Genomic mapping of the MHC transactivator CIITA using an integrated ChIP-seq and genetical genomics approach

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Abstract

Background: The master transactivator CIITA is essential to the regulation of Major Histocompatibility Complex (MHC) class II genes and an effective immune response. CIITA is known to modulate a small number of non-MHC genes involved in antigen presentation such as CD74 and B2M but its broader genome-wide function and relationship with underlying genetic diversity has not been resolved.

Results: We report the first genome-wide ChIP-seq map for CIITA and complement this by mapping inter-individual variation in CIITA expression as a quantitative trait. We analyse CIITA recruitment for pathophysiologically relevant primary human B cells and monocytes, resting and treated with interferon-gamma, in the context of the epigenomic regulatory landscape and DNA-binding proteins associated with the CIITA enhanceosome including RFX, CREB1/ATF1 and NFY. We confirm recruitment to proximal promoter sequences in MHC class II genes and more distally involving the canonical CIITA enhanceosome. Overall, we map 843 CIITA binding intervals involving 442 genes and find 95% of intervals are located outside the MHC and 60% not associated with RFX5 binding. Binding intervals are enriched for genes involved in immune function and infectious disease with novel loci including major histone gene clusters. We resolve differentially expressed genes associated in trans with a CIITA intronic sequence variant, integrate with CIITA recruitment and show how this is mediated by allele-specific recruitment of NF-kB.

Conclusions: Our results indicate a broader role for CIITA beyond the MHC involving immune-related genes. We provide new insights into allele-specific regulation of CIITA informative for understanding gene function and disease.

Background

The transcriptional regulation of the CIITA gene (also referred to as C2TA or MHC2TA) on chromosome 16p13, and the impact of genetic variation involving this gene for disease, has been extensively characterised following the identification of rare loss-of-function mutations in CIITA resulting in the bare lymphocyte syndrome and severe immunodeficiency due to lack of expression of Major Histocompatibility Complex (MHC) class II genes [1]. CIITA was found to encode a critical non-DNA binding factor, the master MHC class II transactivator, which is recruited to the class II enhancer complex and plays a critical role in expression of MHC class II genes and, as a result, in the adaptive immune response through antigen presentation to CD4+ T cells [2].

CIITA is expressed in a variety of antigen presenting cells both constitutively and following induction, notably after interferon-gamma (IFNγ), with transcriptional regulation of CIITA found to be complex and highly context specific. This includes the occurrence of four different promoters, each with a unique first exon, conferring considerable cellular specificity with, for example, the CIITA class III promoter important for constitutive expression in B cells while the class IV promoter is critical to inducible expression [2-5]. A number of enhancer elements have also been identified, including at least five elements over a 110 kb region spanning the gene [6].
CIITA regulates MHC class II gene expression through complex mechanisms including chromatin remodelling, transcriptional initiation and elongation [2]. However it does not directly bind DNA. Rather, it is recruited to the proximal promoter regions of the classical MHC class II genes (HLA-DR, HLA-DR and HLA-DQ), and to HLA-DM, HLA-DO and the CD74 gene (encoding the molecular chaperone invariant chain which associates with the MHC class II complex) through protein-protein interactions with other components of the MHC class II enhanceosome. These include the regulatory factor X complex (RFX5, RFXANK and RFXAP), the cAMP responsive element binding protein (CREB1) and activating transcription factor 1 (ATF1), and nuclear factor Y (NFYA/B/C subunits) which bind DNA through the SXY module, a highly constrained series of sequences (S-X-X-Y boxes) spanning 59 to 68 bp and typically located within 300 bp of the site of transcriptional initiation [2,7,8]. In addition, more distal SXY modules with enhancer activity have been identified within the MHC class II region [9]. SXY modules are also found in the MHC class I region where CIITA is recruited but has an ancillary role, the major trans-activator here being NLRC5 [10]. In addition to CD74 and B2M, a small number of non-MHC target genes for CIITA have been identified following chromatin immunoprecipitation (ChIP) for CIITA analysed using promoter arrays [8] and differential gene expression in cell lines deficient in CIITA [11] including RFX5, TPP1, RAB4B, PSMD3 and MYBPC2 which were found to depend on RFX recruitment to a promoter X-box sequence.

