Genetic studies in rheumatoid arthritis

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Chapter 17

Summary & Discussion

Rheumatoid Arthritis (RA) is a complex disease with unknown etiology. Genetic as well as environmental risk factors are both thought to play a role in disease onset and/or progression[1, 2].

At the immunological level, the role of the adaptive immune system in autoimmune diseases is largely accepted. Based on both mouse models and data from human studies, it has become clear that T cells and B cells, amongst other immune cells, are likely to play a major role in RA. Examples of such studies include the clear role of T cells in the development of RA in the SKG mouse model where a mutation in the ZAP-70 molecule (a key signal transduction molecule in T cells) leads to spontaneous arthritis[3]. Additional evidence for the relevance of T cells in arthritis comes from the very strong association of the *human leukocyte antigen* (HLA) region with RA in human genetic studies[4, 5]. The products of the HLA-DRB1 alleles that associate the strongest with RA share a 5 amino-acid sequence in a peptide-binding pocket called Shared Epitope (SE). The prediction has been that these DRB1 molecules would bind and allow the presentation of RA inducing peptide(s) to T cells[6]. Although these peptides remain elusive, the well-established association of the SE with RA provides evidence of the role of T cells in RA development. However it is becoming increasingly clear that it is not simply a case of SE-positive alleles predisposing to disease. The presence of the SE alleles is highly correlated with the presence of autoantibodies indicating that SE alleles likely do not predispose to the development of the disease as such[7, 8]. Additionally, there may also be HLA-DRB1 protective alleles and also a number of other important associations to polymorphisms in this gene-rich region of the genome6, 9-11.

The role of B cells in RA is highlighted by the presence of autoantibodies in patients. These autoantibodies including rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) have been consistently described12, 13. The emergence of ACPA years before onset of clinical symptoms indicates that it possibly lies in a causal pathway for the development of the disease although no conclusive evidence could be obtained from mouse models14. In 2006, Kuhn and colleagues showed that ACPA can exacerbate disease progression in a collagen-induced mouse model15. In 2009, a recent report suggests that these autoantibodies specific to citrullinated proteins do play a role in both the onset and progression of autoimmunity in the same mouse model16. However, it still takes years before clinical symptoms are apparent in patients who already harbour these autoantibodies, indicating that other genetic as well as non-genetic factors likely play a role in the onset of the disease.

Despite the rather large genetic component in RA (approximately 60%), it has been very difficult to identify the precise genetic markers responsible for the onset of the disease4, 17. The increased risk of disease in siblings of patients with RA compared with that of the general population (λs) has been estimated to be between 2 and 174. However, recent developments in genetics and genomics, heavily influenced by technological innovations, are overcoming the prior limitations within the field.

Genetic variations are sometimes referred to as “polymorphisms”, meaning that the gene or locus (the gene region) occurs in several “forms” within the population. Most loci that are polymorphic have no direct influence on disease risk or human traits, while those that are associated with a difference in risk of disease or a human trait (however subtle) are termed “disease-associated polymorphisms”. Although the clinical significance and causality of these disease-associated polymorphisms are currently difficult to establish, these variations may account for a genetically susceptible individual predisposed to an aberrant immune profile, who,

combined with other non-genetic factors eventually develops diseases like RA[18]. In line with this concept, human genetic studies offer *prima facie* evidence that a biological pathway is critical in disease pathogenesis. Since there are no drugs yet proven to cure RA or other chronic inflammatory/autoimmune diseases, research into the principle mechanisms underlying the disease process are crucial for disease management, new diagnostics and ultimately cure.

In advance of large-scale techniques, which have now become widely available, we took a hypothesis-driven approach to determine whether candidate genes, namely complement component 5 (C5) and interleukin 10 (IL10), both immune related genes, may be involved either in the development and/or progression of RA. This approach has been highly rewarding as exemplified identification of one of the few widely-confirmed genetic risk factors for RA, namely the *Tumour Necrosis Factor (TNF) receptor associated factor 1* (*TRAF1*) – *Complement component 5* (*TRAF1/C5)* locus on chromosome 9q33 (**part I of this thesis)** and by our functional characterization of the mRNA regulation at the *interleukin 10* (*IL10*) locus (**part II of this thesis**). In **part III**, we have also investigated the role of several candidates in relation to disease as well as specific disease phenotypes.

