

IL-10 Autoantibodies and HLA-DRB1*01:03 in Inflammatory Bowel Disease

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Abstract

Background: Neutralizing autoantibodies against interleukin-10 (IL-10) phenocopy monogenic defects of IL-10 signaling in children and may be associated with inflammatory bowel disease (IBD). HLA-DRB1*01:03 is the strongest genetic risk factor for ulcerative colitis.

Methods: We assessed neutralizing IL-10 autoantibodies in sera of IBD and controls using cellular IL-10 reporter assay and confirmatory competitive ELISA. An in-vitro cytokine-release bioassay was performed in a subset of patients. We performed HLA association analysis using imputation and high-resolution sequencing.

Results: IL-10–neutralizing autoantibodies were detected in 173 of 4909 patients with IBD and in none of 1006 controls (3.5%; 95% CI, 3.1–4.1% versus 0%, $p < 0.0001$). Anti-IL-10 seropositivity was strongly associated with HLA-DRB1*01:03 based on imputed (Oxford and UK IBD BioResource (odds ratio=50.0, 95% CI:16.4-152.3, $P = 6.14 \times 10^{-12}$ and odds ratio=24.7, 95% CI:14.5-42.1, $P = 6.20 \times 10^{-32}$, respectively) and high-resolution sequencing (Oxford Cohort) (odds ratio=29.5, 95% CI:12.2-71.1; $P = 4.85 \times 10^{-14}$) analysis.

Conclusions: Neutralizing IL-10 autoantibodies are present in a subgroup of patients with IBD and are strongly associated with HLA-DRB1*01:03.

INTRODUCTION

Inflammatory bowel disease (IBD) is linked to more than 300 genetic susceptibility loci identified through genome-wide association studies.¹ One of the strongest and most consistent genetic associations is located within the human leukocyte antigen (HLA) region.^{2,3} The allele HLA-DRB1*01:03 is the strongest genetic risk factor not only for ulcerative colitis (UC) susceptibility but also for complicated phenotypes, including acute severe UC (ASUC) and an increased likelihood of requiring surgical resection.²⁻⁵ However, the underlying pathogenic mechanism linking this HLA allele to disease remains unclear.

Interleukin-10 (IL-10) is an anti-inflammatory cytokine essential for maintaining immune homeostasis in the gastrointestinal tract.⁶ IL-10 is produced by monocytes, macrophages, regulatory T cells and subsets of B cells, and its signaling in monocytes and macrophages is required to control inflammation.⁷ Autosomal recessive, loss-of-function variants in genes encoding IL-10 or its receptor (IL-10R, encoded by *IL10RA* and *IL10RB*) cause infantile-onset IBD.^{8,9} The *IL10RA* variant p.Pro295Leu (c.884C>T; ~0.1% frequency) can contribute to adult-onset IBD.^{10,11} We previously described two children with neutralizing autoantibodies against IL-10 (anti-IL-10)¹². In one child anti-IL-10 titers and disease activity responded to B cell depleting anti-CD20 therapy.¹² This exemplifies a phenomenon whereby acquired immune dysfunction caused by anti-cytokine autoantibodies can phenocopy inborn errors of immunity.^{13,14,15} The contribution of anti-IL-10 to the spectrum of Crohn's Disease (CD) and UC in adolescents and adults is unknown.

In this study, we measured the seroprevalence of anti-IL-10 in several cohorts of patients with IBD and controls, and establish a genetic association between anti-IL-10 positivity and HLA-DRB1*01:03.

Materials and methods

Design

We investigated the seroprevalence of anti-IL-10 in patients with IBD and controls (**Figure 1**). Neutralization capacity was assessed by a cellular IL-10 reporter assay and a confirmatory

competitive ELISA. A cytokine release assay was performed in a subset of patients and HLA association studies were conducted (**Figure 1**).

Patient cohorts and ethics

We analyzed two IBD cohorts that included a total of 4909 IBD patients (Oxford IBD Discovery, N=847; Oxford IBD Validation, N=715; UK IBD Bioresource (UK-IBD-BR), N=3347) and compared with 1006 individuals without IBD (Pediatrics Control, N=13 and Adult Control, N=993) (**Figure 1 and 2, and Supplementary Appendix**).

Informed written consent to the individual studies was provided by all patients (or parents of pediatric participants) in the UK studies.

IL-10 autoantibody neutralization assays

Sera were classified as IL-10-neutralizing when they showed concordant inhibition of recombinant IL-10 (rIL-10) signaling in two independent assays. This included an *in vitro* IL-10 reporter assay to measure a cellular response downstream of the IL-10 receptor (HEK-Blue IL-10 reporter), and a confirmatory competitive ELISA to measure rIL-10. Complementary assays were performed to assess the functional consequences of blocking endogenous human IL-10 by a cytokine release assay (**Supplementary Appendix**).

Binding of human IgG to recombinant rIL-10 and other cytokines was performed to assess specificity (**Supplementary Appendix**).

Genetic analysis

HLA haplotyping was performed in a subset of the Oxford cohort and the UK-IBD-BR cohort based on HLA imputation of high resolution genotyping data using a multi-ancestry HLA imputation reference panel; High-resolution HLA sequencing was performed in an ISO15189/EFI-accredited NHS laboratory (**Supplementary Appendix**).

Statistical Analysis

Continuous variables are expressed as means $\pm$ standard deviations (SD) or medians with interquartile ranges (IQR), according to distributional characteristics. Categorical variables are presented as counts and percentages with 95% confidence intervals (CI). Comparisons between independent groups used the Mann–Whitney U test for continuous variables (with Benjamini–Hochberg false discovery rate adjustment for multiple testing) and the chi-square test for categorical variables. For genetic analysis, we estimated p values using logistic regression and applied a genome-wide significance threshold of 5×10^{-8} which accounts for genome-wide comparisons at a 5% error rate. All hypothesis tests were two-sided and a p-value <0.05 was considered statistically significant. Figures were generated using GraphPad Prism and Python and R-based visualization packages.

Results

Detection of neutralizing antibodies against IL-10 in patients with inflammatory bowel disease

In the Oxford IBD Discovery cohort, we tested a set of 5 different assays to detect IgG class anti-IL-10 neutralizing autoantibodies. This included detection of IL-10 binding human IgG in a particle-based flow cytometry assay, a competitive ELISA to assess the IL-10 epitope blockade of patient serum, two cellular IL-10 reporter assays to measure the IL-10 neutralizing capacity of patient serum, and a cytokine release assay (**Figure S1-S4 and Table S1**).

We subsequently defined the frequency of patients with anti-IL-10 neutralizing autoantibodies (anti-IL-10+) by a sequential use of a cellular IL-10 neutralization assay (HEK-Blue IL-10 reporter assay) and competitive ELISA. In the cellular reporter assay, a significantly increased proportion of patients with IL-10 neutralization was observed in IBD cohorts compared to non-IBD controls (0% of pediatric control, 0.7% of adult control, 6.5%, 4.4%, and 4.6% in the Oxford IBD Discovery cohort, the Oxford IBD Validation cohort and the UK-IBD-BR, respectively) (**Figure 2A**).

Based on the combined results of the cellular IL-10 neutralization assay and the confirmatory competitive ELISA assay, no individuals with neutralizing anti-IL-10 were found in 1006 control individuals while 173 IBD patients with positive anti-IL-10 (IBD-anti-IL-10) were identified among 4909 IBD patients (0% vs. 3.5%, 95% CI 3.1-4.1%; p-value <0.0001). The frequencies of anti-IL-10 neutralizing autoantibodies were similar among the Oxford IBD discovery cohort, the Oxford IBD validation cohort, and the UK-IBD-BR cohort (5.3%; 95% CI 4.0–7.1%; 3.1%; 95% CI 2.0–4.6%; and 3.2%, 95% CI 2.6-3.8%, respectively) (**Figure 2A-B, Figure S2**).

Neutralizing anti-IL-10 autoantibodies were detected in patients with CD, UC and IBDu (2.5%, 4.4%, 5.8%, respectively), having a slightly older age of IBD disease onset compared to patients without anti-IL-10 (31.8 ± 13.5 , versus 30.8 ± 15.8 years) (**Table S2-S3**).

Neutralizing anti-IL-10 exaggerates inflammatory responses by peripheral blood cells

To evaluate the impact of anti-IL-10 on human immune cell derived endogenous IL-10 and inflammatory responses, we treated peripheral blood mononuclear cells (PBMC) with Lipopolysaccharide (LPS) in the presence of patient serum and measured the induced cytokine secretion (**Figure 2C**). The presence of serum with high anti-IL-10 activity was associated with a reduction in detectable IL-10, consistent with IL-10 epitope masking. This was associated with an exaggerated increase in the secretion of pro-inflammatory cytokines, particularly IL-6, IL-1 β , TNF- α , and IL-23p19 (**Figure 2C**), while other cytokines including IL-17A, IL-8, MCP-1, IFN α , IL-33 did not appear to be associated (**Figure S3**). These changes did not appear to be attributable to pre-existing raised cytokine levels in the sera, which were typically low (**Figure S3**). The cytokine profile induced by LPS in the presence of anti-IL-10-containing patient sera is similar to that generated in the presence of neutralizing monoclonal antibodies against human IL-10 (**Figure 2C, Figure S3**). These findings suggest anti-IL-10 autoantibodies in IBD patient serum potentially neutralize endogenous secreted IL-10 after LPS stimulation, leading to an amplified pro-inflammatory response by immune cells.

There was a correlation between the results of the bead-based anti-IL-10 screening assay and multiple functional measures of IL-10 neutralization, including cell-based neutralization assays, the competitive ELISA (expressed as percentage of IL-10 neutralization), and cytokine responses in the PBMC secretion assay (**Figure S2**). In particular, higher anti-IL-10 IgG binding measured by the bead assay correlated with greater percent neutralization of rhIL-10 in HEK-Blue STAT3 reporter assays and with increased IL-23 and IL-1 β secretion following LPS stimulation. There was also an apparent correlation among the different assays assessing anti-IL-10 neutralizing capacity, demonstrating that sera positive for anti-IL-10 not only bind IL-10 but functionally neutralize recombinant and endogenous IL-10 in vitro, comparable to commercially available neutralizing monoclonal antibodies directed against human IL-10 (**Figure S2-4**).

