Human SNP Links Differential Outcomes in Inßammatory and Infectious Disease to a FOXO3-Regulated Pathway

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This knowledge imbalance is exempli ed in Crohn's disease (CD), a chronic, relapsing-remitting form of in ammatory bowel disease thought to be driven by aberrant immune responses to intestinal bacteria. CD has been a major bene ciary of GWAS technology, with over 140 risk loci having been identi ed (Jostins et al., 2012), though only one of these has been shown to correlate with clinical outcome in CD (Heliö et al., 2003). Such observations have led to criticisms of the utility of GWAS results in translational medicine (McClellan and King, 2010) and raise questions as to whether genetics meaningfully contributes to prognosis in complex disease.

We have recently shown that common transcriptional differences involving the IL-2 and IL-7 cytokine-signaling pathways correlate with prognosis in several diseases, including CD (McKinney et al., 2010; Lee et al., 2011). Here, we perform a candidate gene study to address whether genetic variation in these immune pathways, which have not been associated with the development of CD, might associate with disease prognosis and, if so, to understand the biological mechanisms responsible. To answer this question, we determined that a "within-cases" association analysis would be required, in which the genetic pro les of patients with contrasting disease courses could be directly compared. To achieve this, we used a subset of genotype data relating to the IL-2 and IL-7 pathways from an existing GWAS data set (Wellcome Trust Case Control Consortium, 2007) and exploited allied phenotypic data to identify groups of patients with either particularly aggressive or indolent CD. This led to the identi cation of a noncoding single-nucleotide polymorphism (SNP) in FOXO3A that associates with prognosis in CD, despite not being a disease-associated variant (Jostins et al., 2012). FOXO3A encodes FOXO3, a member of the forkhead box O family of transcription factors, which also includes FOXO1 and FOXO4 (Accili and Arden, 2004). These proteins are widely expressed and regulate diverse transcriptional programs including cell-cycle control, metabolism, regulatory T cell development, and apoptosis (Burgering and Kops, 2002; Modur et al., 2002; Nakae et al., 2008; Harada et al., 2010; Kerdiles et al., 2010; Ouyang et al., 2010). Although many of these roles are redundant between FOXO family members (Accili and Arden, 2004), FOXO3 has been reported to have nonredundant roles in suppressing in ammatory cytokine production by dendritic cells (Dejean et al., 2009; Watkins et al., 2011) and in limiting the in ammatory sequelae of viral infections (Litvak et al., 2012). Here, we show that during in ammatory responses, in which FOXO3 is exported from the nucleus to the cytoplasm, its reaccumulation in the nucleus is dependent on transcription and de novo protein production, and demonstrate that allelic variation at the prognosis-associated SNP regulates this reaccumulation by controlling FOXO3A transcription. Earlier recovery of nuclear FOXO3, which occurs if the indolent disease-associated allele is present, initiates a TGFb1-dependent pathway in monocytes that reduces production of proin ammatory cytokines, including TNFa, and increases production of the anti-in ammatory cytokine, IL-10—changes consistent with a more indolent disease course. Furthermore, we show that genetic variation at this SNP is also associated with prognosis in rheumatoid arthritis and malaria—other diseases in which these cytokines are implicated. These associations were consistent with the role for these cytokines in each disease, and suggest that this pathway may be generally important in diseases in which these cytokines are involved. Collectively, therefore, these data reveal a pathway by which FOXO3 can abrogate in ammatory responses, and uncover a shared genetic contribution to the prognosis of distinct diseases that impacts upon this pathway and is distinct from the genetic contribution to disease development.