Genetic Risk factors in RA

Prior to 2007, only two genetic risk factors had been identified. The first and most robust genetic risk factor to date was identified three decades ago and is confined to the *HLA* region on chromosome 6[4, 5]. The second largest risk factor in RA, the *protein tyrosine phosphatase non-receptor type 22* (*PTPN22)* gene, was identified by using a largescale approach in 2004 and has now been widely replicated as an autoimmune locus[19-22].

In collaboration with our Swedish and American colleagues, we successfully identified a novel genetic risk factor in RA in 2007 in the 9q33 region of the genome containing *TRAF1/C5* by taking a candidate gene approach (**chapter 2**)[23]. Interestingly, this region was also concurrently detected in a genome-wide approach by Plenge and colleagues[24]. Although the original genome wide association study (GWAS) performed by the Wellcome Trust Case-Control Consortium (WTCCC) in 2000 patients and 3000 controls did not originally identify this locus, a follow-up post-genome study performed in the UK now also provides evidence of association at this region[25, 26].

Our data revealed that although the case–control allele frequency increase in different sample sets ranged from 4% to 9%, the frequency ranged from 38% to 46% in control groups of European ancestry. Data from HapMap (www.hapmap.org) support these observations with the G allele (minor allele in Caucasians) frequency of rs3761847 (perfect proxy of rs10818488) varying from 48% in Caucasians of European descent, 31% in Gujarati Indians in Texas, 42% in Japanese in Tokyo, 66% in Mexicans in Los Angeles, 59% in Yorubans in Nigeria and 74% in Luhya in Kenya. Given that association studies compare the frequencies in patients versus healthy individuals, unknown biases in control frequencies may lead to spurious associations. To address this issue and to further complement our data, we have also reproduced this association in 1356 individuals from 452 trio families with RA (chapter 3)27. Interestingly, an RA association of the opposite allele has been reported in the Japanese population but no association has been found in a Korean population, reflecting possible ethnic diversity in the associations at this locus28, 29. A more recent fine-mapping of this region now indicates that a

third gene, PHD finger protein 19 (PHF19), may be part of the haplotype block involved in disease susceptibility30. While TRAF1 and C5 being immune-related molecules represent ideal candidates as biological mediators in RA, relatively little is currently known about PHF19 to infer its possible role in the disease process.

Our work has now also shown that the *TRAF1/C5* region is not only relevant for RA but is also relevant in patients with a polyarticular form of juvenile arthritis (*Odds ratio* 1.46, *p*=0.004) (**chapter 4**)[31]. Behrens and colleagues have also independently reported an association of a perfect proxy to JIA further supporting our findings[32]. In recent years, several common variants associated with RA have been convincingly associated with multiple autoimmune diseases including the PTPN22[33] and *signal transducer and activator of transcription 4* (*STAT4*)[34] genes.

In line with these observations, one widely accepted pattern across the genes loci identified is the overlap of genetic risk factors across various autoimmune diseases. To test this hypothesis we investigated the *TRAF1/C5* locus in a well-powered study including four additional autoimmune diseases including 735 Type I Diabetes (TID), 1049 Celiac Disease (CD), 367 Systemic Sclerosis (SSc), 746 Systemic Lupus Erythematosus (SLE) patients and a common set of controls consisting of 3494 healthy individuals. In combination with the previously unpublished data from the SLEGEN consortium, we observe an overall association to SLE in 1577 patients and 4215 healthy individuals (OR 1.22, $p=1.02x10^{-6}$). We found suggestive evidence for T1D and did not observe any evidence of association to CD and SSc (**chapter 5**)[35].

With these studies, we have provided considerable evidence that the *TRAF1/C5* region is not only relevant to RA but that the frequency of the same allele is increased in JIA and SLE. It is therefore highly likely that the *TRAF1/C5* region is a genetic risk factor involved in a shared pathway underlying multiple autoimmune diseases. It now seems that most of the RA susceptibility loci identified so far have an association with other autoimmune diseases[36]. Interestingly, the association with SLE is absent in a well-powered Japanese case-control study and small study in the Columbian population[37, 38]. As with many genetic loci, it is highly likely that ethnic differences exist in the contribution of this locus to SLE. Since, *TRAF1* is suggested to be a negative regulator of TNF-Receptor and/or CD40 signaling and *C5* is a central component of the complement pathway and they are both highly involved in the immune system, they both represent likely candidates for further functional studies. Current efforts are geared towards the functional relevance of the genetic variants in the *TRAF1/C5* region by determining the expression levels and function of each gene in primary lymphocytes from healthy individuals to tease apart the influence of these genetic variants on biological function.