Anti-IL-10 neutralizing autoantibodies represent a distinct autoimmune signature

Patients with anti-IL-10 lacked detectable autoantibodies against other cytokines, other than anti-IL-6 in one serum and anti-TNF in two sera reflecting patient treatment with infliximab and adalimumab (**Figure S4**).

Anti-IL-10 neutralizing autoantibodies persist long-term

In a subgroup of 10 patients with positive anti-IL-10, repeat serum samples enabled reassessment of anti-IL-10 neutralizing capacity after an average follow up period of 8.6 ± 4.5 years (range 0.3-14 years), demonstrating long-term persistence of the neutralizing anti-IL-10 antibody level in 7 (70%) patients (**Figure 2D**).

Anti-IL-10 autoantibodies are associated with HLA-DRB1*01:03

We next investigated the association between anti-IL-10 and HLA genotype (**Figure S5**). We applied a multi-ancestry HLA imputation¹⁶ to 404 individuals of European ancestry from the Oxford cohort for whom genotyping data were available. We identified the strongest anti-IL-10-associated

allele in the HLA-DRB1 locus as HLA-DRB1*01:03 (**Figure 3A-B**, OR 50.0, 95% CI 16.4-152.3; $P=6.14 \times 10^{-12}$). After conditioning on DRB1*01:03, no other allele had a P-value below the genome-wide significance threshold.

This association between anti-IL-10 positivity and HLA-DRB1*01:03 was validated by multi-ancestry HLA imputation genotyping data from the UK-IBD-BR cohort (OR 24.7, 95% CI 14.5-42.1, $P=6.20 \times 10^{-32}$) (**Figure 3B**).

We next performed high-resolution HLA haplotype sequencing of patients of the Oxford cohort. Possession of the HLA-DRB1*01:03 allele in patients with IBD was associated with anti-IL-10 with an odds ratio of 29.5 (95% CI 12.2-71.1, $P=4.85 \times 10^{-14}$) (**Figure 3B**).

Using all available haplotyped data, the HLA-DRB1*01:03 carriage was higher in the Oxford IBD cohort compared to the English population (carrier rate 13.6%, 95% CI 11.1-16.6% versus 4%, 95% CI 3-5.6%; $p<0.001$). Additionally, HLA-DRB1*01:03 carriage was highly enriched in anti-IL-10+ IBD patients (carrier rate 81.1%, 95% CI 68.4-89.6%, allele frequency= 0.42; 41 heterozygote and two homozygote) compared to anti-IL-10 negative IBD patients (carrier rate=7%, 95% CI 5.1-9.5%, allele frequency=0.04, no homozygotes observed, chi-square test $p<0.001$, **Figure 3C**).

The formation of anti-IL-10 autoantibodies in individuals lacking HLA-DRB1*01:03 might reflect alternative HLA-DRB1 alleles with HLA sequence similarity or peptide presenting an HLA supertype.¹⁷ Based on imputation or clinical sequencing, the strongest anti-IL-10-associated amino acid variant, is glutamic acid (E) at position 71 ($P_{\text{omnibus}} = 1.1 \times 10^{-8}$) (**Figure 3D and Figure S6**). While non-HLA-DRB1*01:03 E71 was contributed predominantly by HLA-DRB1*13:02, we did not observe an independent association of anti-IL-10 autoantibodies with HLA-DRB1*13:02, or any other HLA allele regardless of E71, or HLA supertypes (**Figure 3 and Figure S6**).

DISCUSSION

Our results challenge the current paradigm of IBD in several areas. Neutralizing anti-IL-10 autoantibodies were identified in 3.5% of patients with IBD, defining an immunologically distinct subgroup. The genetic association between HLA-DRB1*01:03 and anti-IL-10 autoreactivity provides mechanistic insight into one of the strongest known genetic IBD susceptibility factors with possible diagnostic, prognostic and therapeutic implications.

Our discovery of IBD-anti-IL-10 in both children¹² and young adults adds to emerging understanding of humoral autoimmunity as a pathogenic mechanism. Anti-IL-10 is associated with an acquired phenocopy of inborn errors of the IL-10 pathway, and is modelled in mice receiving IL-10-neutralizing antibodies.¹⁸ Our *in-vitro* data show blocking IL-10 amplifies pro-inflammatory responses via IBD-relevant cytokines, just as occurs in inborn errors of the IL-10 pathway. While epithelial, phagocytic, T cell and cytokine defects have been widely studied in the pathogenesis of IBD, the contribution of B cell responses and humoral immunity has been less clear.¹⁹ Single cell sequencing has highlighted the involvement of B and plasma cell responses in UC.²⁰⁻²² Autoantibodies have previously been described against a spectrum of autoantigens and microbial antigens in patients with IBD²³⁻²⁴ as disease-associated biomarkers, but anti-IL-10 appears likely to play a pathogenic and disease-modifying role in IBD.

The identification of anti-IL-10 autoantibodies in 173 of 4909 IBD patients indicates IL-10 neutralization by autoantibodies as a common and under-appreciated disease mechanism. In contrast, the proportion of IBD patients reported globally with monogenic Mendelian biallelic loss of function defects of *IL10* (5 patients), *IL10RB* (27 patients) and *IL10RA* (175 patients) in the last 15 years is magnitudes smaller.²⁵ Monogenic IL-10 signalling defects tend to present during infancy with colonic and penetrating disease, poor response to IBD therapies, high inflammatory activity with notably elevated CRP levels and a high incidence of postoperative complications.²⁶ It will be important to establish the extent to which anti-IL-10 seropositivity associates with a similar disease pattern. Notably, we identified a strong association between anti-IL-10 autoantibody formation and HLA-

DRB1*01:03. This rare HLA haplotype has been associated previously with IBD, especially with severe or extensive disease (pancolitis) and the need for colectomy^{2,3,5}, and with extraintestinal manifestation²⁷.

The diagnosis of IBD-anti-IL-10 may have implications for clinical decision-making. Our data highlight the need for research into therapeutic maneuvers to reduce anti-IL-10 titers, for example via B cell and plasma cell depletion (e.g. anti-CD19, anti-CD20, anti-CD38, or CD19 CART cell therapy)²⁸⁻³⁰, plasma exchange, or blockade of the neonatal Fc receptor.³¹ The development of human chimeric autoantibody receptor T cells has been reported to facilitate the depletion of anti-cytokine-specific B cells in pre-clinical models, offering promise for future targeted therapies with potentially lower side effect profiles.³²

Our findings are in line with an emerging conceptual disease model of unified pathogenic mechanisms across rare inborn errors of immunity, acquired autoimmunity and immune-mediated diseases such as IBD.^{15,33,34} Loss of tolerance towards IL-10 might be driven by environmental factors including gastrointestinal infections or IBD flares or by infections that trigger autoimmunity or molecular mimicry due to pathogen encoded IL-10 homologs.³⁵ Recognizing that neutralizing IL-10 autoantibodies are present in a subgroup of patients with IBD and are strongly associated with HLA-DRB1*01:03 may open up new diagnostic, prognostic and therapeutic opportunities.

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Conflicts of interest

No conflict of interest related to this project: **PY, LC, HG, CJ, AF, AYC, AW, OB, KB, HK, YL, MGB, KV, MC, LK, MB, SD, BK, AA, DF, LF, RD, MVV, JS, FP, AS, RD, AS, BN, CD, RDL, SF, AR, JP.**

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Figure legends

Figure 1. Study flow diagram

Study design illustrating IBD and control cohorts, serotyping procedures, and genotyping.

Figure 2. Neutralizing anti-IL-10 autoantibody is associated with IBD

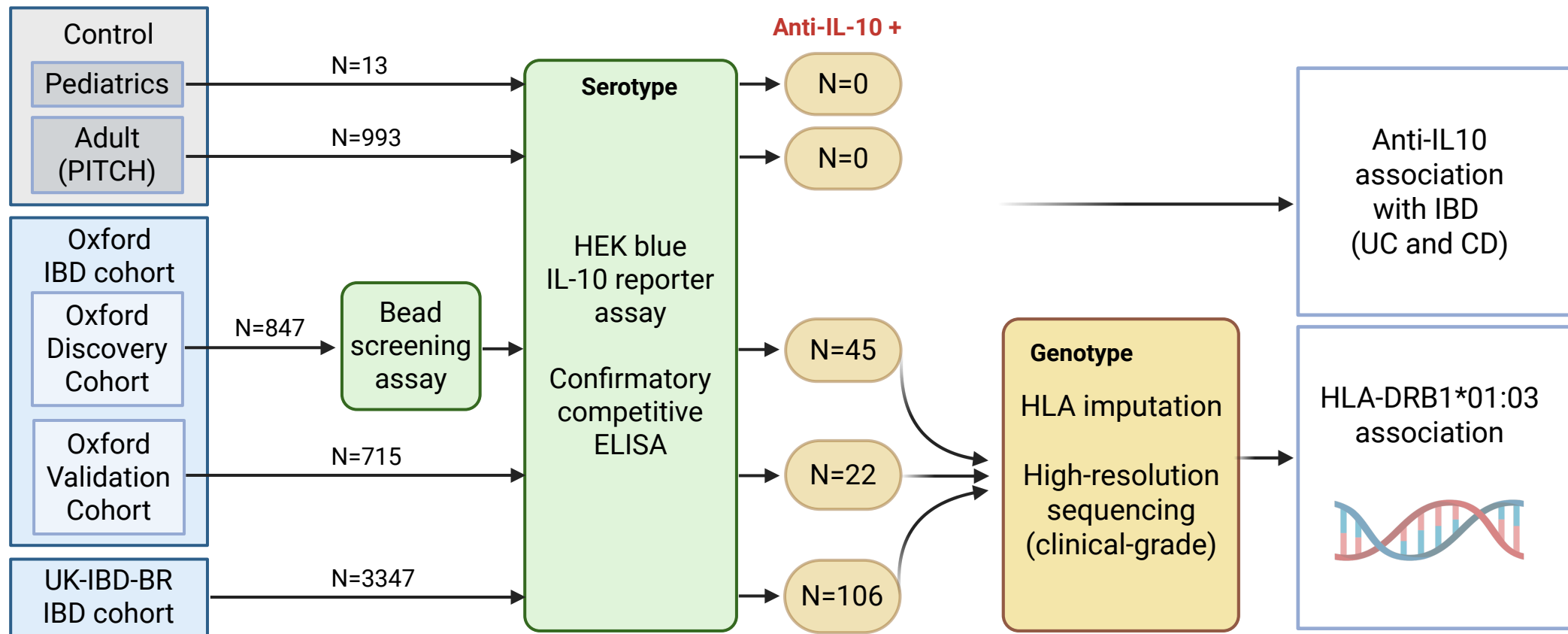
A. Neutralization of IL-10 signaling measured by an IL-10 reporter assay (HEK-Blue). Serum was incubated with exogenous rhIL-10 (50 ng/mL), and reporter activity (secreted alkaline phosphatase) is shown as percent of the IL-10 standard. The horizontal dashed line indicates the neutralization threshold. Neutralization-positive sera were more frequent in IBD (total n=4909) than in controls (total n=1006) (Mann–Whitney U test, * $p < 0.05$).

