Published in final edited form as:
Nat Genet. 2009 September ; 41(9): 1011–1015. doi:10.1038/ng.434.

Cell-specific protein phenotypes for the autoimmune locus *IL2RA* using a genotype-selectable human bioresource

Calliope A. Dendrou[1], **Vincent Plagnol**[1], **Erik Fung**[1], **Jennie H. M. Yang**[1], **Kate Downes**[1], **Jason D. Cooper**[1], **Sarah Nutland**[1], **Gillian Coleman**[1], **Matthew Himsworth**[1], **Matthew Hardy**[1], **Oliver Burren**[1], **Barry Healy**[1], **Neil M. Walker**[1], **Kerstin Koch**[2], **Willem H. Ouwehand**[2,3], **John R. Bradley**[4], **Nicholas J. Wareham**[5], **John A. Todd**[1], and **Linda S. Wicker**[1,6]

[1]Juvenile Diabetes Research Foundation/Wellcome Trust Diabetes and Inflammation Laboratory, Cambridge Institute for Medical Research, University of Cambridge, Addenbrooke's Hospital, Hills Road, Cambridge, CB2 0XY, UK.

[2] Department of Haematology, University of Cambridge and NHS Blood and Transplant Cambridge, Long Road, Cambridge CB2 2PT, UK.

[3] Human Genetics Department, Wellcome Trust Sanger Institute, Hinxton, Cambridge CB10 1SA, UK.

[4] Respiratory Medicine Division, Department of Medicine, University of Cambridge School of Clinical Medicine, Addenbrooke's and Papworth Hospitals, Cambridge CB2 2QQ, UK.

[5] Medical Research Council Epidemiology Unit, Institute of Metabolic Science, Addenbrooke's Hospital, Hills Road, Cambridge, CB2 0QQ, UK.

Abstract

Genome-wide association (GWA) studies have identified over 300 regions associated with more than 70 common diseases1. However, identifying causal genes within an associated region remains a major challenge1,2. One approach to resolving causal genes is through the dissection of gene-phenotype correlations. Here we use polychromatic flow cytometry to show that differences in surface expression of interleukin-2 (IL-2) receptor alpha-chain (IL-2RA, or CD25) protein are restricted to particular immune cell types and correlate with several haplotypes in the *IL2RA* region that have previously been associated to the autoimmune diseases type 1 diabetes (T1D) and multiple sclerosis2-4. We confirm our strongest gene-phenotype correlation at the RNA level by allele-specific expression (ASE). We also define key parameters for the design and implementation of post-GWA gene-phenotype investigations, and demonstrate the usefulness of a

[6]Corresponding author FAX number: +44-(0)-1223-762102 linda.wicker@cimr.cam.ac.uk.

Author Contributions C.A.D. contributed to the conception, design and coordination of the study, performed and analyzed all experiments except those involving monocytes and the allele-specific expression assays, and drafted the manuscript. V.P. contributed to the design of the study, statistical analysis of immunophenotyping data and graphical displays. E.F. contributed to the conception and design of the study and performed the monocyte activation experiments. J. H. M. Yang contributed to peripheral blood mononuclear cell manipulation and stimulations, and performed the allele-specific expression assays. K.D. and J.D.C. contributed to the genetic data analysis. G.C. and M.H. contributed to blood sample preparation. M. Hardy performed the genotyping and K. D. contributed to genotype double scoring. S. N. coordinated collection of the blood samples for this study. N.M.W. contributed to the coordination of the study. S.N., K.K., W.H.O., J.R.B., N.J.W. and J.A.T. are members of the Cambridge BioResource (CBR) Management Committee who had a primary role in the creation and management of the CBR. N.M.W., O.B. and B.H. managed the data. J.A.T. and L.S.W. participated in the conception, design and coordination of the study, data analysis and drafting of the manuscript.

Competing Interests Statement The authors declare no competing financial interests.

URLs. Cambridge BioResource, http://www.cambridgebioresource.org.uk; R, http://www.r-project.org/; T1Dbase, http://www.t1dbase.org/; FlowJo, http://www.treestar.com/flowjo/.

Europe PMC Funders Author Manuscripts

large bioresource of genotype-selectable normal donors from whom fresh, primary cells can be analyzed.

We have previously localized the T1D association in the region containing the IL-2RA gene to three independent SNP groups (haplotypes) found in noncoding regions spanning 18 kb directly 5′ of *IL2RA* to 25 kb into intron 1 of the gene2-4. The most significantly associated T1D SNPs that define the four common disease-associated *IL2RA* haplotypes are rs12722495(Ref3), rs11594656(Ref2), and rs2104286(Ref4) (Fig. 1; Supplementary Table 1a-c). Although none of the SNPs are located within known regulatory regions, we hypothesized that the causative variants may alter CD25 protein expression on the surface of immune cells. The existence of multiple *IL2RA* haplotypes, as well as a distinct pattern haplotype association with multiple sclerosis4, predicts a complex, haplotype-directed CD25 expression pattern. CD25 is expressed constitutively on numerous immune cell types5 such as $CD4^+$ $FOXP3^+$ regulatory T cells (Tregs) and $CD4^+$ memory T cells6 (Fig. 2), and is upregulated upon activation in many cells including certain innate immune cell subsets7. We therefore investigated haplotype-dependent, cell subset-specific variation in surface CD25 levels on peripheral blood $CD4^+$ memory, naïve, and regulatory T cells (as well as stimulated monocyte subsets) by single-cell polychromatic flow cytometry (Table 1). This allows multiple cell types to be studied simultaneously even if they represent only a small peripheral blood subpopulation, as with Tregs6.