In addition to the identification of the *TRAF1/C5* locus, the year 2007 saw the discovery and replication of two additional risk loci, more than doubling the number of genes known to play a role in RA[39]. The *STAT4* gene region on chromosome 2q was identified following a mapping of genes under a linkage peak and so far represents the only linkage study to have successfully pinpointed the gene of interest in RA in Caucasians[40]. The other locus, identified by the WTCCC as well as Plenge and colleagues using a genome wide association study (GWAS) with 100,000 markers is located on chromosome 6q23 and encompasses a very interesting candidate gene *TNF-α induced protein 3* (*TNFAIP3)*[25, 41].

Unprecedented progress however has been witnessed in 2008 when a meta-analysis of three genome-wide association studies from the US, Sweden and the UK was performed (**chapter**

15)[42]. Comprised of 3,393 patients who were predominantly ACPA positive and 12,460 healthy individuals, our study enabled the confirmation of previously associated RA loci (*HLA*, *PTPN22*, *TRAF1/C5*, *STAT4* and *6q23* containing *TNFAIP3*)[39] and also provided suggestive evidence towards previous associations with *Cytotoxic T Lymphocyte associated 4 (CTLA4)* and *interleukin 2/interleukin 21* (*IL2/21)*[43, 44]. By genotyping these variants in our Dutch cohort, we have now independently replicated three of these loci (*STAT4*, *IL2/21* and *CTLA4*) in the Dutch population further confirming the suggested role of *IL2/21* and *CTLA4* in RA (**chapter 12**)[45]. It is of note to mention that an overall estimate of the effect size of the *CTLA4* locus results in an odds ratio of ~1.10, highlighting that lack of power of most previous studies is the likely culprit in the controversy of this locus in RA over the past few years. Both *IL2/21* and *CTLA4* have now been reported in a large UK cohort[36]. In addition, our data also provides evidence of the association of *IL2/21* in juvenile arthritis (**chapter 13**)[46].

The above-mentioned meta-analysis of three GWAS of European-ancestry populations identified 31 regions of interest outside of the known RA risk loci ($P<10^{-4}$). Following replication in 3,929 autoantibody positive individuals (ACPA and/or RF) and 5,807 matched controls, six additional loci were identified with the most significant finding localized in the CD40 gene region, more recently complemented by independent replication[47]. The five additional signals were found in regions harboring the following genes, *membrane metallo-endopeptidase-like 1-Tumor necrosis factor receptor superfamily member 14-TNF receptor superfamily 14* (*MMEL1-TNFRSF14)*, *cyclin-dependent kinase 6* (*CDK6)*, *Chemokine (C-C motif) ligand 21(CCL21)*, *Protein kinase C theta (PRKCQ)*, and *Kinesin family member 5A- Phosphatidylinositol-5-phosphate 4-kinase type II gamma* **(***KIF5A-PIP4K2C)*. A parallel study investigating suggestive hits from the WTCCC study provides additional evidence for associations with *PRKCQ*, *KIF5A-PIP4K2C* and *MMEL1-TNFRSF14* regions in an independent UK sample set[48]. Despite the fact that *CDK6* and *CCL21* have not as yet been independently replicated by other groups, recent data from our group suggests that *CDK6* predisposes to a more severe disease course in ACPA positive patients (Manuscript in Press,A&R). However, additional replication from independent groups will be required to establish whether the *CDK6* and *CCL21* regions are genuine RA loci. Interestingly a recent UK study provides suggestive evidence for *CCL21* but no association with *CDK6*[47]. Barton and colleagues have also suggested a possible role of *IL2RA* and *IL2RB*[48]. We have now confirmed these two loci in our independent Dutch sample set (**chapter 16**) leading to a three-fold increase in the number of RA risk loci in 2008 (Table 1). It will be highly relevant to determine whether gene-gene or gene-environment exists among these loci.