RESULTS

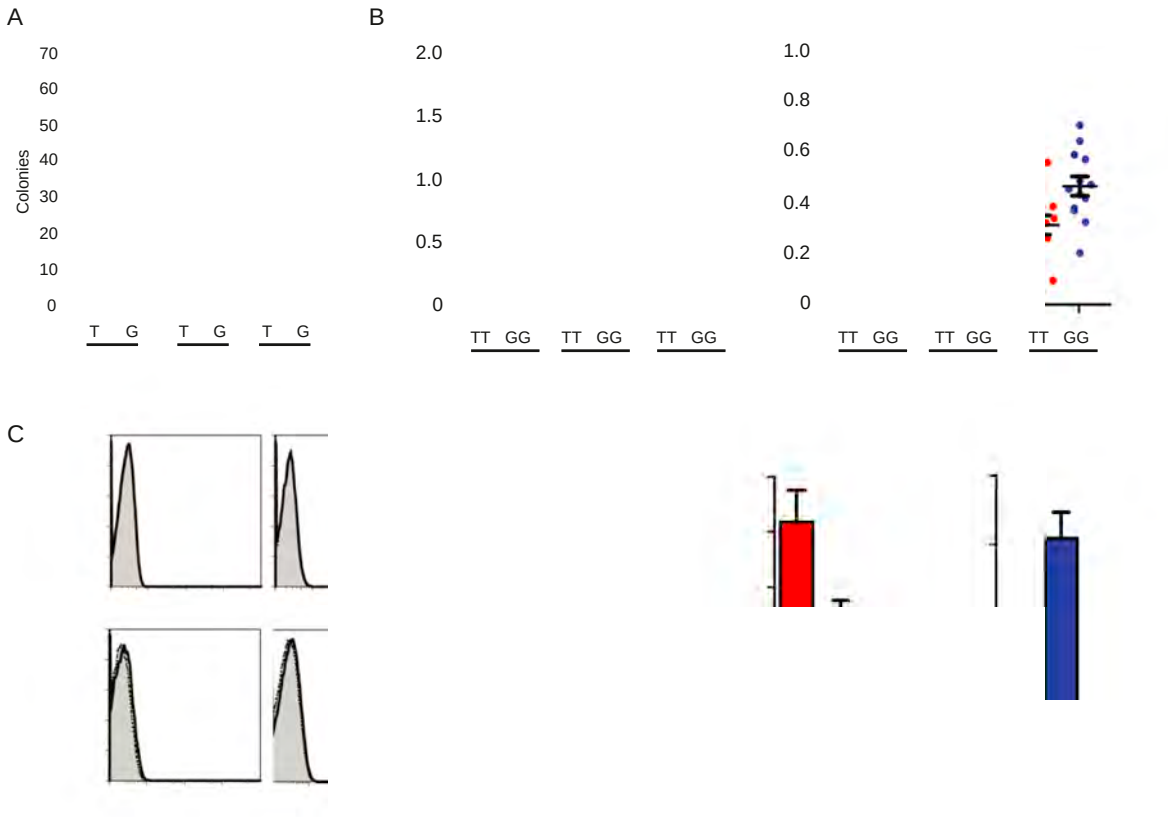
Prognosis-Based Association Study in Crohn's Disease

Subgroups of patients with either particularly aggressive or indolent CD were identi ed within an existing GWAS cohort (Wellcome Trust Case Control Consortium, 2007) using phenotypic data. Aggressive CD (n = 668) was de ned as that for which two or more immunomodulator therapies and/or intestinal resections had been required (treatments reserved for patients with frequently aring or complicated CD). Indolent CD (n = 389) was de ned as disease of greater than 4 years duration, for which immunomodulators or intestinal resections had never been required. Eighty-one genes involved in IL-2 or IL-7 signaling were identi ed from published literature and pathway libraries (KEGG, Kanehisa and Goto, 2000; Biocarta, http://www.biocarta.com) (Table S1 available online). These pathways were selected because we have previously shown that differences within them correlate with prognosis in several diseases, including CD, and yet unlike a number of other disease-associated cytokines, they have not been implicated in disease development (McKinney et al., 2010; Lee et al., 2011). The allele frequency at 1,134 SNPs within these genes was then compared between the indolent and aggressive CD subgroups (Figure S1). The effect of population structure on the results was assessed by three separate methods and shown to be minimal (genomic control in ation factor 1.04, see Extended Experimental Procedures). Replication of the three most signi cant associations was then sought in three independent cohorts, each similarly divided into indolent and aggressive cases (Table S2). The prognostic association of rs12212067, an intronic SNP within FOXO3A, was replicated in all three cohorts, with the minor (G) allele being consistently more common in patients with indolent CD (combined p value = 2.1 3 10 ^{8}, Tables 1 and S3). Notably, this SNP was not associated with risk for CD either in the GWAS used for this study (p = 0.88)—a sample of 1,748 cases and 2,938 controls (Wellcome Trust Case Control Consortium, 2007)—or in a larger meta-analysis (p = 0.99) of 6,333 cases and 15,056 controls (Franke et al., 2010), which suggests that if it does in uence disease risk, its effect size is negligible.