To increase the likelihood of identifying gene-phenotype correlations, study participants were specifically selected on the basis of *IL2RA* haplotype. We used the Cambridge BioResource, a collection of ≈5,000 volunteers living in the Cambridge region who donated blood and saliva samples for research purposes and can be invited to participate in specific research studies based on genotype. The four common *IL2RA* haplotypes comprise (i) susceptibility alleles at all three SNPs; (ii) a protective rs12722495 allele, which predominantly co-occurs with a protective rs2104286 allele; (iii) a protective rs11594656 allele; and (iv) a protective rs2104286 allele (Fig. 1a; Supplementary Table 1d). We aimed to obtain ~50 individuals homozygous or heterozygous for each of the three protective haplotypes, and 50 individuals homozygous for the susceptible haplotype. Individuals heterozygous for two different protective haplotypes were excluded, and thus all heterozygous individuals studied had one protective and one susceptible haplotype. Of the 2,212 individuals who met our recruitment criteria, we contacted ~100 from each haplotype group (Supplementary Table 2) and 179 individuals were analyzed in total (Fig. 1b). Notably, using this haplotype-specific approach to select samples, we succeeded in analyzing 16 individuals homozygous for the protective rs12722495 allele, even though the frequency of such individuals in the Cambridge BioResource is only 1%. All donors selected were of self-reported non-autoimmune disease status. We assessed healthy donors because genetic effects on a phenotype could be confounded by the presence of disease8, particularly as CD25 is known to be upregulated under inflammatory conditions5.

We found that individuals with one or two protective rs12722495 alleles show 27% higher mean CD25 levels on their $CD4^+$ memory T cells compared to fully susceptible individuals or to donors with protective rs11594656 or rs2104286 alleles ($P = 4.30 \times 10^{-9}$; Fig. 3a). Furthermore, there is a gene dosage effect at rs12722495 ($P = 1.16 \times 10^{-10}$; Fig. 3b; see Fig. 2 for representative profiles), with heterozygous and homozygous protective individuals having 22% and 33% higher mean CD25 levels, respectively, compared to homozygous susceptible individuals. This gene dosage effect is consistent with the T1D allelic risk following a conventional multiplicative model whereby the risk with two doses of an allele is the square of the risk with one allele. No dosage effect was found for rs11594656 or rs2104286 (Supplementary Fig. 1a-c), and this phenotype was not associated with donor age

Europe PMC Funders Author Manuscripts

or sex (Table 1). The genetically variable surface CD25 levels on $CD4^+$ memory T cells likely reflect haplotype-dependent transcriptional differences. We found that in total $CD4^+$ T cells and $CD4^+$ memory T cells from donors who were heterozygous for both the protective rs12722495 and fully susceptible haplotypes there were ~30% more pre-mRNA transcripts from the protective haplotype, as determined by cloning-based ASE assays9 designed for SNPs in intron 1 of *IL2RA* (Supplementary Table 3, Supplementary Fig. 2).

Notably, the surface CD25 levels of $CD4^+$ memory T cells were highly consistent over time ($r^2 = 0.997$; Supplementary Fig. 1d), as demonstrated in 15 donors recalled 3-7 months after their initial donation. This repeatability was compromised, however, when 14 peripheral blood mononuclear cell (PBMC) samples were analyzed 9–10 months after cryopreservation for CD25 levels on the $CD4^+$ memory T cells, as these levels were 28–62% lower than those found on the $CD4^+$ memory T cells analyzed in fresh whole blood on the day the PBMCs were isolated and frozen (Supplementary Fig. 1e). The *IL2RA* genotype was estimated to account for 18% of CD25 variation in fresh peripheral blood $CD4^+$ memory T cells. Inter-individual variation in donors with homozygous protective rs12722495 alleles was lower than in homozygous susceptible donors (F-test $P = 0.003$). Given the phenotype repeatability, this indicates that other *IL2RA* SNPs, variants at other genes or environmental factors alter CD25 expression on $CD4^+$ memory T cells, but that these effects are only observable in individuals having rs12722495 susceptibility alleles. We note that no correlation was observed between mean surface CD25 levels on $FOXP3^+$ T cells and *IL2RA* haplotype (Supplementary Fig. 3a-d).

In contrast to the rs12722495 genotypic correlation for increased CD25 on $CD4^+$ memory cells, the percentage of $CD4^+$ naïve cells that are $CD25^+$ is lower in individuals with protective alleles at rs2104286 ($P = 4.25 \times 10^{-6}$; Fig. 3c and d; Supplementary Fig. 3e-h), regardless of whether these alleles co-occur on the same haplotype as protective rs12722495 alleles. This phenotype also had good repeatability ($r^2 = 0.669$). We also obtained evidence for a genotype-phenotype correlation for CD25 upregulation on a stimulated monocyte subset (Table 1; Supplementary Fig. 4), which was analogous to that for $CD4^+$ naïve cells.

Considering our results collectively (Table 1), relative to the susceptible haplotype, the protective rs12722495 haplotype (OR = 0.57; G, protective, minor allele at rs12722495), which is also protective at rs2104286 (G allele), is correlated with three phenotypic changes (in $CD4^+$ memory and naive T cells and in stimulated $CD14^+$ $CD16^+$ monocytes). In comparison, the protective rs2104286 haplotype (OR = 0.88; G, protective, minor allele at rs2104286) is correlated with only two phenotypic changes (in $CD4^+$ naive T cells and stimulated $CD14^+$ $CD16^+$ monocytes). This may, in part, explain their differing strengths of association to T1D. We propose that the $CD4^+$ memory T-cell phenotype is critical for protection from T1D but not from multiple sclerosis, in which rs2104286 (OR = 0.85; G, protective, minor allele at rs2104286) is the most strongly associated SNP4,10, whereas the naïve T-cell and stimulated $CD14^+$ $CD16^+$ monocyte phenotypes are implicated in both diseases.

