infection and Immunity
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Infection and Immunity

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IAI
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Infection and Immunity
IAI
Infection and Immunity

Accepted Manuscript Posted Online

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.

Infection and Immunity
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Figure 1. Fungicidal activity of monocyte-derived macrophage according to *IFNγ*[A], *IL12B* [B], *IL4R* [C] and *IL8* [D] genotypes.

Figure 2. *IFNγ*, *TNFα* and *IL12p70* cytokine levels on stimulated PBMCs according to the $IFN\gamma_{rs2069705}$ genotypes.

PBMCs from healthy donors were stimulated with Zymosan (5μg/ml) and PHA (2 μg/ml) alone or in combination with LPS (100 ng/ml). Supernatants were harvested for *IFNγ*, *TNFα* and *IL12p70* analysis at 72 and 96h.

Figure 3. *IFNγ* [A and B] and *IL4R* mRNA [C-F] and protein [G] expression levels according to the $IL4R_{rs2107356}$ genotypes.

Correlation between $IFN\gamma_{rs2069705}$ genotypes and *IFNγ* mRNA expression levels were analysed in PBMCs from healthy donors at baseline or stimulated with *Aspergillus* conidia [A and B]. Correlation between $IL4R_{rs2107356}$ genotypes and IL4R mRNA expression levels were analysed in blood samples from healthy donors [C and D] and in monocyte-derived macrophages at baseline or stimulated with *Aspergillus* conidia [E and F]. Correlation between this promoter variant and IL4R protein levels were also analysed in different immune cells (CD_{19}^+, CD_{14}^+ and CD_3^+) by flow cytometry [G].

Figure 4. Receiver operating characteristics (ROC) curve analysis.

ROC curves summarize the accuracy of prediction for each particular model. The model including SNPs significantly associated with IA and demographic and clinical variables (marked in blue) showed a significantly improved predictive capacity comparared with a reference model including only demographic and clinical variables (marked in red).
AUC=0.659 vs. AUC=0.564.