Table 1. Non-HLA susceptibility genes for RA

Gene locus	Chromosomal region	Year of confirmation	Method of identification
PTPN22	1p13	2004	Largescale missense SNP screen
TRAF1-C5	9q33	2007	Candidate Gene Study, GWAS
STAT4	2q33	2007	Linkage
TNFAIP3-OLIG3	6q23	2007	GWAS
IL2-IL21	4q27	2008	Candidate Gene Study
CTLA4	2q33	2008	Candidate Gene Study
CD40	20q13	2008	GWAS
MMEL1-TNFRSF14	1p36	2008	GWAS
KIF5A	12q13	2008	GWAS
PRKCQ	10p15	2008	GWAS
CCL21	9p13	-	GWAS
CDK6	7q21	-	GWAS
IL2RA	10p15	2008	GWAS
IL2RB	22q13	2008	GWAS

Genetic risk factors in phenotypes of RA

RA, which remains a clinical diagnosis based on classification criteria, is most likely not a single entity. This point has been clearly illustrated by dichotomy of ACPA-positive versus ACPA-negative RA with the disease course being more severe in ACPA-positive individuals as compared to ACPA-negative individuals[49]. From a genetic perspective, loci identified seem to also differ between these two disease categories. For example, HLA SE alleles seem to predispose to the development of these autoantibodies themselves while the *PTPN22* locus predominantly predisposes to the development of ACPA-positive disease[7, 50]. However, most studies have so far been underpowered to detect a conclusive distinctive pattern between the two subgroups. The HLA-DR3 alleles have been consistently associated with ACPA-negative RA[51, 52]. Similar associations have now been suggested for the *interferon regulatory factor 5* (*IRF5*) locus, a gene region identified as a risk factor for SLE. In our studies we have shown that *IRF5* has a stronger association with ACPA-negative disease (**chapter 14**), a finding which is also supported by a recent report by Eguez-Gonzalez and colleagues[53, 54]. A recent report suggests a considerable genetic component in the development of ACPA-negative RA as well[55]. It is therefore anticipated that with increasing collaborations between groups this particular question can be addressed with relative ease in the near future.

From these studies it is clear that multiple loci of modest effect are involved in the onset of RA. However, despite our understanding that the subclassification of RA patients based on their autoantibody profile results in a more homogeneous subgroup, RA remains a syndrome comprising several phenotypes. For example, following diagnosis of early RA, approximately 10% of patients undergo natural remission while others develop either mild or severe joint damage over time[56].

Data linking newly identified genetic polymorphisms to disease outcome in RA are only beginning to emerge. Since the early arthritis cohort in Leiden possesses a wealth of information on these particular disease phenotypes, we can ask the clinically relevant question regarding genetic loci that associate with remission as well as severity of joint damage. One of the interesting candidates that has been consistently investigated in radiographic damage in RA is the TNF-α locus. Historically, this cytokine has been of high relevance in RA with TNF-α blockers in use for years already[57]. However at a genetic level, several inconsistent reports exist between this locus and both the susceptibility and severity of RA. Recent GWAS have not identified this region as a susceptibility factor for RA and our recent investigation reveals that it does not predispose to a more severe disease course after stratification for autoantibody status (**chapter 10**)[42, 58]. This is most likely due to the linkage disequilibrium between *TNF-α* and *HLA-DR3* with the latter being highly correlated to ACPA-negative RA generally following a less severe disease course.

More recently we have highlighted the relevance of taking into account these disease phenotypes in genetic studies of RA (**chapter 11**). Two SNPs on chromosome 6q23 near *TNFAIP3* have been associated with susceptibility to rheumatoid arthritis (RA)[25, 41, 59]. While the initial associations were detected in patients with long-standing RA, no association was found in a Swedish early arthritis cohort. Since these sample sets are well controlled for population stratification issues, a likely explanation for this discordance could be the overrepresentation of patients with severe disease in cohorts with long-standing RA. To this end, we analyzed the effect of the 6q23 region (*TNFAIP3*) on the rate of joint destruction in our early arthritis cohort with a mean duration of follow-up of 5 years. Our data are unique as they cover a long period of radiographic follow-up and have been scrutinized for artefacts such as secular trends in treatment intensity. Albeit based on relatively low patient numbers, our data suggest a contribution of the 6q23 region to the rate of joint destruction in ACPA-positive RA, thereby further refining our understanding of the effects exerted by this locus. Replication of our findings in other cohorts is needed. Nonetheless, this is the first study demonstrating such an effect for genetic polymorphisms located outside the HLA-region in ACPA-positive RA patients. More recently, one additional independent protective allele has been described at this locus and requires further confirmation[60]. It would be highly relevant to investigate this additional signal in relation to the joint damage of RA patients.