The *IL2RA* haplotype-specific CD25 expression patterns in $CD4^+$ naïve and memory T cells are consistent with these cells being distinct cell types, as determined by gene expression changes that occur with T-cell maturation and differentiation11, including those mediated by chromosome remodeling and altered transcription and splicing factors. According to the quantal theory of immunity, T-cell responses depend on cells receiving a critical number of T cell receptor (TCR)- and IL-2R-mediated stimuli12. For $CD4^+$ naïve T cells, the protective rs2104286 genotype reduces the probability of this cell type expressing CD25 thereby reducing the likelihood of activation. Indeed, we found that this genotypic effect persisted upon T cell activation, such that a lower proportion of $CD69^+$ $CD4^+$ naïve T cells

upregulated CD25 in donors carrying the protective rs2104286 allele compared to fully susceptible donors (Supplementary Fig. 5). In contrast, because individuals with protective rs12722495 alleles have higher CD25 levels on their $CD4^+$ memory cells, these cells are likely more responsive to IL-2 and to TCR-mediated activation. In mice it has been hypothesized that these nonregulatory $CD4^+$ $CD25^+$ T cells are the primary source *in vivo* of the IL-2 required for $FOXP3^+$ Treg homeostasis and competitive fitness13-15. This is consistent with the correlation between an ~50% reduction in IL-2 abundance and reduced Treg function in mice carrying two T1D susceptibility alleles at *Idd3*(Ref16,17). By analogy, it is possible that when human nonregulatory $CD4^+$ memory T cells express higher levels of CD25 due to the protective rs12722495 haplotype, homeostatic IL-2 production is increased; this would be consistent with observed defects in IL-2 production shown by individuals with T1D18,19. In support of this hypothesis we have found that upon stimulation, a higher percentage of $CD69^+$ $CD4^+$ memory T cells from protective rs12722495 homozygous donors secrete IL-2 compared with those from homozygous susceptible donors ($P = 5.74 \times 10^{-4}$; Fig. 4). An alternative, or additional, mechanism to reinforce self-tolerance that would be improved with higher CD25 levels on activated, anti-self effector T cells is activation-induced cell death20,21. Modest increases in IL-2 production enable mice to more efficiently establish transplant tolerance, presumably via this mechanism22.

This study represents the first description gene-phenotype correlations between protein levels on specific subsets of primary human immune cell types and autoimmune disease-associated variants. We provide evidence for three immune cell type-specific quantitative trait effects on protein expression associated with two protective *IL2RA* haplotypes, providing further support for *IL2RA* as a T1D-causative gene at the 10p15.1 locus. We also delineate important parameters for the design and practical implementation of gene-phenotype correlation studies. The establishment of a panel of thousands of healthy donors recallable by genotype and resident near the study location was critical to the success of our approach, as was the analysis of fresh rather than frozen primary immune cells. Given the necessity for advances in the field of human immunology23,24, our findings illustrate that although the human population is genetically diverse and individuals are subjected to immune challenges, gene-phenotype correlations can indeed be dissected. Our results will also direct further studies to examine the functional consequences of *IL2RA* genetic variation *in vitro* and in animal models of autoimmune disease.

Methods

Subjects

The Cambridge BioResource is funded by the Wellcome Trust, the Juvenile Diabetes Research Foundation (JDRF) International and the National Institute for Health Research Cambridge Biomedical Research Centre. The Cambridgeshire 2 Research Ethics Committee gave ethical approval for the recruitment of 1,000 local volunteers to the Cambridge BioResource, which was formed as a collaboration between the University of Cambridge and the Medical Research Council (MRC). In collaboration with the National Health Service Blood and Transplant (NHSBT) Centre, another 4,000 blood donors were recruited to the Cambridge BioResource, with the ethical approval of the Cambridgeshire 1 Research Ethics Committee. Informed consent was obtained from all volunteers upon recruitment to the Cambridge BioResource for the collection and use of DNA samples for genotyping.

Subjects were selected by genotype from the Cambridge BioResource as part of the ‘Genes and Mechanisms of Type 1 Diabetes in the Cambridge BioResource’ study and were of self-reported white ethnicity. Ethical approval for this study was given by the Peterborough and Fenland Local Research Ethics Committee, and informed consent was obtained for the

collection and use of the peripheral blood samples. Subjects were of self-reported non-autoimmune disease status; we specifically excluded individuals with T1D to avoid any confounding due to T1D status or to insulin treatment.

We aimed to obtain samples from ~200 donors enriched for less common *IL2RA* genotypes and including at least one donor from each of our four haplotype groups on each sample donation day to minimize any day-of-collection effects. The immunophenotyping was conducted in a genotype-blinded fashion. We designed our study to be able to detect an effect size as small as 50% of the genotype-related difference in CTLA-4 expression observed in previous work25. Given these prior estimates a power study shows that a random sampling of the population would provide 10% power to detect a gene-phenotype correlation at $P \simeq 10^{-5}$. With the same total number of donors and the allele distribution resulting from our genotype-specific sampling, we have obtained 87% power at the same significance level.

Genotyping

SNPs were genotyped using Custom TaqMan® SNP Genotyping Assays or Taqman (Applied Biosystems) in accordance with the manufacturers' protocols. All genotyping data were double scored by a second operator to minimize error.

Antibodies and whole blood immunostaining

The anti-human monoclonal antibodies used for T cell surface immunostaining were allophycocyanin (APC)-conjugated anti-CD25 (clones M-A251 and 2A3; BD Biosciences), Alexa-Fluor®700-conjugated anti-CD4, Alexa-Fluor®488-conjugated anti-CD127, and Pacific Blue™-conjugated anti-CD45RA (BioLegend). The isotype control antibodies used were APC-conjugated mouse IgG1 (BD Biosciences) and Alexa-Fluor®488-conjugated mouse IgG1 (BioLegend). To minimize potential variation due to antibody batch differences, all antibodies were obtained prior to the start of the experiment and all vials of antibody derived from the same clone and labeled with the same fluorochrome were pooled prior to usage.

To better visualize lower-level surface CD25 expression, we increased CD25 detection sensitivity by simultaneously using two anti-CD25 monoclonal antibodies, labeled with the same fluorochrome (from clones 2A3 and M-A251, BD Biosciences), that do not cross-compete and that recognize distinct epitopes on the CD25 molecule26,27.

Samples were stained within 5 h after venesection. After blocking, samples were stained for 40 min and then lysed with freshly prepared 1X BD FACS™ Lysing Solution (BD Biosciences). After erythrocyte lysis, samples were incubated at 4°C and were washed with BD CellWASH™ (BD Biosciences). The samples were fixed with freshly prepared 1X BD CellFIX™ (BD Biosciences). The samples were stored at 4°C until analysis by flow cytometry or intracellular staining. For intracellular staining, the PE (phycoerythrin)-anti-human FOXP3 Staining Set from eBioscience was used, according to the manufacturer's instructions.