B. Rates of anti-IL-10 positivity as defined by neutralization in the IL-10 reporter assay (HEK-Blue) and neutralization in the competitive ELISA in controls and IBD (Chi-square test, **** $p < 0.0001$).

C. PBMC cytokine-release assay. Cytokine concentrations ($\log^{10}$) are shown for IBD patients with anti-IL-10–negative versus anti-IL-10 positive groups (Multiple Mann–Whitney U test using the Benjamini–Hochberg false discovery rate adjustment, ** $p < 0.01$, *** $p < 0.001$). **D.** Follow up levels of neutralization of IL-10 signaling indicated by HEK Blue IL-10 reporter assay over time (10 IBD patients with positive anti-IL-10 and 7 IBD patients with negative anti-IL-10).

Figure 3. Association of anti-IL-10 and HLA-DRB1*01:03

A. HLA-wide association analysis of anti-IL-10 positivity on 404 genotyped patients with of the Oxford IBD cohort. Genomic positions of chromosome 6 (genome build 37), and $-\log_{10}$ P-value obtained from two-sided regression analyses for SNPs (grey) and classical HLA alleles. Logistic regression model testing association with biallelic variant alleles (counts of presence versus absence), including sex and age as fixed effect covariates. **B.** Odds Ratio (OR) for HLA-DRB1*01:03 carriage and anti-IL-10 status on the Oxford IBD cohort and the UK-IBD-BR cohort. **C.** Carrier percentage in European descent IBD and non-IBD patients in the English population where non-disease frequencies were derived from allelefrequencies.net. **D.** Circos plots and sequence logos illustrating HLA-DRB1 allele pairing and amino acid composition in 234 Oxford patients stratified by anti-IL-10 autoantibody status. The sequence logos depict the relative frequency of amino acids at positions 60 to 80 of the HLA-DRB1 molecule within each group. The color scheme for the Circos plots corresponds to the amino acid at position 71.



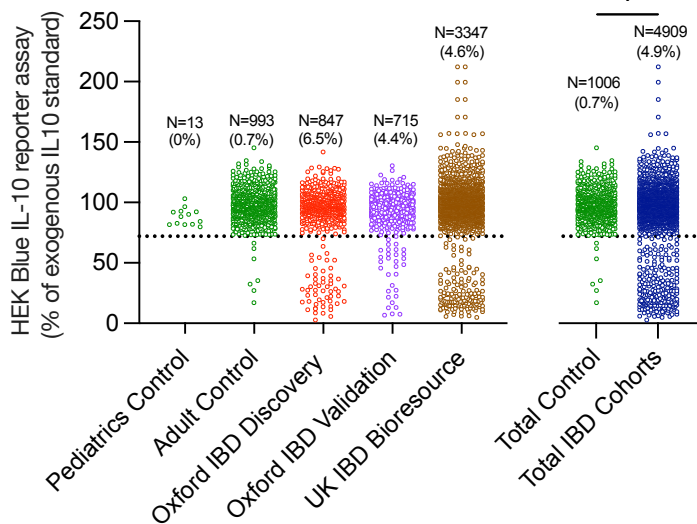
Different Cohorts

Meta Analysis

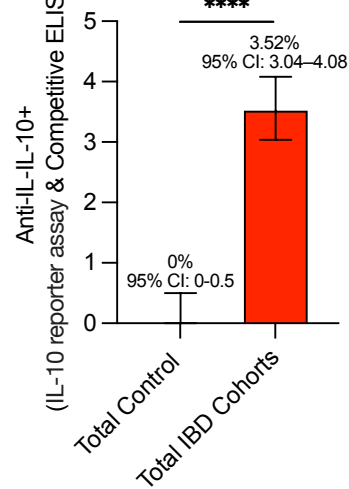
IBD Subtypes

Anti-IL-10+

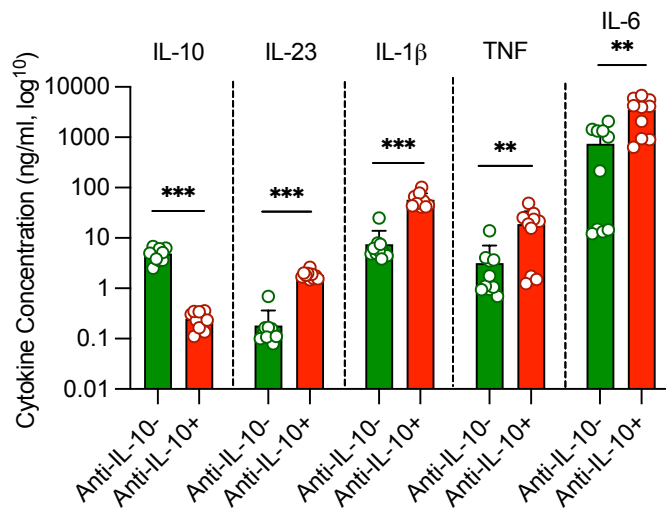
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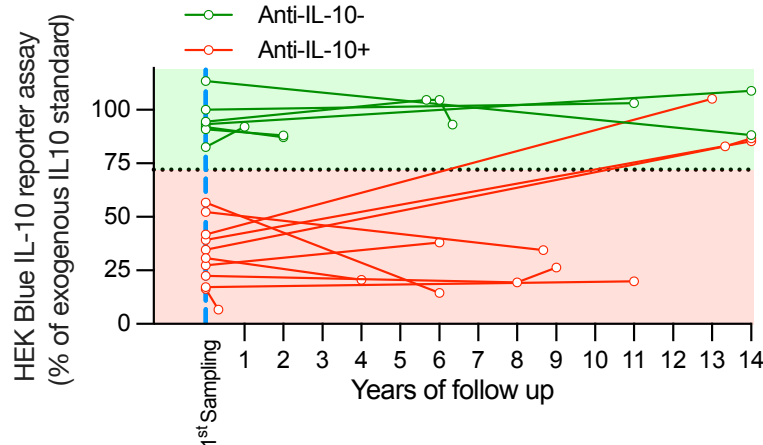
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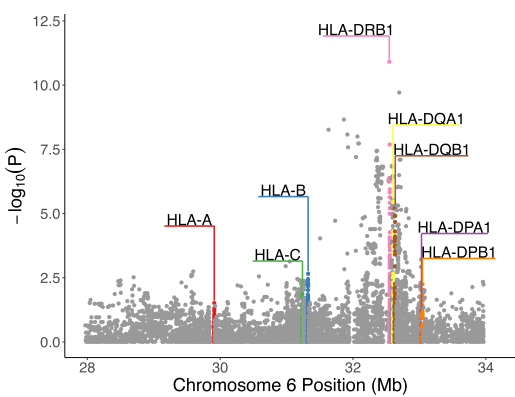
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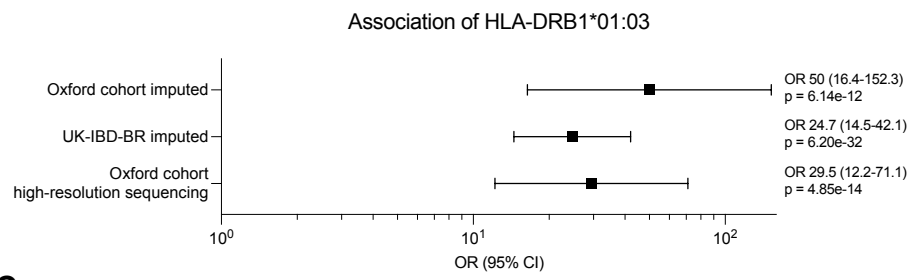
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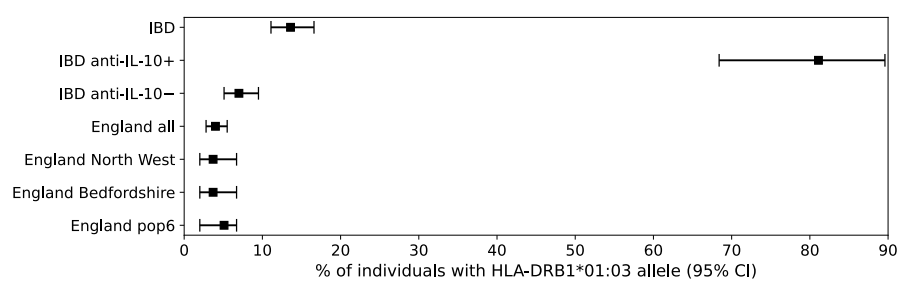
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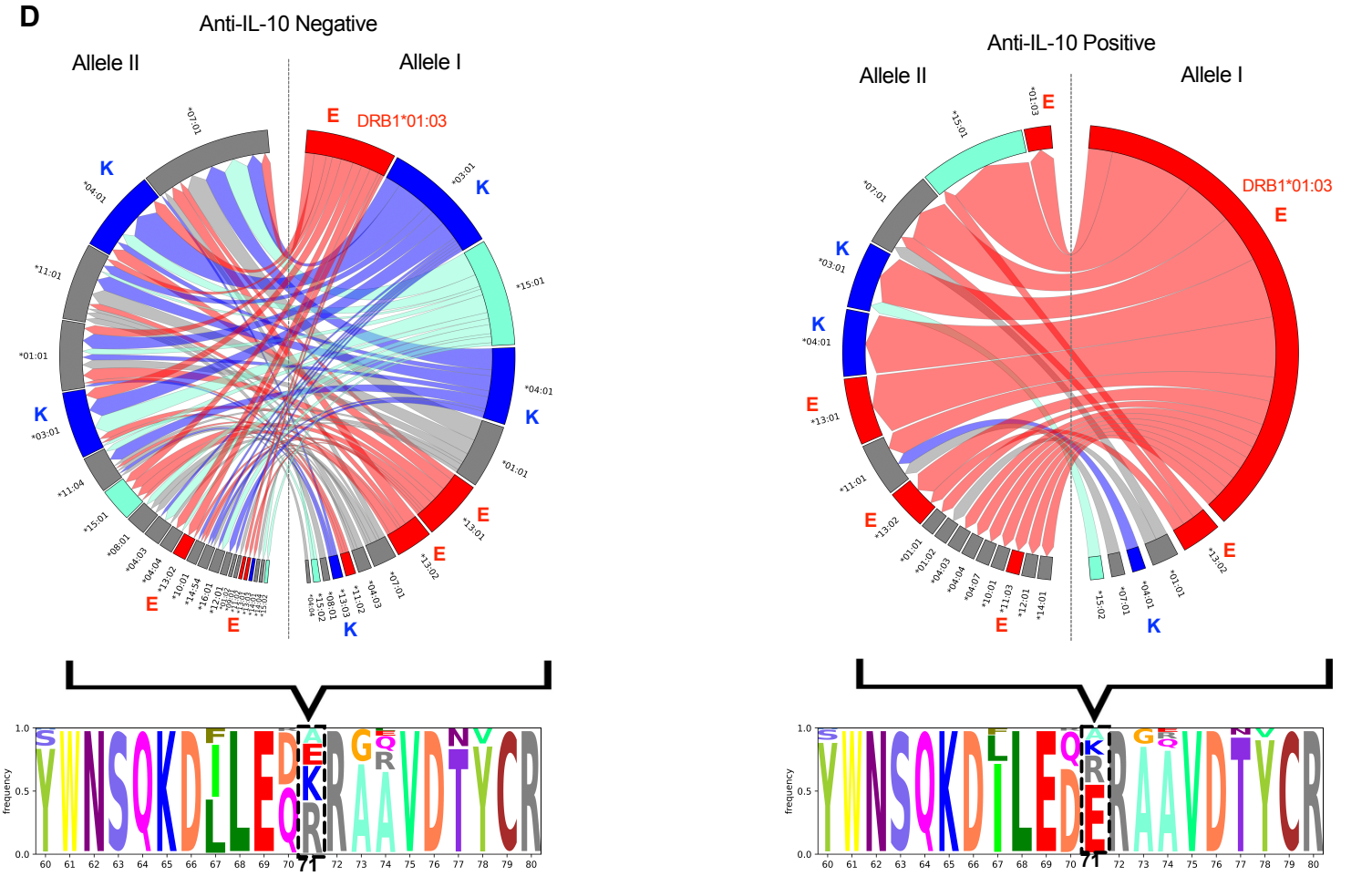
B