In **part II** of this thesis, we have also revisited an old cytokine, repeatedly suggested to be associated with radiographic progression in RA. IL10 is a cytokine with key regulatory and anti-inflammatory function involved in the pathogenesis of various diseases[61-64]. Although the large interindividual differences in the production of IL10 have been extensively associated with polymorphisms and haplotypes of the *IL10* gene, surprisingly little evidence existed that this variation was actually dictated by *IL10* haplotypes[65].

Using the technique of allele-specific transcript quantification, the ratio between two alleles (A and G) of the *IL10* gene was characterized in 15 healthy heterozygous individuals. Two groups were identified whereby donors in group 1 exhibited a 1:1 ratio, whereas those in group 2 exhibited a ratio>1 (P<0.0017). We found that donors heterozygous for haplotype *IL10.2* (one of the four ancient *IL10* haplotypes) were only prevalent in the group that showed higher allelic expression ratios. In our study we show that *IL10* alleles are indeed differentially transcribed in cells from heterozygous individuals and that *IL10* haplotypes dictate production of IL10. These findings showed, for the first time, that interindividual differences in IL10 protein levels could be partially explained at the allele-specific transcriptional level (**chapter 7**)[65]. More recently, Sharma and colleagues have reported the post-transcriptional regulation of IL10 via the binding of microRNA hsa-miR-106a to the 3' UTR in several cell lines[66]. It would be interesting to determine whether this microRNA has an allele-specific effect in primary cells and whether this post-transcriptional regulation of IL10 is functionally relevant in diseases like RA.

In a previous study, an increased risk of familial osteoarthritis (OA) at multiple site was detected in subjects with a low innate production of *IL10* as measured by the same *ex vivo* whole blood assay using lipopolysaccharide stimulation as in several other studies[67, 68]. This finding implicating IL10 in cartilage destruction led us to investigate whether genetic variation in *IL10* contributes to the susceptibility of OA (**chapter 6**). Our study has failed to detect those differences. However, due to the highly limited power of the study, we can only preclude a large effect size of this locus in the development of osteoarthritis. To enable more conclusive results with regards to modest effect sizes, more extensive studies with larger sample sizes will have to be conducted.

In RA, the -A2849G (rs6703630), an *IL10* promoter SNP, was shown to be associated with differences in titres of autoantibodies (RF and ACPA). Moreover the rate of joint destruction in RA patients from the early arthritis cohort was twice as high in patients that were –2849G carrier to those who were not (median rate per year 8 versus 4 Sharp van der Heijde units on X-rays of hand and feet)[69]. However, none of the known *IL10* promoter polymorphisms alone could be a causal variant as shown from our functional study. A more likely possibility of causal mutations is either unknown or untyped SNP(s) located on haplotype *IL10.*2. As the length of the haplotype block around *IL10* is highly relevant to the search for the functional polymorphism(s), we characterized the level of linkage disequilibrium in a region of 217 kb, encompassing *IL10* as well as its neighbouring homologues (*IL19*, *IL20* and *IL24*). We successfully excluded the neighboring genes as potential candidates for harboring the functional cis-acting variants (**chapter 8**)[70]. We further fine-mapped the immediate *IL10* region by genotyping 43 SNPs in 57 healthy unrelated individuals of European descent. One haplotype block was identified restricted to 17 kb encompassing the coding region of the *IL10* gene (5kb) as well as 5'and 3' untranslated regions. HapMap data showed similar linkage disequilibrium patterns. Six tagging SNPs, explaining 93% of the variation in the *IL10* region were identified in our study. We have now genotyped these SNPs in our extensive and clinically well-defined RA cohort to determine their relevance to phenotypes of RA. However, our data show no significant differences in the rate of joint damage and remission with respect to the *IL10* genotypes and haplotypes irrespective of their ACPA status (**chapter 9**). These data suggest that *IL10* polymorphisms most likely do not play a major role in phenotypes of R

More recently however, a GWAS in ulcerative colitis and a meta-analysis of T1D GWAS has identified a signal in the *IL10* gene (rs3024505) located in the 3' UTR[71, 72]. Unfortunately, this polymorphism was not tagged in our fine-mapping approach. While it is clear from the lack of association of this region in the recent GWAS meta-analysis that *IL10* most likely does not predispose to the development of RA as such, it remains important to perform a more extensive sequencing and genotyping effort of this region to definitively exclude any potential role of this locus in RA phenotype.