Flow cytometry and data analysis

Immunostained samples were analyzed using a BD™ LSRII Flow Cytometer with BD FACSDiVa Software (BD Biosciences). The flow cytometry data obtained were analyzed using FlowJo (Tree Star, Inc.). Each day donor samples were evaluated, FluoroSpheres were also analyzed (Blank Beads and Calibration Beads, Dakocytomation). The Calibration Beads contain a mixture of bead populations of different fluorescence intensities that have been assigned specific 'molecules of equivalent fluorochrome' (MEF) values; thus they

allow the transformation of mean or median fluorescence intensity (MFI) into mean or median MEF, which is a quantity proportional to the average number of molecules on a cell. This transformation is linear, MEF=*a*.MFI, where the slope '*a*' is a measure of the number of molecules equivalent to one unit of fluorescence.

Statistical analysis of flow cytometry data

All statistical tests were performed using the R statistical software package. Regression analysis and analysis of variance (ANOVA) were used to test for correlations between genotypes and phenotypes, controlling for age and sex. Genotype-phenotype correlations were analysed assuming a linear genotype-phenotype relationship (1-d.f. F-test). A conservative significance threshold was estimated (P=5.56×10^{-4}) using a Bonferroni correction for multiple testing (using a P-value of 0.01 and assuming six potential CD25-related phenotypes and three *IL2RA* SNPs, giving a total of 18 independent hypotheses). The percentage of *IL2RA* genotype accounting for the variation in CD4$^+$ memory T-cell CD25 levels was estimated by least-squares regression analysis. One donor was excluded from the CD4$^+$ T cell data and another was excluded from the monocyte data, on the basis that the CD25 levels of these individuals differed from the mean of the rest of the donors by at least 5 standard deviations, and these individuals were thus considered to be outliers.

Peripheral blood mononuclear cell (PBMC) isolation, cryopresesrvation and thawing

PBMC isolation, cryopreservation and thawing were performed as previously described28. PBMC isolation was carried out using Lymphoprep (Fresenius Kabi Norge AS for Axis-Shield PoC AS). PBMCs were cryopreserved in heat-inactivated, filtered human AB serum (Sigma-Aldrich) + 10% DMSO (Hybri-MAX™, Sigma-Aldrich) at a final concentration of 10×10^6 ml^{-1} and were stored in liquid nitrogen. Cryovials were thawed in a 37°C water bath. PBMCs were washed by adding 5 ml of room-temperature (20-25°C), heat-inactivated, filtered human AB serum and then 5 ml of RPMI-1640 + GlutaMAX™ I + 25 mM HEPES (GIBCO®, Invitrogen) per 10×10^6 cells, in a drop-wise fashion.

Cloning-based allele-specific expression assay

PBMCs were isolated from three donors heterozygous for the protective rs12722495 and fully susceptible haplotypes. CD4$^+$ T cells were obtained from PBMCs using the Dynabeads® Untouched™ Human CD4 T Cells kit (Dynal, Invitrogen). CD4$^+$ memory T cell enrichment was carried out using the Dynabeads® Untouched™ Human CD4 T Cells kit with the inclusion of unconjugated anti-CD45RA and anti-HLA-DR antibodies (BD Biosciences), to remove CD4$^+$ naïve T cells and the majority of CD4$^+$ CD127$^{\text{int-low}}$ CD25$^{\text{hi}}$ regulatory T cells along with non-CD4$^+$ T cells. Cell purity was estimated by flow cytometry.

Genomic DNA was obtained from PBMCs by chloroform extraction. Total RNA was extracted from the negatively selected cells using TRIzol® Reagent (Invitrogen) and further purified using the RNeasy Mini kit (QIAGEN), according to the manufacturers' instructions. RNA was either left unfragmented or fragmented29 to provide better coverage throughout the gene30. Complementary DNA synthesis was then carried out using Superscript™ III RT kit and oligo dT or random hexamer primers (Invitrogen). After cDNA synthesis using fragmented RNA, 150-350 bp long cDNA molecules were gel-purified using QIAquick® Gel Extraction Kit (QIAGEN). Primers flanking the rs12722495 and rs2104286 SNPs were used to amplify the cDNA and genomic DNA by PCR. PCR products were gel-purified and ligated into pCR®4-TOPO® Vector and transformed chemically into One Shot® Top 10 competent *Escherichia coli* cells (Invitrogen).

Bacterial colonies were picked and PCR amplification was performed using an AmpliTaq Gold™ protocol (ABI). PCR products were screened by TaqMan genotyping (ABI). Alleles were scored on SDS v2.1 software (ABI) manually and counted. Statistical analysis was performed using a 2×2 contingency table to calculate one-tailed *P*-values for T1D-associated SNPs, while a two-tailed *P*-value was used for rs12244380 as the phase for the alleles of this SNP relative to the T1D-associated SNPs could not be determined. Assuming a 30% allelic imbalance between the protective rs12722495 and fully susceptible haplotypes, we calculated that 350 colonies needed to be picked to provide 80% power to detect a 30% allelic imbalance at the RNA level at $P=0.05$, while 1,000 colonies would provide 90% power at $P=0.001$; hence we aimed to pick ~350 bacterial colonies per assay.

Monocyte whole blood activation

Aliquots of whole blood, collected in CPDA VACUETTE® tubes (Greiner Bio-One) were stimulated with 200 U/ml GM-CSF and 200 U/ml IFNγ (PeproTech) for 6 hours at 37°C with shaking at 200 rpm. Unstimulated samples were treated identically except that GM-CSF and IFNγ were not added to the whole blood samples. After activation the blood samples were immunostained. The anti-human monoclonal antibodies used were PE-conjugated anti-CD25 (clones M-A251 and 2A3; BD Biosciences), Pacific Blue™-conjugated anti-CD16 (BioLegend), Pacific Orange™-conjugated anti-CD14 (Invitrogen), and PerCP-conjugated anti-HLA-DR (BD Biosciences).