C



D



Supplementary Appendix

Supplement to: IL-10 Autoantibodies and HLA-DRB1*01:03 in Inflammatory Bowel Disease

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1. Extended material and methods

Anti-IL-10 autoantibody bead assay

As described previously¹, recombinant human IL-10 (rhIL-10; ImmunoTools, #11340107), were covalently coupled to magnetic beads (Luminex, Netherlands). Beads were first activated with 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (Pierce / Thermo Fisher Scientific, #11851335) in the presence of N-hydroxysuccinimide (Pierce / Thermo Fisher Scientific, #10391314), according to the manufacturer's instructions, to form amine-reactive intermediates. The activated beads were incubated with rhIL-10 (20 µg/mL) for 3 hours at room temperature on a rotator. Beads were washed and stored in blocking buffer (10 mM PBS, 1% BSA, 0.05% NaN₃). Successful coupling of the cytokine to its bead set was verified with specific mAb (R&D Systems, #BAF217).

IL-10-coupled beads were incubated with patient sera for 1 h in 96-well F plates (Greiner Bio One #655096.) at room temperature in the dark on an orbital shaker. The plate was placed on a magnet and buffers were aspirated. Beads were washed three times with 10 mM PBS/0.05% Tween 20. Beads were then incubated for 30 min with a PE-labeled anti-human IgG-Fc antibody (Leinco/Biotrend; #I-127), washed and resuspended in 100 µl PBS/Tween 20. Sera were analyzed using the Luminex assay.

Competitive ELISA assay

A competitive ELISA assay was developed to assess the neutralizing activity of serum-derived autoantibodies against rhIL-10, using the colorimetric ELISA MAX™ IL-10 kit (BioLegend, #430601) as the assay platform. This method quantifies the degree of epitope masking by measuring residual unbound IL-10 that remains available to bind the capture antibody. 96-well flat-bottom plates were coated overnight at 4°C with the IL-10 capture antibody provided in the kit, diluted in the recommended coating buffer. Plates were then washed three times with PBS containing 0.05% Tween-20 and blocked with assay diluent for 1 hour at room temperature to reduce nonspecific binding. rhIL-10 (125 pg/mL; PeproTech, #200-10) was pre-incubated with 10% patient serum for 1 hour at 37°C to allow potential neutralizing autoantibodies to bind and mask IL-10 epitopes. After incubation, 100 µL of the IL-10/serum mixture was added to each well and incubated for 2 hours at room temperature. Plates were washed and incubated with the detection antibody for 1 hour, followed

by incubation with avidin-HRP conjugate for 30 minutes. The signal was developed using tetramethylbenzidine (TMB) substrate solution and stopped with the stop solution provided in the kit. Optical density was measured at 450 nm using a microplate reader. Assay validation included titration of rhIL-10 and the use of IL-10–specific monoclonal blocking antibodies (Biolegend, #506802, clone: JES3-19F1) as positive controls for neutralization. Reduced signal indicated effective neutralization of IL-10 by serum autoantibodies.

Recombinant human IL-10 STAT3 reporter assay

The HEK-blue™ IL-10 cell line (InvivoGen #hkb-IL-10) responds to human IL-10 by secreting alkaline phosphatase which can be measured colorimetrically at OD 620. This cell line is designed to minimize the response to different types of cytokines, in particular IL-1 β , IL-2, IL-6, IL-12, IL-22, IL-23, IFN- α , IFN- β , IFN- γ and TNF- α . Cells were cultured in growth medium, prepared with Dulbecco Modified Eagle Medium (DMEM, Gibco®, #10313021) supplemented with 10% fetal bovine serum (FBS, Sigma #F2442), 2mM L-Glutamine (Gibco® #25030081), 100 units/mL penicillin, 100 μ g/mL streptomycin (Gibco® #15140122) and 100 μ g/mL of Normocin™ (InvivoGen #ant-nr-1) for two passages. After the second passage the growth medium was replaced with selection medium plus HEK-blue™ selection antibiotics (InvivoGen #hb-sel). Following 48 hours in selection medium, cells were harvested and washed once with growth medium without Normocin™. The cells were re-suspended in growth medium without Normocin™, counted, and their viability assessed. Using a 96 well, flat bottom, sterile plate, harvested cells were stimulated with 50 ng/mL rhIL-10 (PeproTech, #200-10) alone or in the presence of 20% patient sera. After 24 hours of incubation, 20 μ L of each supernatant were added to 180 μ L of QUANTI_Blue™ solution (InvivoGen #rep-qbs). The reactions were incubated for 60 minutes at 37°C. Following the incubation, the optical density at 620 nm was measured to determine SEAP (secreted embryonic alkaline phosphatase) levels. Reduced reporter signal reflected effective neutralization of rhIL-10 by serum autoantibodies, resulting in diminished activation of intracellular STAT3 signaling. The experimental validation included

titration of rhIL-10 stimulation and the use of IL-10 blocking monoclonal antibodies (Biotechne, #MAB217-100, clone IgG2B 23738 and Biolegend, #506802, clone: JES3-19F1).

A second STAT3 dual luciferase reporter assay was based on IL10RA non-expressing HEK293 cells that were transfected with IL10RA and STAT3 dual luciferase reporter plasmids. Cells were cultured at a density of 30×10^3 cells per well in 96-well plates using DMEM (Sigma-Aldrich, #D6429) supplemented with 10% FBS (Sigma-Aldrich, #F9665), 1% penicillin (100 IU/mL), and streptomycin (100 µg/mL) (Sigma-Aldrich, #P0781). Cells were co-transfected with a vector encoding wild-type *IL10RA* (100 ng plasmid) and a STAT3 reporter (10 ng plasmid, CCS-9028L, Cignal Reporter Assay Kit, #336841, QIAGEN) using Lipofectamine™ 3000 Transfection Reagent (#L3000001) in OPTI-MEM (reduced serum medium, Gibco, #31985-062). After 24-hours, transfected cells were exposed to rhIL-10 (Peprotech, #200-10) 200 ng/mL, in the presence or absence of control sera (1:10) or sera of patients (1:10). Luciferase activity was assessed using the Dual-Luciferase® Reporter Assay System (Promega, E1960) using a luminescence reader (FLUOstar OPTIMA, BMG LABTECH) as per the manufacturer's instructions.