rs12212067 Regulates Transcription of FOXO3A during In ammation

To investigate how genetic variation at this locus might in uence prognosis in CD, we rst examined whether rs12212067 was in linkage disequilibrium (LD) with a coding variant that could affect protein structure or function. Using sequence data from the 1000 Genomes Project Consortium et al. (2010) we identi ed all of the SNPs in LD ($r^2 > 0.5$) with rs12212067, but none of these were exonic (Table S4 and Figure S2). Indeed, of the 45 coding variants that have been described within FOXO3A (dbSNP,

http://www.ncbi.nlm.nih.gov/projects/SNP) none were in any demonstrable LD with rs12212067: T > G (r2 < 0.001, data not shown). This implies that noncoding variation is likely to drive the association. The majority of complex disease-associated SNPs are also noncoding and are assumed to affect gene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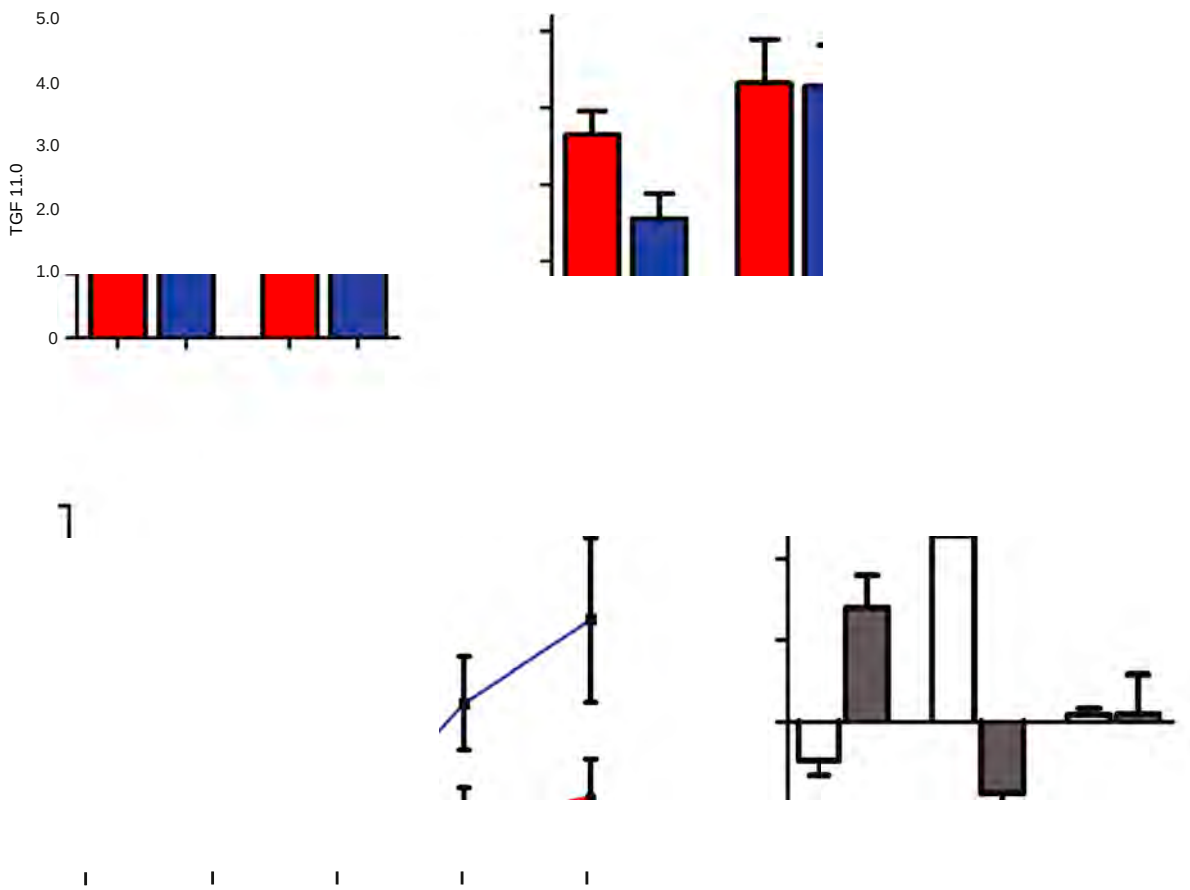
Differences in FOXO3A Transcription during Inflammation Determine when FOXO3 Reaccumulates within the Nucleus