PBMC activation

Eight pairs of donors were recalled such that in each pair one donor was homozygous for the protective rs12722495 haplotype and one donor was homozygous for the fully susceptible haplotype (and had $CD4^+$ memory cells with CD25 levels <800 MEF). Freshly isolated PBMCs were cultured in 24-well flat-bottom plates at a concentration of 2×10^6 ml^{-1} per well in X-VIVO™ 15 (Lonza) supplemented with 1% heat-inactivated, filtered human AB serum (Sigma-Aldrich). Cells were stimulated with 5 μg/ml of SEB (Toxin Technology, Inc.) for 4 hours. Following stimulation, T-cell subsets secreting IL-2 were identified using a PE IL-2 Secretion Assay (Miltenyi Biotec) and immunostaining with fluorescein isothiocyanate (FITC)-conjugated anti-CD25 (clones M-A251 and 2A3; BD Biosciences), APC-conjugated anti-CD69 (BD Biosciences), Alexa-Fluor®700-conjugated anti-CD4, and Pacific Blue™-conjugated anti-CD45RA (BioLegend). Data were analyzed using a paired, one-tailed student's t-test and a 5% significance level.

T-cell whole blood activation

Pairs of donors were used as described for "PBMC activation". Aliquots of heparinized whole blood were diluted 1:1 with RPMI-1640 + GlutaMAX™ I + 25 mM HEPES (GIBCO®, Invitrogen). Samples were stimulated with 10 μl of BD FastImmune™ CD2/CD2R (BD Biosciences) per 1 ml of diluted blood at 37°C for 21 hours. Samples were stained with PE-conjugated CD69 (BD Biosciences), APC-conjugated anti-CD25 (clones M-A251 and 2A3; BD Biosciences), Alexa-Fluor®700-conjugated anti-CD4, and Pacific Blue™-conjugated anti-CD45RA (BioLegend), and the FITC anti-human FOXP3 Staining Set from eBioscience was used according to the manufacturer's instructions. Data were analyzed using a paired, one-tailed student's t-test and a 5% significance level.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge the participation of all Cambridge BioResource subjects. We thank R. Bailey, P. Clarke, H. Stevens, S. Duley, D. Harrison, M. Maisuria, T. Mistry, N. Taylor, for DNA sample preparation and D. Smyth for genotype double scoring. We thank J. Casey, W. Herring, K. Hodge, H. Lloyd-Jones, R. Walker, and M. Wiesner for donor coordination and blood sample obtainment and J. Clark for help with immunostaining. We thank T. Attwood for his contribution to sample management. We thank the other members of the Cambridge BioResource Management Committee: D. Dunger, S. O'Rahilly, D. Savage, J. Sambrook, A. Calder, W. Marslen-Wilson, K. Chatterjee, and C. Saunders. We acknowledge use of the DNA from the British 1958 Birth Cohort collection, funded by the Medical Research Council and Wellcome Trust. We thank the Avon Longitudinal Study of Parents and Children laboratory in Bristol and the British 1958 Birth Cohort team, including S. Ring, R. Jones, M. Pembrey, W. McArdle, D. Strachan and P. Burton for preparing and providing the control DNA samples. We also thank the Wellcome Trust, the Juvenile Diabetes Research Foundation (JDRF) International, and the National Institute for Health Research Cambridge Biomedical Research Centre for funding. This work was also supported by a National Institutes of Health (USA) grant P01 AI39671 and a grant from the JDRF UK Centre for Diabetes Genes, Autoimmunity and Prevention (D-GAP; 4-2007-1003). The Cambridge Institute for Medical Research is the recipient of a Wellcome Trust Strategic Award (079895). LSW is a Juvenile Diabetes Research Foundation/ Wellcome Trust Principal Research Fellow.

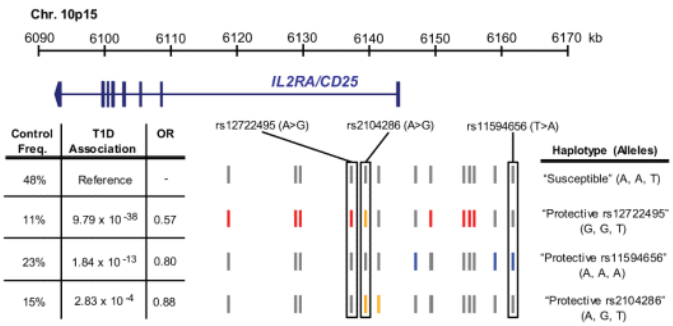
References

1. Donnelly P. Progress and challenges in genome-wide association studies in humans. Nature. 2008; 456:728–731. [PubMed: 19079049]
2. Lowe CE, et al. Large-scale genetic fine mapping and genotype-phenotype associations implicate polymorphism in the *IL2RA* region in type 1 diabetes. Nat. Genet. 2007; 39:1074–1082. [PubMed: 17676041]
3. Smyth DJ, et al. Shared, distinct and opposing genetic factors in type 1 diabetes and celiac disease. N. Engl. J. Med. 2008; 359:2767–2777. [PubMed: 19073967]
4. Maier LM, et al. *IL2RA* genetic heterogeneity in multiple sclerosis and type 1 diabetes susceptibility and soluble interleukin-2 receptor production. PLoS Genet. 2009; 5:e1000322. doi:10.1371/journal.pgen.1000322. [PubMed: 19119414]
5. Dendrou CA, Wicker LS. The IL-2/CD25 pathway determines susceptibility to T1D in humans and NOD mice. J. Clin. Immunol. 2008; 28:685–696. [PubMed: 18780166]
6. Baecher-Allan C, Wolf E, Hafler DA. Functional analysis of highly defined, FACS-isolated populations of human regulatory $CD4^+$ $CD25^+$ T cells. Clin. Immunol. 2005; 115:10–18. [PubMed: 15870015]
7. Kniep EM, Strelow I, Lohmannmatthes ML. The monocyte interleukin-2 receptor light chain - production of cell-associated and soluble interleukin-2 receptor by monocytes. Immunology. 1992; 75:299–304. [PubMed: 1551692]
8. Maier LM, et al. Soluble IL-2RA in multiple sclerosis subjects and the effect of soluble IL-2RA on immune responses. J. Immunol. 2009; 182:1541–1547. [PubMed: 19155502]
9. Rainbow DB, et al. Commonality in the genetic control of Type 1 diabetes in humans and NOD mice: variants of genes in the IL-2 pathway are associated with autoimmune diabetes in both species. Biochem. Soc. Trans. 2008; 36:312–315. [PubMed: 18481948]
10. Hafler DA, et al. Risk alleles for multiple sclerosis identified by a genomewide study. N. Engl. J. Med. 2007; 357:851–862. [PubMed: 17660530]
11. McKarns SC, Schwartz RH. Biphasic regulation of *Il2* transcription in $CD4^+$ T cells: Roles for TNF-alpha receptor signaling and chromatin structure. J. Immunol. 2008; 181:1272–1281. [PubMed: 18606681]
12. Smith KA. The quantal theory of how the immune system discriminates between "self and non-self". Med. Immunol. 2004; 3:3. [PubMed: 15606917]
13. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A function for interleukin 2 in Foxp3-expressing regulatory T cells. Nat. Immunol. 2005; 6:1142–1151. [PubMed: 16227984]
14. Setoguchi R, Hori S, Takahashi T, Sakaguchi S. Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. J. Exp. Med. 2005; 201:723–735. [PubMed: 15753206]