Neutralization assay of endogenous human IL-10 in peripheral blood cells (cytokine release assay)

To assess the effect of anti-IL-10 on endogenous IL-10 produced by human immune cells, a cytokine release assay in peripheral blood cells was used. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy control donors using Ficoll and standard density gradient centrifugation. The cells were washed with PBS and resuspended in RPMI-1640 medium (RPMI Gibco supplemented with 10% fetal bovine serum (Sigma-Aldrich, #F9665) and 1% penicillin (100 IU/mL) and streptomycin (100 µg/mL; Sigma-Aldrich, #P0781)). The cultured cells were incubated with 300 ng/ml lipopolysaccharide (LPS, Merck Life Science, L2630-25MG) and 20% serum obtained from patients with either high and low anti-hIL10 antibody levels. Control samples were left unstimulated. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. After 6 and 18 hours of incubation, supernatants were collected from each well and analyzed using the bead-based multiplex assay (Biolegend, 740809) coupled with flow cytometry (LSRII, BD

Biosciences). As positive controls for blocking IL10 signaling, we used two monoclonal anti-IL-10 antibodies (Biotechne, #MAB217-100, clone IgG2B 23738 and Biolegend, #506802, clone: JES3-19F1).

Anti-cytokine autoantibody screening

Serum autoantibodies against a broad panel of cytokines were measured using two complementary platforms. First, a chemiluminescent immunoassay (Randox Laboratories) was used according to the manufacturer's instructions to detect immunoglobulin G (IgG) binding to immobilized cytokine antigens. Cytokines include G-CSF, GM-CSF, IFN-alpha2a, IFN-gamma, IFN-omega, IL-1alpha, IL-1 beta, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12p40, IL-12p35, IL-17A, IL-17F, IL-18, IL-22, TNF. Signals were reported as relative light units.

A bead-based multiplex assay (Luminex) was performed to assess cytokine-specific IgG binding in parallel¹. External positive-control sera were included where available to confirm assay performance. Positive-control samples from patients with IPEX² and PAP³ have been described previously. Patients with APS-1 were recruited through the Troubled Immune System study (REC reference 20/NE/0044).

Definition of positivity in the HEK-blue™ IL-10 reporter assay

We apply pre-specified thresholds from an independent adult non-IBD control cohort and apply them unchanged to all IBD cases, enabling unbiased estimates of positivity. We fixed a single threshold at 72% of exogenous IL-10 detection.

Neutralizing activity against IL-10 was quantified using a HEK-Blue IL-10 reporter assay, in which IL-10-induced reporter activity is expressed as the percentage of IL-10 detection relative to control. In this assay, lower percentages indicate stronger IL-10 neutralization. For assay calibration, we analyzed 993 control samples from individuals without known autoimmune or inflammatory disease. Because the distribution of percentage IL-10 detection in controls was non-normal, we used a nonparametric, percentile-based approach to define the lower reference limit, in accordance with Clinical and Laboratory Standards Institute (CLSI) recommendations for reference-interval

estimation.⁴ The prespecified cut-off was set at 72% IL-10 detection, corresponding to the 0.7th percentile of the control distribution (7 of 993 controls below this value), thereby providing a stringent, high-specificity threshold.

As a robustness check, we also estimated dispersion around the median using a robust standard deviation derived from the median absolute deviation.⁵ Among controls, the median percentage IL-10 detection was 96.6%, and the robust standard deviation was 10.52%, such that the selected cut-off of 72% corresponds to approximately median – 2.3×(robust standard deviation). On the basis of this threshold, serum samples with percentage IL-10 detection of 72% or less were classified as anti-IL-10 neutralizing antibody positive, and those with values above 72% were classified as negative.

HLA haplotyping

HLA haplotyping was performed on immunochip genotyping. Based on genotype data from UKIBDGC (N =22,588 with EUR ancestry), HIBAG⁶ with pre-fitted model was used to impute the alleles of HLA genes including *A*, *B*, *C*, *DRB1*, *DQA1*, *DQB1*, *DPB1*. The pre-fitted model developed for HumanCoreExome-12v1.0 array with samples from EUR ancestry was downloaded from https://hibag.s3.amazonaws.com/hlares_index.html. The alleles were imputed at both 2- and 4-digit resolutions. Among the 67 patients with positive anti-IL-10 in the Oxford cohort, 21 were genotyped in the 22,588 patients. Those 21 patients were treated as cases and the others as controls, performing logistic regression to check whether the imputed HLA alleles showed allele frequency differences between cases and controls.

HLA high-resolution long-range sequencing

High-resolution genotyping of all HLA loci, including class I and class II genes (and their respective alpha chains), was performed using next-generation sequencing (NGS). Clinical-grade HLA typing was conducted with the AllType FASTplex™ NGS kit (Thermo Fisher Scientific) in a laboratory accredited by ISO 15189:2022, the United Kingdom Accreditation Service (UKAS), and the European Federation for Immunogenetics (EFI).

HLA allele and amino acid analysis

Genotyping and quality control of the 404 patients (from Oxford discovery and validation cohorts) with IBD and European ancestry was previously described⁷. For this study, we extracted genotyped variants within the extended MHC region (Chr6: 28–34 Mb) and performed HLA imputation using the multi-ancestry HLA reference panel (v2) hosted on the Michigan Imputation Server⁸.

HLA imputation yielded 570 classical HLA alleles, 3,449 HLA amino acid polymorphisms, and 18,742 SNPs. To assess association, we conducted univariate logistic regression, adjusting for age and sex, under an additive genetic model. For the conditional analysis, we included the genotype of the previously defined top signal (HLA-DRB1*01:03) as a covariate in the regression model.

High-resolution HLA genotyping of 234 samples (from both discovery and validation Oxford cohorts) yielded 26 unique HLA-DRB1 alleles after quality control removing samples with unresolved alleles at two-field resolution. Unresolved alleles were treated as follows: if possible, alleles separated by “/”, we take the first allele listed; if a range of alleles is denoted using “-”, and the possibilities result in unambiguous amino acid in position 71, we take the first allele in the range, otherwise we exclude the sample from analysis.

For each amino acid position, we tested a multi-allelic association between the anti-IL-10 status (binary) and a haplotype matrix (of the position) with covariates, including age and sex. That is

$$y = \beta_0 + \sum_i \beta_{1i} x_i + age + sex$$

where x_i is the amino acid haplotype formed by each of the m amino acid residues that occur at that position. We then estimated the effect of each amino acid by assessing the significance of the improvement in fit by calculating the in-model fit, compared to a null model following an F-distribution with $m - 1$ degrees of freedoms. This is implemented using an ANOVA test in R. The most frequent haplotype was excluded from a haplotype matrix as a reference haplotype for association.

For each possible amino acid in position 71 (A, E, K and R), we tested association of amino acid dosage with anti-IL-10 status using a logistic regression model with sex and age of diagnosis as

covariates, implemented using statsmodels in Python^{9,10}. For conditional analysis, we applied a similar logistic regression approach for HLA-DRB1 allele dosage, with sex, age of diagnosis and HLA-DRB1*01:03 as covariates. Visualization of clinical grade HLA genotyping results include plots made using pycirclize¹¹, logomaker¹², and matplotlib¹³ in Python. Chord diagram displays arrows from allele 1 to allele 2. We therefore designate allele 1 and allele 2 as follows: if one allele has E in position 71 such as in HLA-DRB1*01:03, that allele is allele 1 and the other is allele 2; otherwise, allele 1 is the allele with position 71 amino acid less frequent in anti-IL-10-negative patients. Where position 71 amino acids are the same in both alleles from an individual, the allele with lower numbering is called allele 1.

English cohorts used in analysis of percentage of persons with HLA-DRB1*01:03 allele

For population studies we report three England population cohorts: Northwest, Bedfordshire, and England Pop6); Northwest (a region in North West England, covering five counties; sample size n=298); Bedfordshire (a county at East England; sample size n=354); England Pop6 (derived from a British Caucasian population study, pop6; sample size n=177). The data are derived from <https://allelefrequencies.net>. The “England, All” population is a sum of the three individual cohorts (n=829) (**Figure 3C**).

2. Patient cohorts and ethics approval

Oxford IBD cohort

For the Oxford IBD cohort, patients were recruited through the Gastrointestinal Illnesses Biobank - Oxford IBD cohort study (REC 21/YH/0206, amended REC 21/YH/0206). The cross-sectional Oxford IBD cohort (n=1562) was comprised of two subgroups (Oxford discovery cohort n=847 and Oxford validation cohort n=715) and cover a broad range of age groups but were recruited at different time periods (**Table S2**).

Adult and pediatric non-IBD control cohorts (PITCH and Oxford GI biobank).

A total of 1006 individuals without IBD were analyzed as the control group. The adult non-IBD control cohort from the PITCH study consisted of 993 individuals, including 271 males. The

adult control was healthcare workers in UK recruited as part of the PITCH (Protective Immunity from T cells in Healthcare workers) study under the Gastrointestinal Illness Biobank study (REC 16/YH/0247, amended REC 21/YH/0206), and the PITCH 2 study (REC 23/NW/0280). Blood samples were collected between 2020 and 2024. The mean age at sampling was 40.7 ± 12.8 years (range: 19–83). The majority of participants were of white ethnicity ($n = 749$, 75.4%, inclusive of white British and white Irish). Reported comorbidities included asthma ($n = 85$), hypertension ($n = 53$), thyroid disease ($n = 27$), diabetes mellitus ($n = 22$), malignancy ($n = 16$), cardiovascular disease ($n = 8$), and others (multiple sclerosis in 2, chronic obstructive lung disease in 1, stroke in 1, and chronic renal disease in 1). HLA analysis was available in 139 individuals, and two of them carried the HLA-DRB1*01:03 allele.