We next considered how differences in FOXO3A transcription might affect cytokine production. Because the downstream transcriptional program of FOXO3 is largely controlled by posttranslational modifications that determine whether it is retained within, or excluded from, the nucleus (Hedrick, 2009), we first examined how the intracellular localization of FOXO3 changed as monocytes were stimulated (Figure 2A). TNFα and IL-10 production were also measured. We showed that in unstimulated monocytes, most FOXO3 was nuclear, and TNFα production was low. Upon LPS stimulation, FOXO3 was translocated out of the nucleus (Figure 2A) and a linear increase in TNFα production was observed. This process occurred similarly in minor and major allele homozygotes, until very little nuclear FOXO3 remained after 4 hr (Figure 2B). Thereafter, a gradual increase in the amount of nuclear FOXO3 was observed (termed "nuclear recovery"), which correlated with reduced TNFα and increased IL-10 production. Strikingly, this nuclear recovery phase occurred considerably faster in minor (G) allele homozygotes than in major (T) allele homozygotes and correlated with an earlier reduction in TNFα production and an earlier increase in IL-10 production (Figures 2B–2D). This difference is consistent with the allele-specific expression differences and implies that, during inflammation, increased FOXO3A transcription in carriers of the minor (G) allele leads to faster recovery of nuclear FOXO3. This also suggests that de novo protein synthesis, rather than nuclear translocation of cytoplasmic FOXO3, is responsible for nuclear recovery; consistent with the observation that once FOXO3 is translocated into the cytoplasm, it is targeted for proteasomal degradation (Yang et al., 2008). This was confirmed by showing that if protein synthesis was inhibited, nuclear recovery of FOXO3 was substantially retarded (Figure S5).