15. Wu YQ, et al. FOXP3 controls regulatory T cell function through cooperation with NFAT. Cell. 2006; 126:375–387. [PubMed: 16873067]
16. Yamanouchi J, et al. Interleukin-2 gene variation impairs regulatory T cell function and causes autoimmunity. Nat. Genet. 2007; 39:329–337. [PubMed: 17277778]
17. Sgouroudis E, Albanese A, Piccirillo CA. Impact of protective IL-2 allelic variants on $CD4^+$ $Foxp3^+$ regulatory T cell function in situ and resistance to autoimmune diabetes in NOD mice. J. Immunol. 2008; 181:6283–6292. [PubMed: 18941219]
18. Zier K, Leo M, Spielman R, Baker L. Decreased synthesis of interleukin-2 (IL-2) in insulin-dependent diabetes mellitus. Diabetes. 1984; 33:552–555. [PubMed: 6609855]
19. Kaye WA, et al. Acquired defect in interleukin-2 production in patients with type 1 diabetes mellitus. N. Engl. J. Med. 1986; 315:920–924. [PubMed: 3531850]
20. Lenardo MJ. Fas and the art of lymphocyte maintenance. J. Exp. Med. 1996; 183:721–724. [PubMed: 8642275]
21. Willerford DM, et al. Interleukin-2 receptor-alpha chain regulates the size and content of the peripheral lymphoid compartment. Immunity. 1995; 3:521–530. [PubMed: 7584142]
22. Pearson T, et al. Islet allograft survival induced by costimulation blockade in NOD mice is controlled by allelic variants of *Idd3*. Diabetes. 2004; 53:1972–1978. [PubMed: 15277375]
23. Hayday AC, Peakman M. The habitual, diverse and surmountable obstacles to human immunology research. Nat. Immunol. 2008; 9:575–580. [PubMed: 18490903]
24. Davis MM. A prescription for human immunology. Immunity. 2008; 29:835–838. [PubMed: 19100694]
25. Ueda H, et al. Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. Nature. 2003; 423:506–511. [PubMed: 12724780]
26. Tkaczuk J, et al. Effect of anti-IL-2Ra antibody on IL-2-induced Jak/STAT signaling. Am. J. Transplant. 2002; 2:31–40. [PubMed: 12095053]
27. Urdal DL, March CJ, Gillis S, Larsen A, Dower SK. Purification and chemical characterization of the receptor for interleukin 2 from activated human T lymphocytes and from a human T-cell lymphoma cell line. Proc. Natl. Acad. Sci. U. S. A. 1984; 81:6481–6485. [PubMed: 6436815]
28. Tree TI, Roep BO, Peakman M. Enhancing the sensitivity of assays to detect T cell reactivity: the effect of cell separation and cryopreservation media. Ann. N. Y. Acad. Sci. 2004; 1037:26–32. [PubMed: 15699490]
29. Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat. Methods. 2008; 5:621–628. [PubMed: 18516045]
30. Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. Nat. Rev. Genet. 2009; 10:57–63. [PubMed: 19015660]

Europe PMC Funders Author Manuscripts

a



b

Subject statistic	"Susceptible" haplotype group	"Protective rs12722495" haplotype group		"Protective rs11594656" haplotype group		"Protective rs2104286" haplotype group	
Donor number	55	36		51		37	
Mean age (yr)	37.9	39.8		37.7		38.2	
Age range (yr)	18-55	19-52		19-55		20-52	
Male:Female ratio	27:28	19:17		22:29		16:21	
	All donors:	Hom (GG)	Het (AG)	Hom (AA)	Het (AT)	Hom (GG)	Het (AG)
Donor number	rs12722495 (AA)	16	20	27	24	20	17
Mean age (yr)	rs11594656 (TT)	38.0	41.2	37.8	37.6	39.8	36.5
Age range (yr)	rs2104286 (AA)	19-50	26-52	19-53	21-55	20-52	21-51
Male:Female ratio		9:7	10:10	10:17	12:12	9:11	7:10

Figure 1.

The four common *IL2RA/CD25* haplotypes and summary of the subject statistics by haplotype group. (**a**) The three independent T1D-associated SNP groups are located in the non-coding, 5′ and intron 1 regions of the IL-2RA gene on chromosome 10. The number of SNPs per associated group is 8, 3 and 2, as previously described, with each SNP being represented by a vertical bar2-4. Gray bars represent susceptibility alleles at these SNPs; the red, blue and orange bars represent protective alleles for each respective SNP group. The most associated SNPs in each group are rs12722495 (A>G), rs11594656 (T>A), and rs2104286 (A>G). The major alleles at all three SNPs are T1D susceptible and minor alleles are protective. Using the genotypes at these SNPs, four common haplotypes can be defined: "Susceptible", "rs12722495", "rs11594656", and "rs2104286". The frequency of these haplotypes in ≈9,000 healthy control individuals is shown, and the T1D association and odds ratios (OR) of the protective haplotypes were calculated relative to the 'Susceptible' haplotype using ≈7,000 case and ≈9,000 control samples (Supplementary Table 1). Chr, chromosome; kb, kilobases; Freq, frequency. (**b**) Statistics for the 179 individuals analysed in total. Individuals in the rs2104286 haplotype group did not have protective rs12722495 alleles. No individuals were heterozygous for two different protective haplotypes. SNP alleles are shown in the brackets. Hom, homozygotes; Het, heterozygotes; yr, year.