Translating genetic findings into functionally relevant pathways

One common theme across most loci identified is the fact that most loci either contain multiple genes or they are located in regions devoid of any known genes. However, most loci do harbor at least one candidate gene involved in immune function and thereby do provide important information in potential biological processes. For example, in RA the role of T and B cell functions in general have been highlighted as well as a significant indication of the involvement of the NF-kB signalling pathway. The genes involved in those pathways are discussed below. We believe that many of the proteins encoded by these genes either represent drug targets that have been or will be of high therapeutic potential in the near future. Given the overlap of genetic risk factors across various autoimmune genes, these drug targets may prove useful for more than one disease entity[18, 73]. I have attempted to outline the potential biological relevance of the genetic regions identified by the likely candidates in each region as we know it now but fully aware that this landscape is rapidly evolving.

T cell function

The strongest genetic risk factor for RA is the **SE** alleles from the HLA locus. Activation of the T cell occurs via the engagement of the T cell receptor (TCR) and CD28 on the T cell to the HLA-complex presenting the specific peptides as well as the B7 molecules. In these initial steps, **PRCKQ**, also known as protein kinase C theta (PKCθ) and involved in the phosphorylation of a wide variety of protein targets may play an essential role in relocating to the immunological synapse between the T cell and the antigen-presenting cell (APC) during antigen specific interactions[74, 75]. **PTPN22**, the second strongest genetic risk factor in RA, is involved in regulating the threshold of T cell activation through increased phosphatase activity of downstream proteins that associate with the TCR[21]. **CTLA4** is known to compete with CD28 to limit the extent of T cell activation[76, 77]. Once a T cell is activated, **IL2RA** is induced, enabling the trimerisation of the IL2RA, **IL2RB** and IL2RG to form the high affinity IL2 receptor[78]. **IL2** binds this receptor and through further downstream signaling enhances the proliferation and survival of T cells[79]. Interestingly, **STAT4** and **IL21** are involved in T helper 1 and T helper 17 cells, which are T cell lineages involved in inflammatory reactions[80-82]. IL21 as well as **CDK6** also seem to play a role in the proliferation of activated T cells[83, 84]. More importantly, in the last couple of years, a key role for IL21 in the differentiation of B cells into antibody secreting cells has been reported as a result of T-B cell interaction[85-87]. Interestingly, **CCL21** is also a molecule involved in the homing of lymphocytes to secondary lymphoid organs[88]. Expression of this chemokine is associated with ectopic lymphoid structures and has been implicated in the organization of lymphoid tissue affected by rheumatoid arthritis[89].

Taken together, it seems that these genetic risk factors now identified in RA play a crucial role in the regulation of T cell receptor activation and proliferation, migration as well as differentiation of T cells. It will therefore be interesting to determine how these genetic variations alter their respective gene functions. In the case of PTPN22, strong indications already exist that the susceptibility allele results in a higher threshold of T cell activation which may reduce the negative selection of autoreactive T cells in the thymus[22]. In line with these findings and the fact that T cells may play a more regulatory role by providing help for the activation of B cells, it is not surprising that therapies targeted towards T cells have so far not been proven to be highly effective in RA patients[90]. However, gaining insight into the biological implications will inevitably lead to a better understanding of disease processes.

B cell function

B cells are an integral part of our immune system which is geared towards responses against invading antigens. The genes identified by human genetic studies have now also highlighted a role of certain molecules that play an important role in the activation of B cells through the regulation of both the B cell receptor (BCR) function as well as the second signal required to activate B cells i.e the CD40 signalling cascade. In addition to its role in the increased threshold for T cell activation, the **PTPN22** susceptibility allele has recently been implicated in the reduction of BCR signaling[91, 92]. Associations near **TRAF1**, **TNFAIP3** (A20) and **CD40** itself already suggest the possibility that the CD40 signaling pathway mediates rheumatoid pathogenesis through NF-kB activation[93]. In particular, TRAF1 binds the CD40 receptor and cooperates with TRAF2 to activate NF-kB[94-97]. TRAF1 also binds TNFAIP3, which is a negative regulator of NF-kB signaling[98, 99]. Furthermore, CD40 stimulation results in B cell proliferation through regulation of **CDK6** expression[100]. The CD40 signaling pathway has been investigated in drug development, and mouse models have demonstrated that its disruption could prevent development of immune-mediated arthritis[101]. In addition, the production of RF autoantibodies have been shown to be dependent on CD40 signalling[102]. More recently, the NF-kB family of transcription factors (also known as REL) has been implicated in RA by an extensive GWAS performed by Gregersen and colleagues[103]. The various members of *REL* are *c-Rel*, *p65* (*Rel-A*), *Rel-B*, *p50* (*NFkB-1*) and *p52* (*NFkB-2*) and they have been shown to play important roles in immune responses and autoimmunity[104]. Notably, c-Rel has been reported to physically interact with nuclear CD40 and results in the transcriptional regulation of several target genes[105]. These data further uphold the crucial role of the NFkB pathway as well as the CD40 pathway in RA.