Pediatric controls (non-IBD-non-celiac, $n=13$) were also included via the Oxford subset of the AbCD (Antibody Diagnostics in Paediatric Coeliac Disease) study (**Table S2, Figure 1**).

UK Inflammatory Bowel Disease BioResource cohort

UK-IBD-BR (Inflammatory Bowel Disease BioResource) is a national research cohort (subset of the UK IBD national cohort) established to support translational studies in inflammatory bowel disease in the UK. It recruits patients with confirmed Crohn's disease, ulcerative colitis, or IBD-unclassified from more than 100 NHS sites across the country. Participants contribute detailed clinical and demographic information, provide blood samples for DNA extraction and biomarker analyses, and consent to be contacted for future recall studies. The cohort is broadly representative of UK IBD demographics, with a predominance of White British and Northern European ancestry but also including minority ethnic groups reflecting the contemporary UK population. Patients are eligible for inclusion upon confirmed diagnosis by an IBD specialist, while individuals unable to provide informed consent or lacking adequate clinical documentation are excluded. All data and biological samples are collected according to standardized operating procedures, and longitudinal clinical updates continue to be incorporated into the database. The genomic data for the IBDBR cohort was

generated at the Wellcome Sanger Institute using standardized pipelines for genome-wide genotyping and quality control. HLA alleles were imputed using validated reference panels optimized for European populations, with stringent filtering to ensure high confidence calls for downstream analyses. The IBDBR operates under ethical approval from the Cambridge East Research Ethics Committee (REC number 15/EE/0283) (n=3551 adult patients with IBD), and all participants provide written informed consent in accordance with the Declaration of Helsinki.

3. Figures

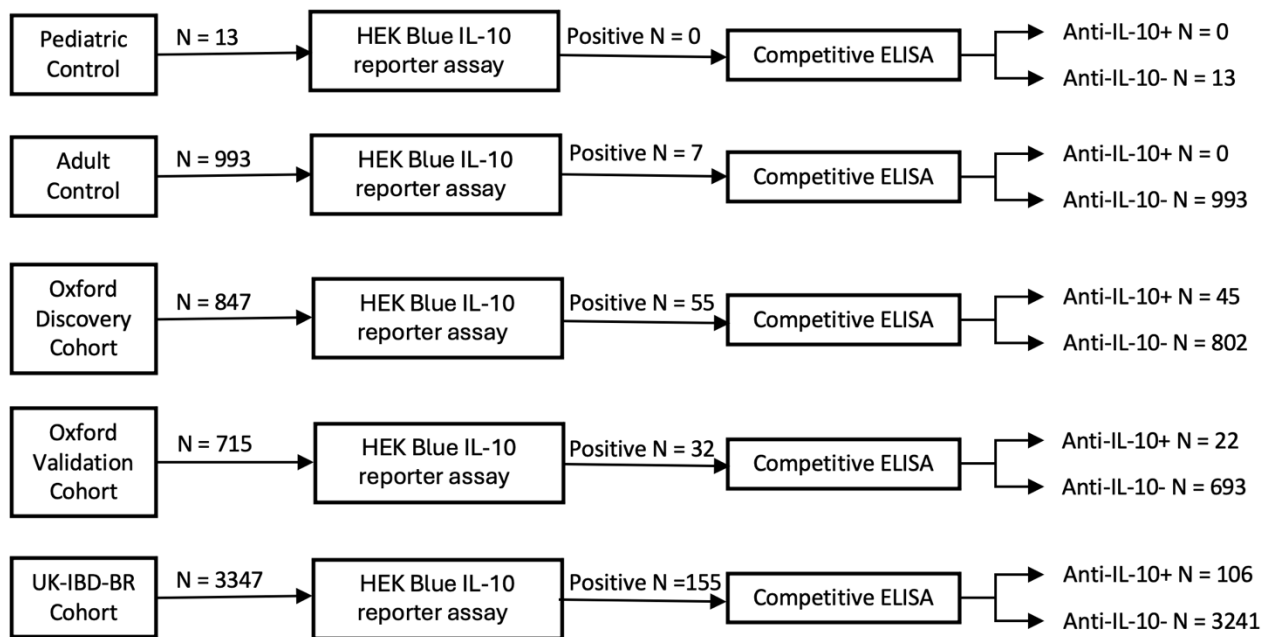


Figure S1. Study diagram of serotyping workflow.

The flow of patient and control cohorts through serologic testing to detect anti-IL-10 autoantibodies, including HEK-Blue IL-10 reporter assay and competitive ELISA.

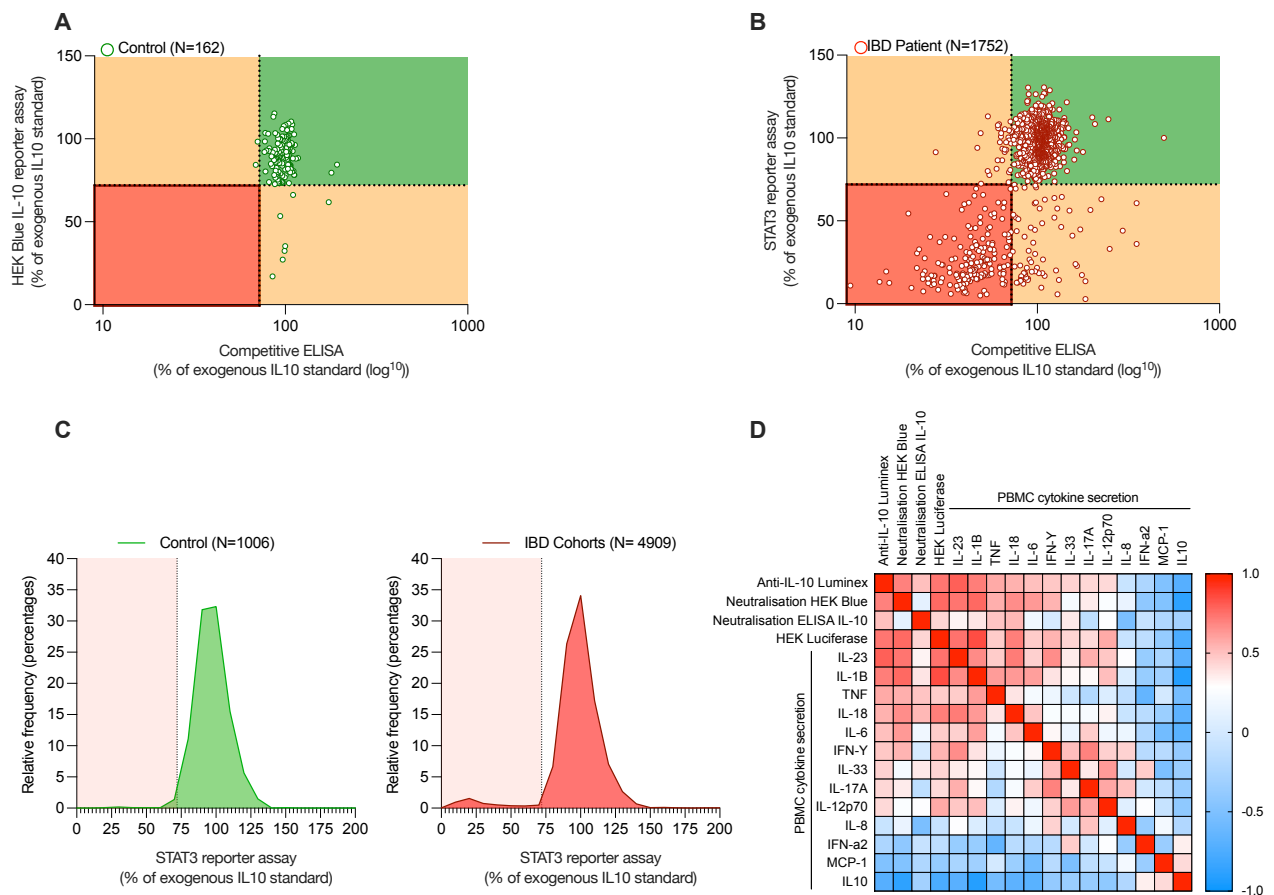
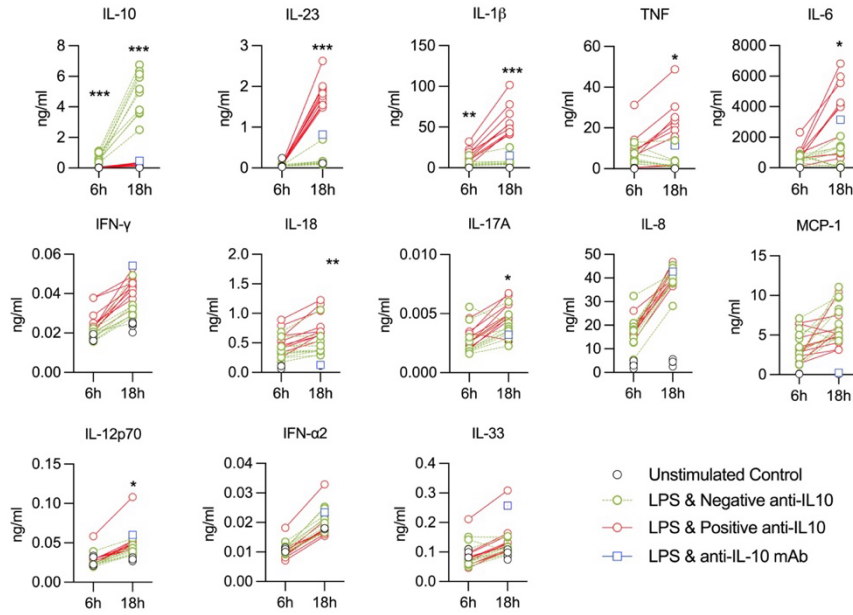
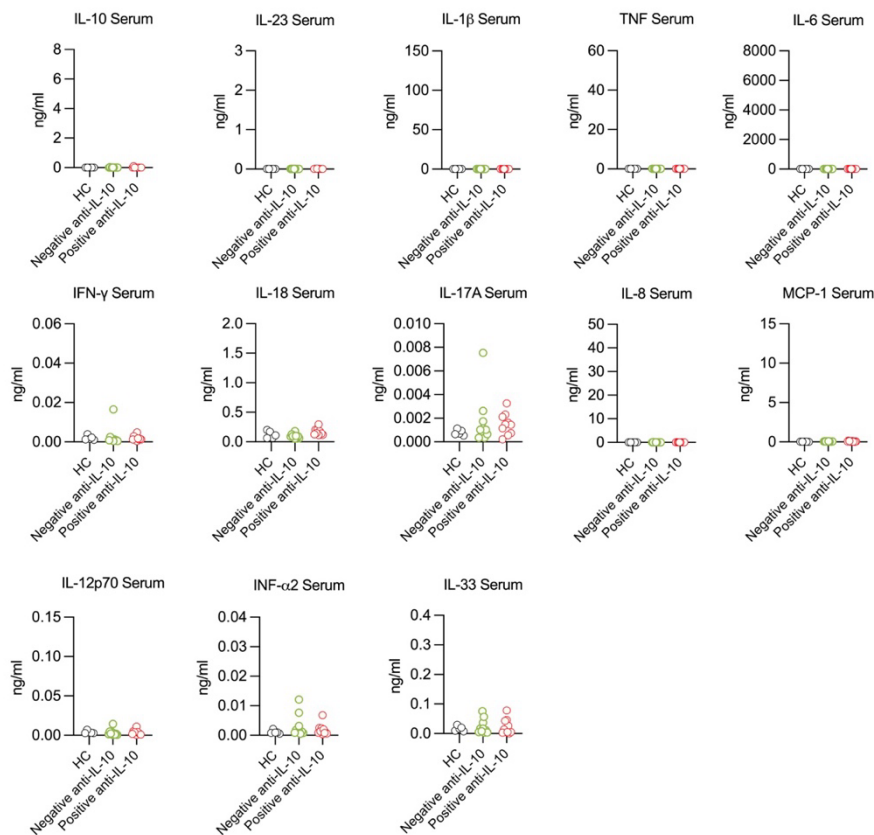