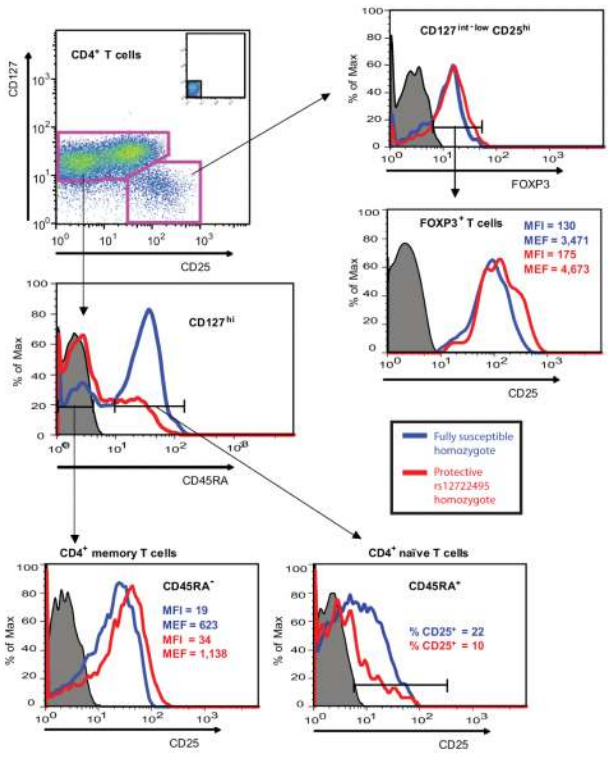


Figure 2.

Gating strategy for CD4+ T-cell subsets. CD4+ T cells were gated based on their expression of IL-7R (CD127) and CD25 (inset plot shows isotype control staining). CD127^{hi} cells were further subdivided by their CD45RA expression, with CD45RA− cells constituting the CD4+ memory T cell subset (14-76% of CD4+ T cells), and CD45RA+ cells constituting the CD4+ naive T cell subset (4-63% of CD4+ T cells). The CD127$^{int\text{-}low}$ CD25^{hi} cells were gated on FOXP3 expression so as to define the FOXP3+ T-cell subpopulation (2-9% of CD4+ T cells). Representative CD25 expression profiles from individuals homozygous for susceptible alleles at all three SNPs (blue) and homozygous for rs12722495 protective alleles (and thus for rs2104286 protective alleles also because of the haplotype structure) (red) are shown for the CD4+ memory, naïve and FOXP3+ T cells. Samples from these individuals were obtained in the same donation session. Gray histograms are isotype control staining profiles. “Percent of max” is the cell number in each bin divided by the cell number in the bin containing the largest cell number; this statistic is calculated to normalize for different numbers of events collected for each sample that is overlaid. MFI, mean fluorescence intensity; MEF, molecules of equivalent fluorochrome.

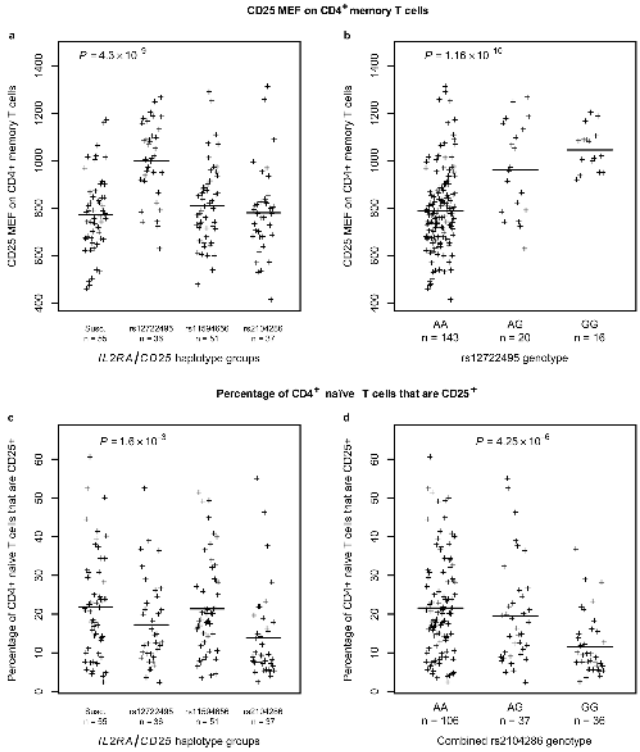


Figure 3.

IL2RA genotype and CD4$^+$ T-cell phenotype correlations. (**a**) Individuals with at least one protective rs12722495 allele have higher CD25 expression on their CD4$^+$ memory T cells (mean CD25 MEF=1,000) than fully susceptible individuals (mean=773) or protected rs11594656 (mean=810) or rs2104286 (mean=783) donors (1-d.f. P=4.3×10^{-9}). (**b**) rs12722495 gene dosage effect on the CD25 MEF of CD4$^+$ memory T cells (1-d.f. P=1.16×10^{-10}). At rs12722495 A>G, where A=susceptible allele, G=protective allele. For AA, AG and GG donors mean CD25 MEF=788, 962 and 1,048, respectively. (**c**) Individuals with one or two protective rs2104286 alleles in the rs2104286 or rs12722495 haplotype groups, have a lower percentage of naïve cells that are CD25$^+$ (for protective rs2104286 and rs12722495 donors mean=14 and 17%, respectively), compared to fully susceptible individuals or protected rs11594656 donors (mean=22 and 21% respectively; 1-d.f. P=1.6×10^{-3}). (**d**) rs2104286 gene dosage effect on the percentage of CD4$^+$ naive T cells that are CD25$^+$ (1-d.f. P=4.25×10^{-6}). At rs2104286 A>G, A=susceptible allele, G=protective allele. For AA, AG and GG donors mean percentage of CD4$^+$ naïve cells that are CD25$^+$=22, 20 and 11%, respectively. For the "combined" rs2104286 genotype, individuals with one or two protective alleles at this SNP are included regardless of allelic status at rs12722495. Susc., susceptible.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