Genome-wide functional genomic approaches provide new opportunities to systematically define such regulatory elements and the impact of genetic variation. Here we describe the first ChIP-seq derived genome-wide map for CIITA occupancy, set in the context of complementary data for other DNA-binding protein members of the CIITA enhanceosome and regulatory features of the epigenomic landscape. We demonstrate how this can be integrated with data mapping the genetic determinants of inter-individual variation in CIITA expression, resolving associated target genes for CIITA both within the MHC and in the larger genomic space outside the MHC. We show how a specific intronic regulatory variant of CIITA is associated in trans with a network of target genes and modulates allele-specific recruitment of NF-KB.

Results
A genome-wide map of CIITA recruitment in human B cells and monocytes
In order to generate a high-resolution genome-wide map of CIITA recruitment, we performed ChIP for CIITA followed by high throughput sequencing (ChIP-seq) in primary human B cells and monocytes. Published work to date suggests that CIITA binding is associated with RFX binding to an X box sequence and our experimental design included generating ChIP-seq data for CIITA and RFX5 using chromatin prepared from the same cells. We analysed CD19+ B cells, which show high constitutive levels of MHC class II expression, together with CD14+ monocytes either resting or treated with IFNγ as an inducible paradigm for MHC class II expression, as this cytokine is known to significantly upregulate gene expression [12].

ChIP-seq peaks for CIITA and RFX5 were scored using the model-based analysis of ChIP (MACS) after mapping to the reference human genome haplotype sequence for the MHC region (chr6: 29,580,000-33,100,000). We defined a set of 1,010 binding intervals (BIs) genome-wide for CIITA and RFX5 across 12 samples derived from highly purified CD19+ B cells and CD14+ monocyte populations from two different healthy individuals (Figure 1A and Additional file 1: Table S1). Within a given cell type or condition, BIs were defined as CIITA and/or RFX5 enriched ChIP-seq peaks present in both individuals. When we performed an analysis of CIITA and RFX5 affinities (differences in read densities) across the BIs, this partitioned the binding profiles for B cells and monocytes into two distinct groupings consistent with cell type (Additional file 2: Figure S1A). For CIITA, we identified 329 BIs in B cells, 81 in resting monocytes and 591 in monocytes treated with IFNγ. Specific known and novel CIITA BIs were validated by ChIP for a third individual using a different CIITA antibody (Additional file 2: Figure S2). We integrated all CIITA and RFX5 BIs across the 12 ChIP-seq datasets (Additional file 1: Table S1) with DNase I hypersensitivity site (DHS) maps for primary B cells and monocytes [13]. This revealed that genome-wide, 95% of CIITA BIs in B cells and 79% in naïve monocytes are co-incident with a DHS (Additional file 1: Table S1). We investigated the overall relationship with components of the canonical CIITA enhanceosome RFX5, CREB1, ATF1, NFYA and -YB for CIITA BIs. The majority of BIs, 81% and 98% for B cells and naïve monocytes, respectively, have data coincidence scores (DCS) greater than 4 (Additional file 1: Table S1 & Additional file 2: Figure S1B) suggesting that genome-wide, CIITA occupancy involves sites of accessible chromatin and that other constituents of the canonical enhanceosome may be relevant to recruitment.

We proceeded to characterise further the relationship of CIITA and RFX5, defining instances where CIITA BIs are co-incident with RFX5 binding as CIITA Enhanceosome marks (CE-marks) (Figure 1A and B). In primary human CD19+ B cells, 329 CIITA and 318 RFX5 BIs were mapped genome-wide (Additional file 1: Table S1) and 69% of CIITA BIs overlapped with RFX5 to form
CE-marks (Figure 1A). Within the classical MHC Class II region (chr6: 32,400,000-33,100,000), we found all CIITA BIs were CE-marks. For primary CD14+ monocytes in a naïve state, there were 81 CIITA and 105 RFX5 BIs of which 81% overlap to form CE marks (Figure 1A) while within the MHC Class II region, there were 17 CIITA BIs that, as observed with B cells, were all CE marks. When monocytes were treated with IFNγ, we observed 591 CIITA BIs (a 7.3-fold increase compared to the naïve state) with 28% of CIITA BIs being co-incident with RFX5 and