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Figure 1

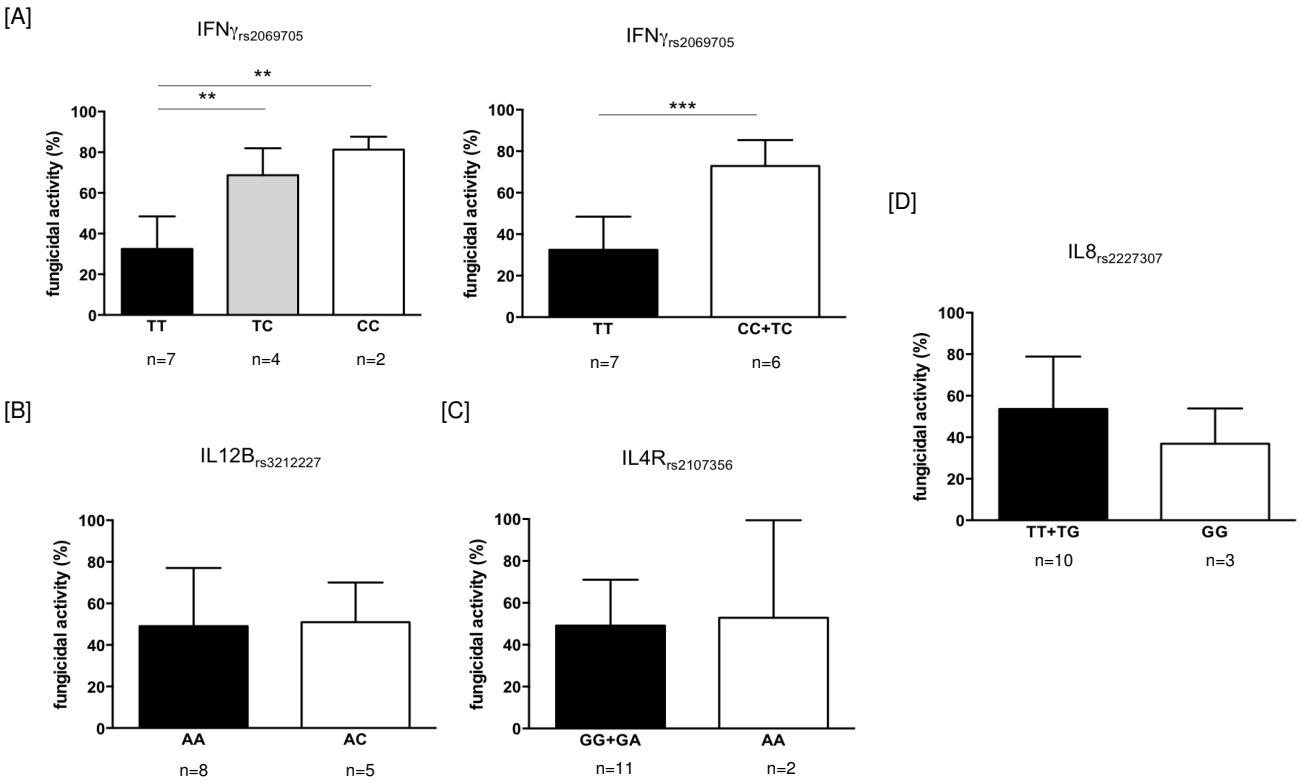

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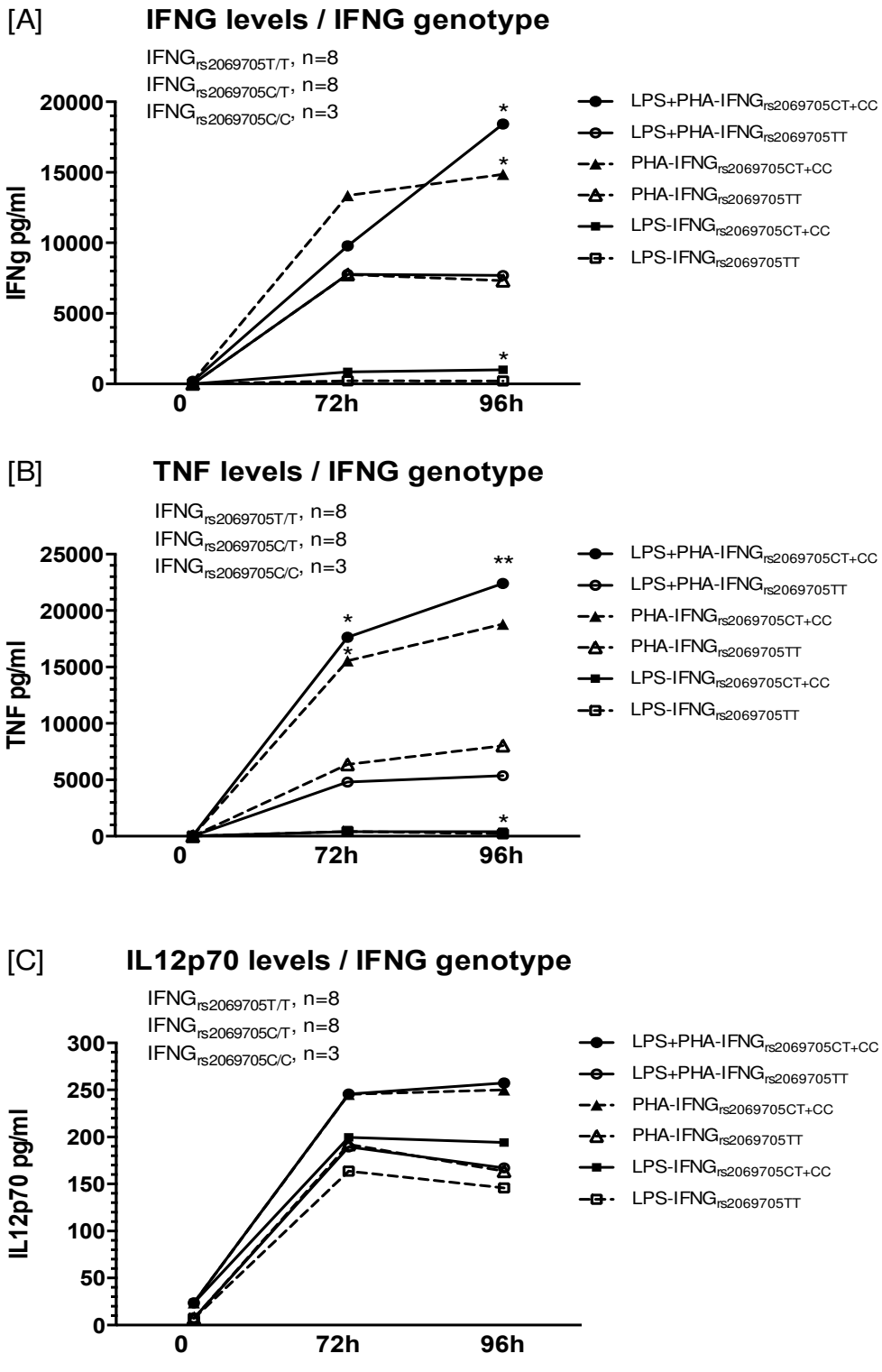
Figure 2