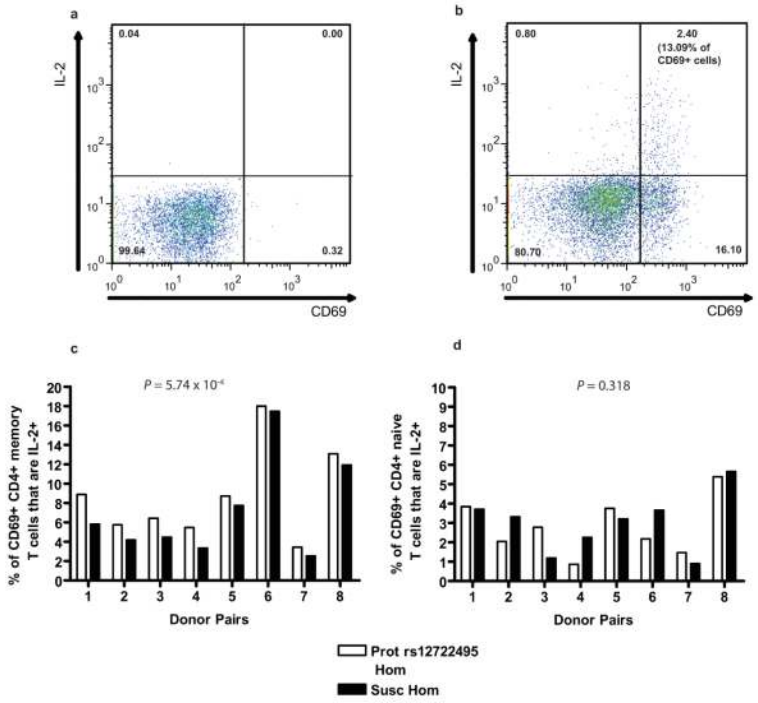


Figure 4.

IL-2 secretion by stimulated CD69$^+$ CD4$^+$ T cell subsets. (**a**) Plot showing that unstimulated CD4$^+$ memory T cells do not upregulate CD69 at their cell surface and do not secrete IL-2. (**b**) Upon stimulation of isolated PBMCs for 4 hours with 5 μg/ml of staphylococcal enterotoxin B (SEB), a proportion of CD4$^+$ memory T cells became CD69$^+$ and secreted IL-2. (**c**) Eight pairs of previously immunophenotyped donors were recalled such that in each pair one donor was homozygous for the protective rs12722495 haplotype and one donor was homozygous for the fully susceptible haplotype (and had CD4$^+$ memory T cells with CD25 levels <800 MEF). Susceptible donors were selected to have low CD25 levels on their CD4$^+$ memory cells in order to test the hypothesis that differential CD25 expression results in differential IL-2 production. A higher proportion of the CD69$^+$ CD4$^+$ memory T cells from protective donors secrete IL-2 compared to fully susceptible donors (P=5.74×10^{-4}). The average difference in CD25 levels of the unstimulated CD4$^+$ memory T cells between the protective and susceptible donors (23.2%) corresponds to the average difference in IL-2 secretion observed for these donors (22.5%). (**d**) A genotypic correlation was not observed for the proportion of CD69$^+$ CD4$^+$ naïve T cells secreting IL-2 (P=0.318).

Europe PMC Funders Author Manuscripts

Nat Genet. Author manuscript; available in PMC 2010 March 01.

Table 1

Summary of *IL2RA* genetic effects, repeatability, age effects and sex effects on $CD4^+$ T-cell and monocyte phenotypes.

Cell Subset	Phenotype	Genetic effect	Repeatability	Age effect	Sex effect
$CD4^+$ memory T cells	CD25 MEF on $CD4^+$ memory T cells	Phenotype ↑ with protective rs12722495 haplotype $P = 1.16 \times 10^{-10}$	$r^2 = 0.997$	None	None
	Percentage of $CD4^+$ memory T cells	None	$r^2 = 0.862$	↑ $P = 8.97 \times 10^{-5}$	None
$CD4^+$ $FOXP3^+$ T cells	CD25 MEF on $FOXP3^+$ T cells	None	$r^2 = 0.336$	None	M>F $P = 0.020$
	Percentage of $FOXP3^+$ T cells	None	$r^2 = 0.417$	↑ $P = 0.017$	None
$CD4^+$ naïve T cells	Percentage of $CD4^+$ naïve T cells that are $CD25^+$	Phenotype ↓ with protective combined rs2104286 genotype $P = 4.25 \times 10^{-6}$	$r^2 = 0.669$	↑ $P = 2.22 \times 10^{-9}$	M<F $P = 0.005$
$CD14^+$ $CD16^+$ monocytes	CD25 median MEF on stimulated $CD14^+$ $CD16^+$ monocytes	Phenotype ↓ with protective combined rs2104286 genotype $P = 4.75 \times 10^{-4}$	Not Done	↓ $P = 0.022$	None
$CD14^+$ monocytes	CD25 median MEF on stimulated $CD14^+$ monocytes	None	Not Done	↓ $P = 0.005$	None
$CD16^+$ monocytes	CD25 median MEF on stimulated $CD16^+$ monocytes	None	Not Done	↓ $P = 0.027$	M>F $P = 0.025$

MEF, molecules of equivalent fluorochrome. "Combined rs2104286 genotype" refers to the rs2104286 genotype regardless of whether the protective rs2104286 alleles co-occur with protective rs12722495 alleles or not. All *P*-values for genotypic effects have been corrected for age and sex effects. Upward arrows by *P*-values for age effects indicate increasing phenotype valueswith increasing donor age; downward arrows indicate the opposite.