The Effect of rs12212067 on Monocyte Cytokine Production Is TGFβ1 Dependent

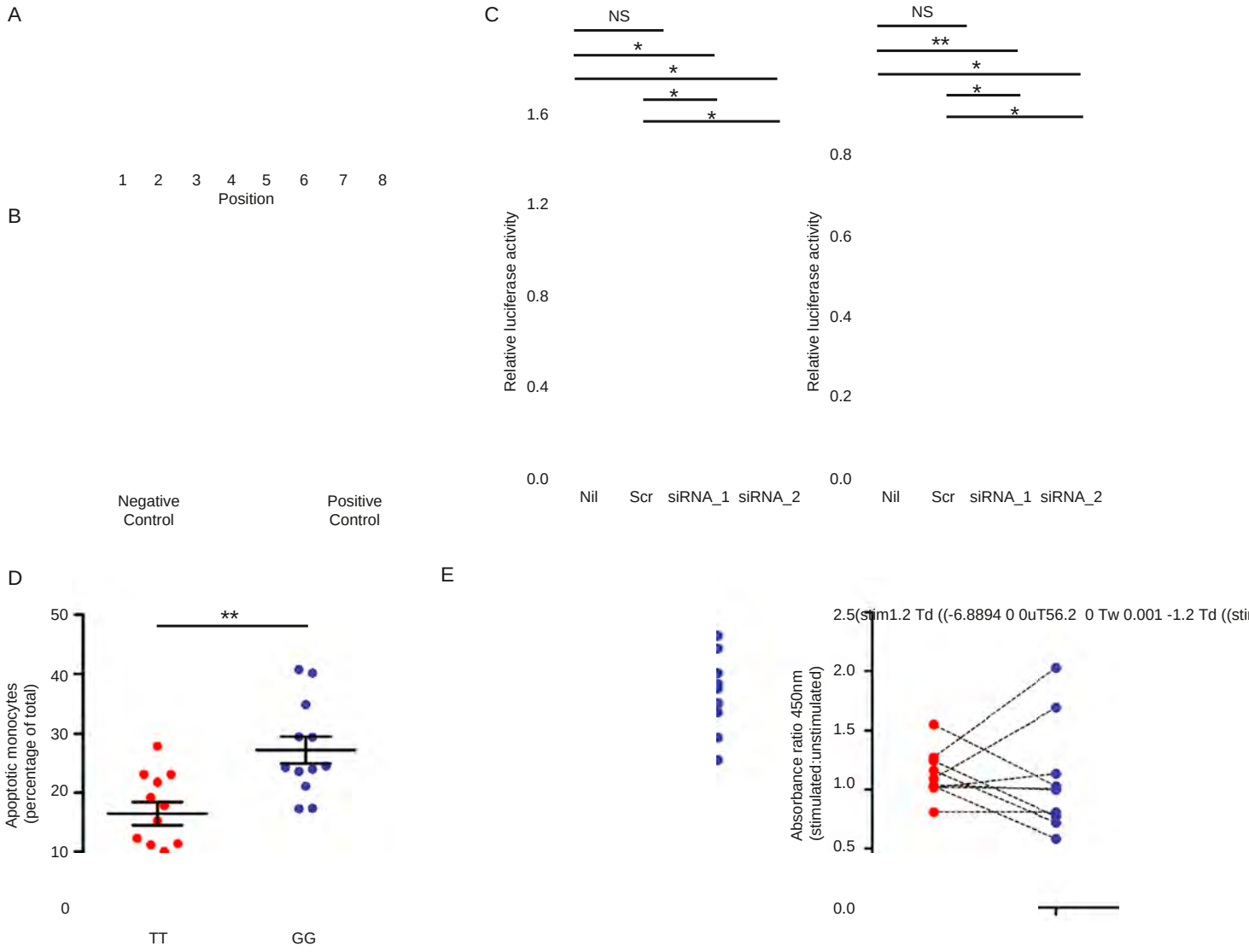
Next we investigated how the earlier recovery of nuclear FOXO3 might alter inflammatory cytokine production. In mice, silencing Foxo3a has been shown to abrogate production of TGFβ1 (Watkins et al., 2011), an anti-inflammatory cytokine that can modulate production of other cytokines (Musso et al., 1990; Fadok et al., 1998). To determine whether TGFβ1 might contribute to the observed differences in cytokine production, we re-examined the supernatants from the earlier experiments. We found that relatively more TGFβ1 had been secreted by PBMC and monocytes from minor (G) allele homozygotes (Figure 3A) with the greatest difference, and highest concentrations, being present in the monocyte supernatants—implicating these cells as the major source of TGFβ1. To determine whether this difference might contribute to the differences in TNFα and IL-10 production, we examined the effects of blocking TGFβ1 signaling. Strikingly, we observed that the genotype-specific differences in TNFα and IL-10 production were abolished if TGFβ1 signaling was inhibited (Figure 3B)—suggesting that a TGFβ1-dependent mechanism was responsible. In support of this, differences in TGFβ1 titers from minor and major allele homozygotes were also detected in the samples from the earlier time-course experiment, with the kinetics of TGFβ1 production correlating with the differences



in nuclear recovery of FOXO3 (Figure 3C). Moreover, the addition of exogenous TGFb1 to stimulated monocytes of either genotype led to less TNFa and more IL-10 production—consistent with the differences observed between the genotypes—whereas blockade of TGFb1 had the opposite effect (Figure 3D). This suggests that endogenous TGFb1 production plays an important regulatory role in monocyte-driven in ammatory responses.