Another potential role of the TNF receptor superfamily has been highlighted by the identification of **TNFRSF14**, the cytoplasmic region of which was found to bind to several TRAF family members, which may mediate the signal transduction pathways that activate the immune response[106]. Although the specific functions of phosphatylinositol 4 phosphate 5 kinase type II γ, **PIP4K2C**, remain largely uncharacterized, it is known that it forms part of a family of kinases which are also thought to play a role in the phosphorylation of proteins downstream of the TNF-receptor signaling cascade[107, 108]. The protein is found in sera from RA patients as well as healthy individuals[109].

In addition to its function as a homing signal for T cells to the lymph nodes, it has been suggested that **CCL21** may also play a role in B cells as it is the high-affinity ligand for CCR7, a

molecule also expressed by B cells, although its precise role in these cells remain to be investigated thoroughly[88].

Taken together, the genes identified seem to play important roles in B cell function, either by regulating the first signal which is the BCR activation or by affecting the second signal which is the signaling cascade resulting from TNF-receptor signaling, in particular via the CD40 receptor.

Altogether, several processes have been highlighted involving antigen recognition, lymphocyte migration, receptor activation, cell fate of T and B cells and NFkB signaling. While the identification of these genetic risk factors have provided considerable insight into the crucial role of the adaptive immune system in RA, we realize that much work lies ahead in the identification of the actual causal variants through sequencing, fine-mapping and functional experiments.

The next step

Common Variants

While pinpointing these gene regions has been a monumental advancement on our previous knowledge of genetic risk factors for RA, the exact common causal mutation(s) is not known for most of these genetic regions. It is therefore paramount to now take steps to characterize the regions of association in more detail, especially since only one out of three common polymorphisms in the human genome have so far been identified[110, 111]. Additionally, recent studies have highlighted the existence of multiple signals at one locus indicating the complexity of associations with disease. One particular example in RA are the multiple alleles at the *TNFAIP3* locus which shows complex haplotypic associations[41, 59, 60] . To resolve these issues across loci identified in RA, we will need to sequence all the regions identified to capture all unknown variations, followed by an in-depth evaluation of association signals conferred by these polymorphisms. Undoubtedly, applying this strategy across multiple autoimmune diseases as well diverse ethnic populations will be invaluable tools in identifying causal alleles and understanding pathways.

Rare variants

The entirety of RA susceptibility variants identified so far does not explain the entire genetic burden of RA, indicating that more remain to be discovered. While current studies in RA are biased towards common variants, increasing evidence points towards the relevance of rare mutations in other complex diseases. Examples of such rare variants influencing common traits exists in Systemic Lupus Erythematosus (*TREX1*)[112], Inflammatory Bowel Disease (*NOD2* and *IL23R*)[113-115], disorders of cholesterol metabolism (*PCSK9*, *ANGPTL4*, among others)[116-119] and deregulated blood pressure levels (SLC12A3, SLC12A1 and KCNJ1)[120]. Notably, numerous examples of non-RA genes exist containing both common mutations of small effect size together with independent rare functional mutations of larger effect size. The most compelling example includes the rare gain- and loss-of-function missense mutations in *PCSK9* which cause deregulated levels of cholesterol while common *PCSK9* variants reproducibly associate with plasma LDL cholesterol levels[116, 121, 122]. An additional 18 loci containing common variants influencing levels of LDL/HDL cholesterol and/or triglycerides was recently reported[123]. Strikingly, rare variants at 9 of these loci have been shown to cause rare Mendelian forms of

dyslipidemia. It is therefore likely that rare mutations contributing to the RA phenotype exist and contribute significantly towards disease pathogenesis. To enable their discovery, sequencing of exon-coding regions in thousands of patients and controls will be required. Compared with common variants, the technologies to detect these rare variants, termed next-generation sequencing, have only recently become available. Hence, our ability to assay them reliably is still maturing but holds great promise for future investigations of our complex genome[124-126].