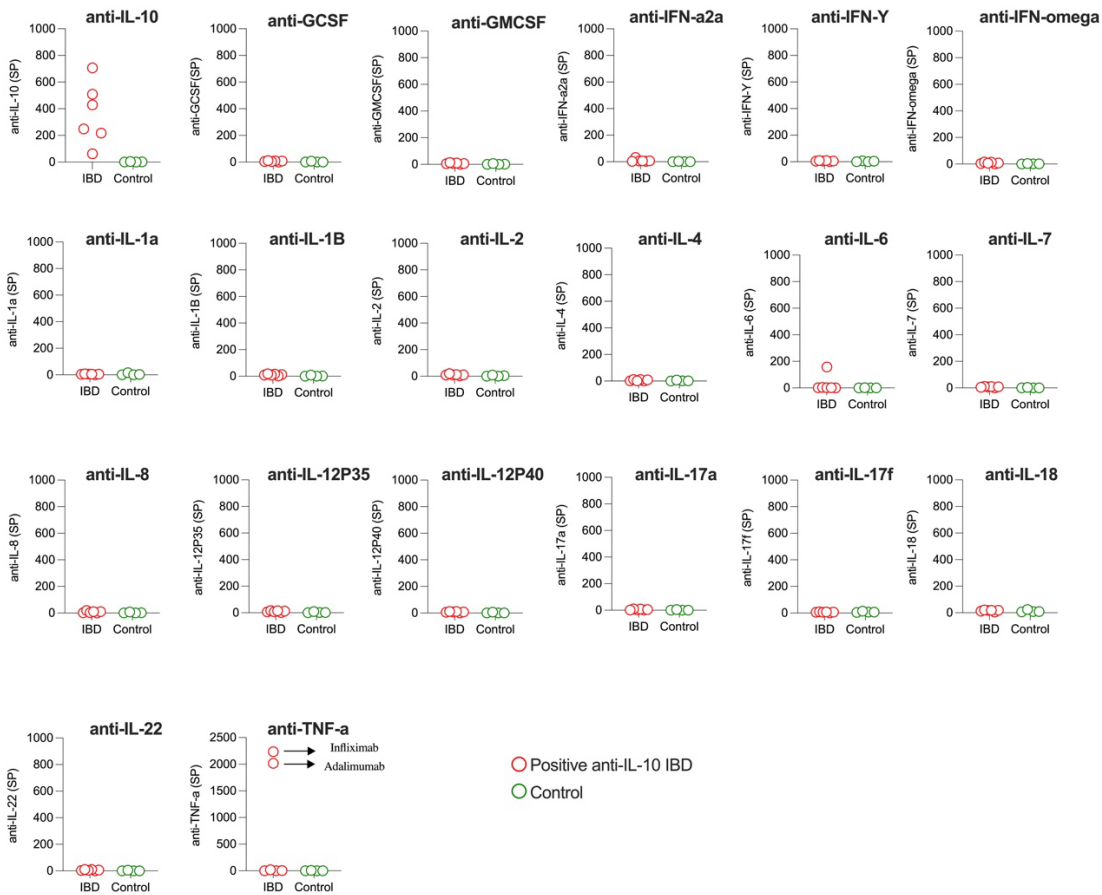
Figure S2. Comparison across assays.

A-B. Relationship between IL-10 reporter assay (HEK-Blue) and competitive ELISA as a marker of anti-IL-10 activity in controls (N=162) and patients with IBD (N=1752). **C.** Distribution of HEK-Blue IL-10 reporter activity in control and IBD cohorts; the vertical dashed line indicates the prespecified neutralization threshold. **D.** Correlation matrix summarizing concordance across assay platforms, including bead-based IL-10-binding IgG (anti-IL-10), competitive ELISA, cellular IL-10 reporter assay neutralization assay HEK-Blue neutralization/HEK luciferase readouts, and PBMC cytokine secretion.

A Functional impact of anti-IL-10 autoantibodies on LPS-induced cytokine secretion**B Baseline serum cytokine profiles****Figure S3. Cytokine secretion assay to assess neutralizing autoantibodies against IL-10.**

A. Impact of serum samples from IBD patients with positive or negative anti-IL-10 antibodies on cytokine secretion from healthy control PBMCs following LPS stimulation. Levels of cytokines either unstimulated or after LPS stimulation in the presence of positive or negative anti-IL-10 serum or anti-IL-10 mAb. **B.** Baseline cytokine levels of cytokines measured in serum from healthy controls (HC), and IBD patients with positive and negative anti-IL-10 antibody levels. Mann-Whitney U test between negative and positive ant-IL-10 groups, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

A Autoantibody Assay (Randox Chemiluminescence)



B Autoantibody Assay (Luminex Fluorescence)

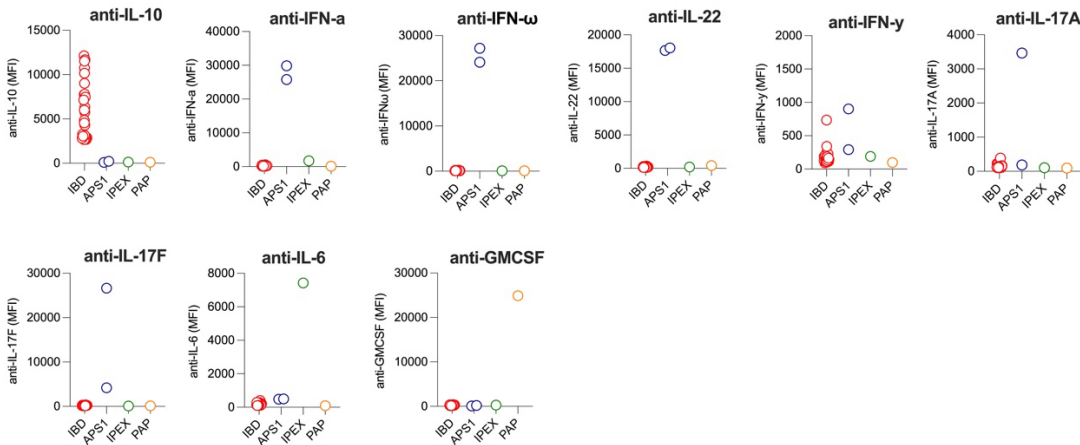


Figure S4. Anti-cytokine autoantibody screening in IBD and controls.

A. Anti-cytokine autoantibody profiling using a chemiluminescent immunoassay. Serum reactivity against a panel of cytokines is shown for IBD patients with anti-IL-10 positive (red, n=6) and non-IBD anti-IL-10 negative (green, controls, n=4). **B.** Anti-cytokine autoantibody profiling using a bead-based multiplex fluorescent assay (Luminex). Reactivity against selected cytokines is shown for anti-IL-10 positive IBD and anti-IL-10 negative control samples. External positive-control sera are included for assay validation; Autoimmune Polyendocrine Syndrome Type 1 (APS1), Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX); Pulmonary Alveolar Proteinosis (PAP).