FOXO3 Regulates TGF b1 Production in Stimulated Monocytes

Although these data implicate a TGFb1-dependent mechanism in driving the genotype-speci c differences in cytokine production, it was unclear how the earlier recovery of nuclear FOXO3 might mediate this. To determine whether FOXO3 directly regulated TGFb1 production, we analyzed the TGFb1 promoter and identi ed several sites at which FOXO3 was predicted to bind (Figure 4A and Table S6). To establish whether this interaction occurred during monocyte activation, we immunoprecipitated FOXO3 from stimulated monocytes and found that the DNA to which it was bound included the predicted binding site from the TGFb1 promoter (Figure 4B), con rming that a direct protein-DNA interaction occurs. To determine the result of this interaction, we silenced FOXO3A in two monocyte cell lines, U937 (Sundström and Nilsson, 1976) and MONO-MAC6 (Ziegler-Heitbrock et al., 1988), and examined the effect on TGFb1 transcription. In both, we showed that when FOXO3A was silenced, transcription from the TGFb1 promoter was reduced, con rming that the FOXO3-TGFb1 interaction promotes TGFb1 transcription (Figure 4C). Together, these data reveal that FOXO3 induces TGFb1 expression via a direct protein-DNA interaction in



stimulated monocytes and explain how the earlier recovery of nuclear FOXO3 could modulate TNFa and IL-10 production in a TGFb1-dependent manner.

TGFb1 has also been implicated in the modulation of immune responses by apoptotic cells (Fadok et al., 1998), whereas FOXO3 is known to be proapoptotic (Brunet et al., 1999). This raised the possibility that earlier nuclear recovery of FOXO3 might also augment TGFb1 production indirectly via apoptosis induction. To examine this, we quanti ed apoptosis in PBMC following LPS stimulation (using Annexin V and 7-AAD) and

observed that more occurred in minor (G) allele homozygotes (Figure 4D), consistent with the earlier recovery of nuclear FOXO3 in this genotype. To exclude a contribution from necrotic cells to this result, we quanti ed mono- and oligonucleosomes in the supernatant (necrosis) and cytoplasm (apoptosis) of PBMC from another cohort of minor and major allele homozygotes and again observed more apoptosis in minor allele homozygotes following stimulation (Figure 4E). We also con rmed that stimulated monocytes produced less TNF a and more IL-10 in the presence of autologous apoptotic cells and that this could be abrogated by TGFb1-blockade (Figure S6). These data imply that FOXO3 might drive TGFb1 production not only via a direct interaction with its promoter, but also indirectly via the control of apoptosis.

FOXO3 has also been shown to mediate some of the downstream components of TGF b1 signaling (Wildey and Howe, 2009), raising the possibility that earlier nuclear recovery might amplify some of the effects of TGF b1 production. However, we did not detect any genotype-speci c differences in the transcription of 11 genes whose activation by TGF b1 is FOXO dependent (Gomis et al., 2006) (data not shown), consistent with reports that this role is redundant between FOXO family members (

in human disease, or even occurred physiologically in humans,

It is noteworthy that the prognostic association of this polymorphism appears to be mediated via monocytes, which are known to be important in these in ammatory and infectious diseases (Malaguarnera and Musumeci, 2002; Khor et al., 2011; Bain et al., 2013; Davignon et al., 2013). CD pathogenesis, for example, is thought to stem from a dysfunctional innate immune response to intestinal bacteria by proin ammatory macrophages and dendritic cells (Khor et al., 2011)—both of which derive from circulating monocytes (Geissmann et al., 2010; Rivollier et al., 2012; Bain et al., 2013). The resulting production of proin ammatory cytokines by these cells not only drives disease activity but is also the target of the most effective therapy (Krygier et al., 2009). Similarly, in RA, disease severity correlates with the extent of synovial in ltration by monocytes/macrophages, and these can be targeted therapeutically (Mulherin et al., 1996; Davignon et al., 2013).

Although our evidence points to an effect in monocytes, T cells are also important in these diseases (

statistical signi cance. Genotype frequencies were in Hardy Weinberg equilib-

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