Structural variations

Unlike single nucleotide polymorphisms, structural variations have largely been ignored in the genetic mapping of common diseases. Although these have been historically difficult to assay until recently, sequencing of several human genomes have now revealed that they comprise over 20% of genetic variants in the human genome[127]. According to Frazer and colleagues, structural variation refers to all base pairs that differ between individuals and that are not single nucleotide variants. These variations include insertion–deletions (indels), block substitutions, inversions of DNA sequences and copy number differences. Interestingly, recent studies that have looked for associations between rare structural variants and autism and schizophrenia have identified specific deletions involved in both of these diseases[128-130]. Additionally, recent technological developments have also generated the possibility of interrogating the genome for copy number variation in disease[131]. Copy numbers in Fc gamma receptor genes have been implicated in SLE as well as RA[132, 133]. We therefore expect that the coming years will provide insight into the relevance of these structural variations in RA and hopefully explain some of the large proportion of the genetic variance in this disease that remains unexplained.

Functional characterization of both common and rare variants

The next frontier lies in the functional characterization of the myriad of sequence variations that influence disease susceptibility. For common variants as well as rare variants, most focus will likely be on candidate protein coding genes and their putative regulatory regions. Experiments are currently being conducted that simultaneously assay global gene expression and genome-wide variation in a large number of individuals to map genetic factors underlying differences in expression levels[134]. While these data sets may undoubtedly be valuable tools for identifying the causative variants and biological bases for many loci associated with a complex trait, they will primarily serve as a prioritization tool for candidate genes and will aid in the hypothesis-generating exercise prior to functional testing. In my opinion, each gene region will require tailor-made experiments in the relevant cell-types to enable a successful translation of genetic findings to biology. Additionally, since each region often encompasses large regions of linkage disequilibrium, there is every possibility that many of these variations will alter the interactions between regulatory (non-coding) RNAs and their targets, a prospect that should not be excluded from future functional analyses[135].

Shared genetic risk factors across diseases and phenotypes – a powerful tool

While sequencing, fine-mapping and identifying common and rare functional variants in RA are the obvious steps the genetic community needs to take, there is one theme in common diseases that can immensely enrich our understanding of the pathways underlying disease. A recent review by Zhernakova and colleagues has recently highlighted the importance of investigating which genetic risk factors are shared by several immune-mediated diseases. The authors investigated genes involved in ankylosing spondylitis, asthma, Graves' disease, celiac disease, Crohn's disease, multiple sclerosis, psoriasis, RA, SLE, type 1 diabetes, and ulcerative colitis and observed that the most likely function of the shared genes were two common pathways involving T cell differentiation and signaling as well as the innate immune response[73]. In addition, a study performed by Smyth and colleagues has highlighted common as well as distinct genetic risk factors between type 1 diabetes and celiac disease[136]. Of the fifteen previously validated susceptibility alleles in type 1 diabetes, at least two contributed to a risk of celiac disease with five showing highly suggestive associations. Interestingly two shared alleles actually had opposite effects in the two diseases: the minor alleles of *IL18RAP* on chromosome 2q12 and *TAGAP* on chromosome 6q25 conferred protection against type 1 diabetes but susceptibility to celiac disease. The authors suggest that common biologic mechanisms, such as autoimmunity related tissue damage and intolerance to dietary antigens may be etiologic features of both diseases.

The concept of shared autoimmunity is now well-established and represents an opportunity not only to study which mechanisms are relevant to several diseases but also which phenotype of a particular disease may have distinct or common pathways. In RA, most of the genetic loci also associate with at least one other autoimmune disease, implying that once a systematic search for these loci has been performed in other immune-related conditions, we can begin to dissect the various overlapping and common mechanisms involved. More recently, suggestions have been made for a multi-step process involving the study of risk factors for specific common sub-phenotypes in diseases, the study of isolated subphenotypes as well as a meta-analysis of these to maximize statistical power in fine-mapping efforts (Huizinga and Grondal, in press at A&R). Therefore combining datasets with well defined phenotypes of each disease may lead to new understanding of disease pathogenesis.

In summary, the advent of technological advances enabling the study of hundreds of thousands of markers across the genome in thousands of cases and controls has significantly changed the genetic landscape of diseases like RA. Now with the sequencing of a thousand genomes (www.1000genomes.org) the genetic community can expect advances on the evaluation of previously unaddressed rare and structural variants in health and disease potentially leading to an even greater understanding of the role of variation in common diseases. In addition, great progress is expected in the coming years in translating these genetic findings into functionally relevant targets for therapeutic interventions.

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