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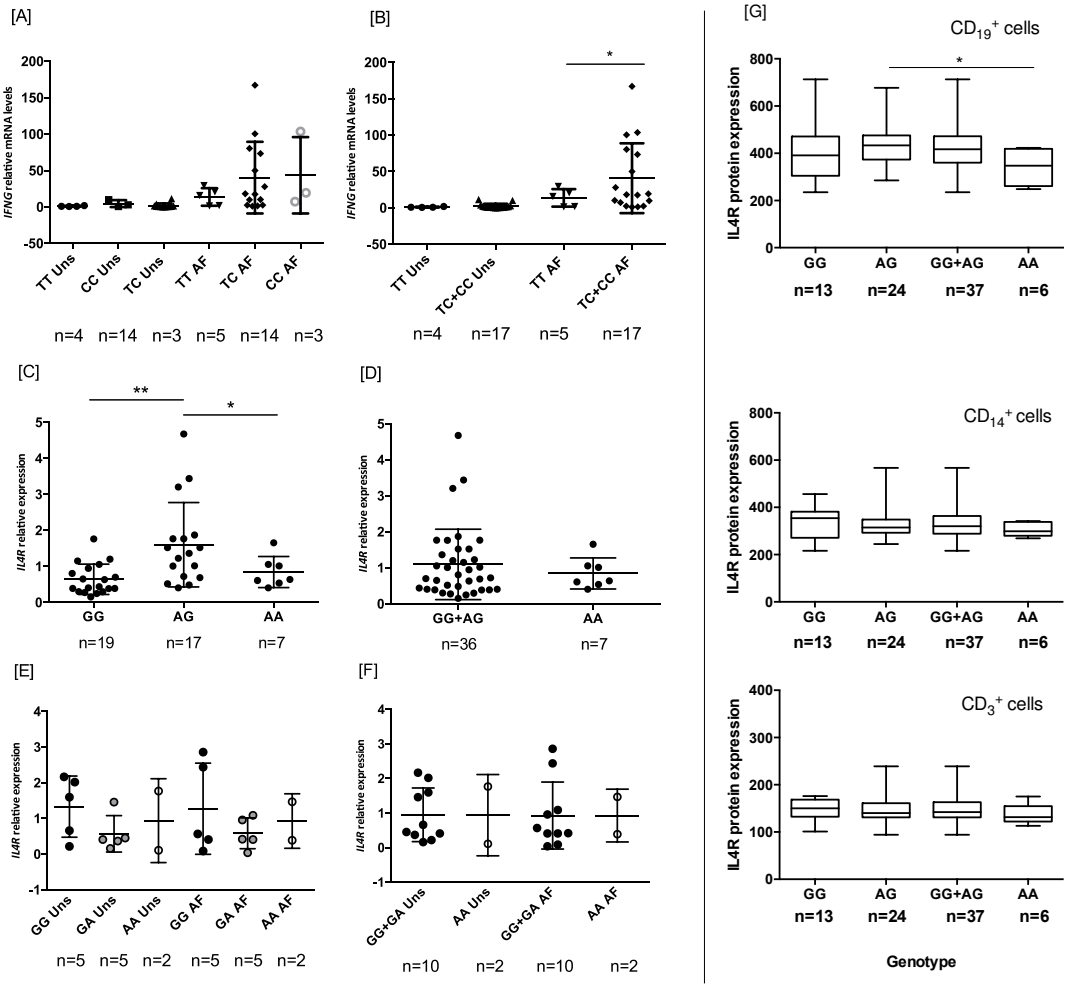
IAI
Infection and Immunity

Figure 3



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Infection and Immunity

Figure 4

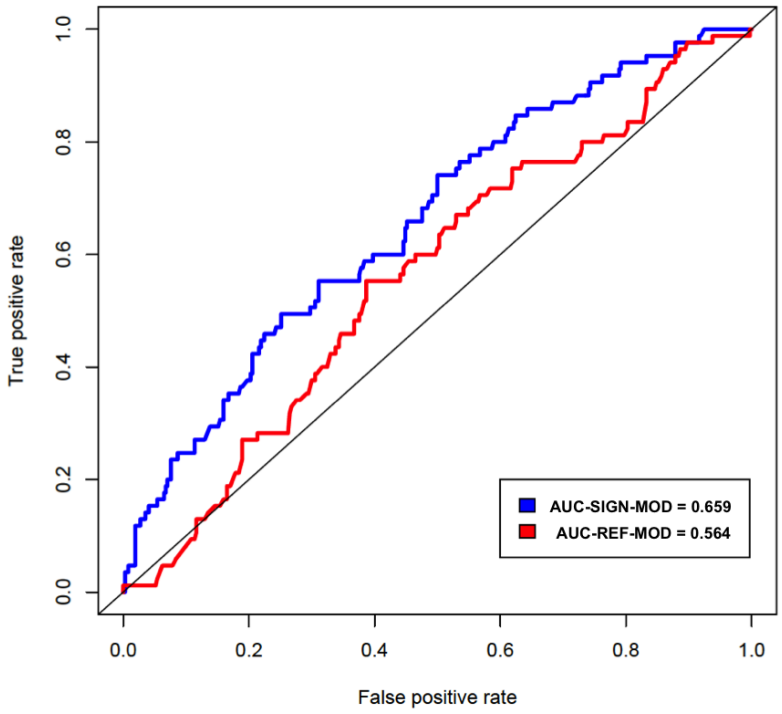

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Table 1. Selected SNPs within immune-modulating genes.

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)

Infection and Immunity
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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.

Infection and Immunity
IAI
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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.

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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).

Infection and Immunity
IAI
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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).

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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 $P_{50.000perm}$=$9.34 \cdot 10^{-05}$.

Infection and Immunity

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Infection and Immunity