	<u>Anti-IL-10 screening</u>	<u>MHC imputation</u>	<u>High-resolution sequencing</u>	<u>Combined MHC imputation & High-resolution sequencing</u>
Oxford discovery cohort 847 IBD patients	Anti-IL-10 + 45 Anti-IL-10 - 802	HLADRB1*01:03 + 16 HLADRB1*01:03 - 5 HLADRB1*01:03 + 23 HLADRB1*01:03 - 324	HLADRB1*01:03 + 24 HLADRB1*01:03 - 6 HLADRB1*01:03 + 18 HLADRB1*01:03 - 79	HLADRB1*01:03 + 27 HLADRB1*01:03 - 8 HLADRB1*01:03 + 27 HLADRB1*01:03 - 363
Oxford validation cohort 715 IBD patients	Anti-IL-10 + 22 Anti-IL-10 - 693	HLADRB1*01:03 + 3 HLADRB1*01:03 - 33	HLADRB1*01:03 + 16 HLADRB1*01:03 - 2 HLADRB1*01:03 + 9 HLADRB1*01:03 - 108	HLADRB1*01:03 + 16 HLADRB1*01:03 - 2 HLADRB1*01:03 + 11 HLADRB1*01:03 - 140
UK-IBD-BR cohort 3347 IBD patients	Anti-IL-10 + 106 Anti-IL-10 - 3241	HLADRB1*01:03 + 32 HLADRB1*01:03 - 35 HLADRB1*01:03 + 81 HLADRB1*01:03 - 2217		HLADRB1*01:03 + 32 HLADRB1*01:03 - 35 HLADRB1*01:03 + 81 HLADRB1*01:03 - 2217

Figure S5. Schematic of genetic analysis in three IBD patient cohorts to investigate the association between anti-IL-10 autoantibody positivity and HLA-DRB1*01:03 carriage. HLA-DRB1*01:03* was markedly more frequent in anti-IL-10-positive patients compared with anti-IL-10-negative patients. Blue column indicated HLA haplotypes inferred by imputation methods, whereas orange column indicated haplotypes determined by high-resolution sequencing. In the Oxford discovery cohort and Oxford validation cohort, immuno-chip-based HLA imputation was performed in a subset of patients, while high-resolution sequencing data were additionally available for a subset of patients in the Oxford cohorts; for analyses of carrier rate and allele frequency, genotyped individuals from both methods were combined, and duplicate samples were removed then summarized in the combined column. In the UK-IBD-BR cohort, HLA genotypes were imputed from UK Biobank Axiom array data using the HIBAG package.

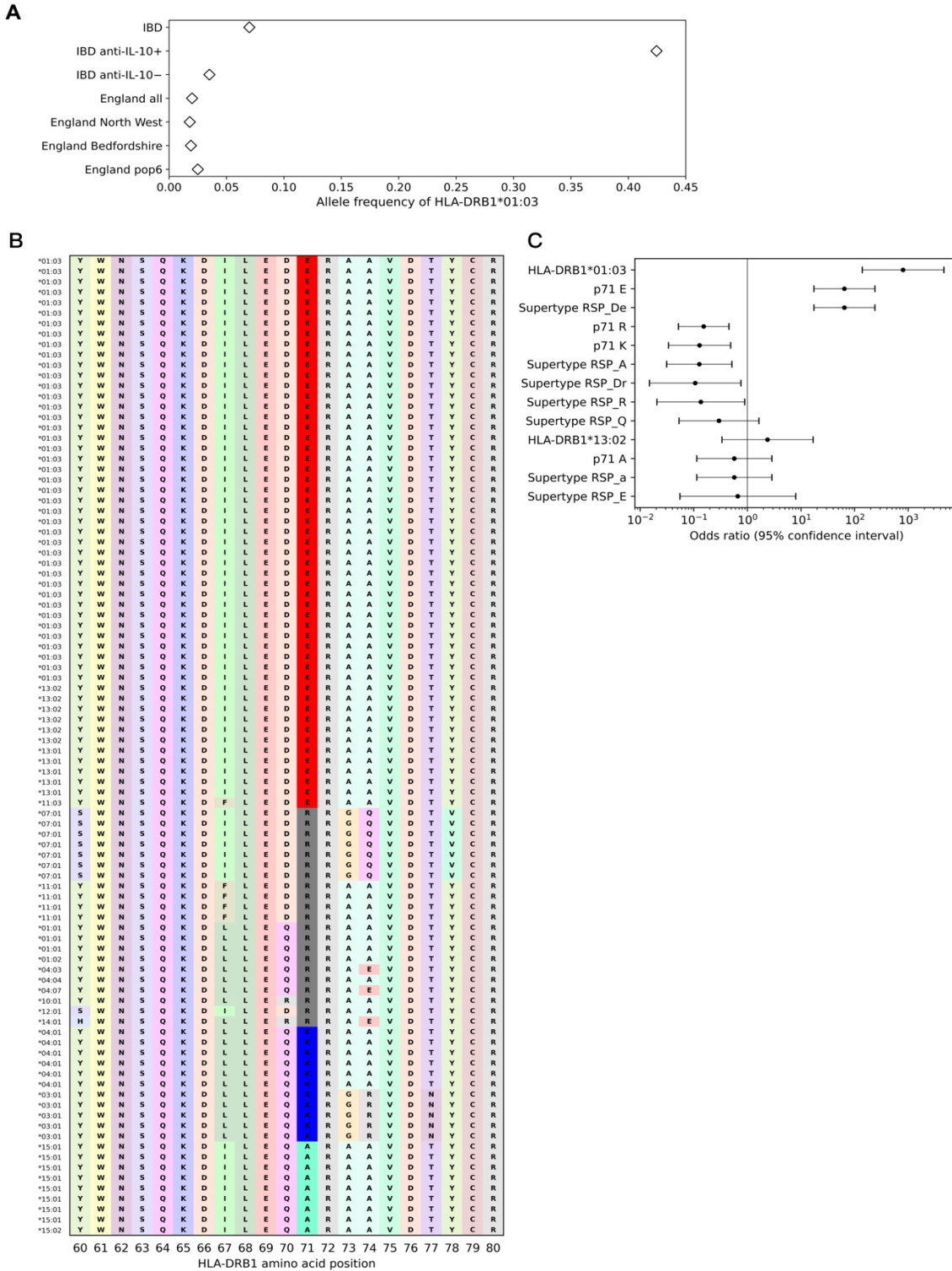


Figure S6. Amino acid association analysis. **A.** Allele frequency of HLA-DRB1*01:03 in European descent IBD and non-IBD patients in the English population where non-disease frequencies were derived from allelefrequenciest.net. **B.** Alignment block plots display the amino acid residue composition for HLA-DRB1 alleles across eligible anti-IL-10+ patients (n=47). Each row represents an individual patient with amino acid residue, and residue E (glutamic acid) at position 71 (highlighted in red) is significantly enriched in anti-IL-10+ patients. **C.** Forest plot of logistic regression model testing the association of various condition with anti-IL-10 positivity, including HLA-DRB1*01:03 (dose-effect considered in this model), supertype (Ou et al. 1998), and amino acid in position 71, sorted by ascending unadjusted p value.

4. Supplementary Tables

Table S1. Primary detection and the interference of all anti-IL10 detection assays used

Assay	Primary detection / read-out	Potential interference / caveats
Luminex bead assay	IgG binding to IL-10-coupled beads (screening of anti-IL-10 antibodies)	Possible signal from non-neutralizing IgG; heterophile antibodies; high total IgG or sample matrix effects
HEK-Blue IL-10 reporter assay	Neutralization of IL-10 signaling measured by loss of STAT3 reporter activation	Janus kinase (JAK) inhibitors, anti-IL-10 receptor (IL-10R) antibodies, cytotoxic sera; matrix effects
Competitive ELISA	Patient IgG competes with capture/detection monoclonal antibodies for IL-10 epitopes (evidence of blocking/epitope masking)	False-negative if autoantibody targets epitopes that do not overlap with capture or detection antibody
Cytokine assay (PBMC ± LPS)	Functional modulation of ex vivo cytokine profile consistent with IL-10 pathway blockade	Sensitive to concomitant immunomodulators (e.g., JAK inhibitors, anti-TNF and anti-IL-23 agents, corticosteroids)

Table S2. Demographics of the screened cohorts

Group	N	Gender	Age at IBD diagnosis (year) Age at anti-IL-10 sampling (year) *			IBD subtype
			Mean ± SD	Median (IQR)	Range	
Adult control (PITCH cohort)*	993	Male 271 Female 708 Not reported 14	40.7 ± 12.8	42 [29.8, 50]	19-83	NA
Pediatric control*	13	Male 7 Female 6	9.4 ± 3.9	11.6 [5.6, 12.8]	3-14	NA
Oxford discovery cohort	847	Male 396 Female 451	28.9 ± 15.1 41.5 ± 17.8 *	25 [18, 38] 39 [27, 55] *	0-84 3-93 *	CD 461 UC 357 IBDu 29
Oxford validation cohort	715	Male 364 Female 351	28 ± 15.6 38.4 ± 17.6 *	25 [13, 43] 36 [25, 51] *	0-83 1-84 *	CD 329 UC 330 IBDu 56
UK-IBD-BR cohort	3347	Male 1696 Female 1651	See table Supp. 3			CD 1638 UC 1572 IBDu 137
Anti-IL-10 positive IBD patients (total)	173	Male 66 Female 107	CD 31.5 ± 12.3 UC 31 ± 12.9 IBDu 39 ± 19.5 Total 31.8 ± 13.5	CD 30 [23, 40] UC 27.4 [21.2, 38] IBDu 33 [23.4, 56.6] Total 29 [22, 39.2]	CD 4-60 UC 9-63 IBDu 5-75 Total 4-75	CD 61 UC 99 IBDu 13
Anti-IL-10 negative IBD patients (total)	4736	Male 2390 Female 2346	CD 28 ± 15.1 UC 33.9 ± 15.6 IBDu 30.5 ± 19.2 Total 30.8 ± 15.8	CD 24 [17, 35.6] UC 30.6 [22, 43.6] IBDu 27 [15, 44] Total 27 [19, 40]	CD 0-83 UC 0-84 IBDu 0-82 Total 0-84	CD 2367 UC 2160 IBDu 209

* Age at anti-IL-10 sampling; NA, not applicable

Table S3. Age bands of the UK-IBD-BR cohort

	Age of anti-IL-10 sampling	Age of IBD diagnosis
<16	0	442
16-24	523	897
25-29	368	426
30-34	375	335
35-39	334	259
40-44	274	250
45-49	271	152
50-54	244	174
55-59	271	112
60-64	217	97
65-69	178	66
>=70	292	67
Unknown	0	70
Total	3347	3347

5. Supplementary References

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