Figure 1 CIITA and RFX5 binding in B cells, naïve and IFNγ treated monocytes. (A) Numbers of CIITA and RFX5 BIs identified as occurring alone or overlapping. Counts shown genome-wide, within the MHC or excluding the MHC (MHC region defined as chr6: 29,580,000-33,100,000). (B) Chromosomal location of CE-marks and of non-overlapping CIITA and RFX5 BIs.
forming CE marks. Within the MHC Class II region the proportion of CE marks increased to 78% and of these, 7/31 were stimulus specific. In contrast, all CIITA BIs within the MHC Class II region of naïve monocytes were also present in treated cells. Similarly, outside of the MHC region we observed a lower proportion of CIITA binding in naïve monocytes as compared with induced (Figure 1A). We observed that some CE marks were identified across all cell types and conditions both within and outside of the MHC region. Overall, we find that for B cells and resting monocytes all CIITA BIs within the MHC class II region were CE marks while only 78% were CE marks following IFNγ treatment of monocytes (Figure 1A). We found that outside the MHC region, in B cells and naïve monocytes 66% and 77%, respectively, of CIITA BIs were CE marks while after IFNγ treatment only 25% of CIITA BIs in monocytes were identified as CE marks.

We next considered the relationship of CIITA BIs with genes and gene networks. We defined a potential functional relationship with a gene when the distance of a CIITA BI from the transcriptional start site (TSS) of a gene was <10 kb (5’ or 3’). Following this definition, we found that 635 of 843 CIITA BIs (75%) were associated with a gene (Figure 2A), with a total of 442 genes identified proximally within 2 kb of the TSS including classical genes associated with a CIITA BI (Additional file 1: Table S1). CIITA BIs in B cells, naïve monocytes and monocytes treated with IFNγ showed association with 242, 40 and 271 genes, respectively (Additional file 1: Table S1). Across all cell types and conditions, a single BI was found within 2 kb of the TSS for 75% to 94% of these genes. For a further 3% to 16% of these genes a single, more distal BI within 10 kb of the TSS was present. Finally, for the remaining 5% to 9% of these genes there was association with both proximal and more distal BIs.

We identified networks enriched for genes associated with a CIITA BI for genome-wide data using DAVID (the database for annotation, visualization and integrated discovery) bioinformatics resources [14,15] and Ingenuity Pathway Analysis tools. The enriched gene networks revealed biological themes related to immunity and inflammation, with antigen presentation the most significantly enriched pathway on global analysis of all CIITA BI genes (Additional file 1: Table S1). Signalling involving OX40, a member of the TNFR superfamily important in T cell activation, was also significantly enriched on analysis of all CIITA BI genes (P = 4.7×10^{-19}) and when CIITA BIs identified in each of the specific cell types were analysed (Additional file 1: Table S1). Signalling involving OX40, a member of the TNFR superfamily important in T cell activation, was also significantly enriched on analysis of all CIITA BI genes (P = 1.8×10^{-10}) and for individual cell types. In terms of disease, we found that autoimmune disease phenotypes were significantly enriched across different cell types in the CIITA BI associated genes. In B cells, RFX5 was the most significant predicted upstream transcriptional regulator (P = 1.4×10^{-9}) together with NLRC5 (P = 3.6×10^{-8}), SRF (P = 3.8×10^{-9}) and CIITA (P = 4.4×10^{-5}) while in naïve monocytes RFX5 (P = 4.6×10^{-9}) and CIITA (2×10^{-7}) were most significant (Additional file 1: Table S1). In induced monocytes, a more complex picture was seen with STAT1 (P = 9.3×10^{-15}), NFκB complex (P = 1.5×10^{-10}) and IRF1 (P = 1.1×10^{-9}) identified as upstream regulators together with RFX5 (P = 3.3×10^{-9}).

We proceeded to examine for possible enrichment of genes associated with a CIITA BI among genes that are differentially expressed in monocytes after IFNγ treatment [16]. We found significant enrichments of such genes, with highest enrichment when a CE mark was present but there was also an enrichment of genes associated with a CIITA BI where RFX5 was absent (Figure 2B). There was a similar enrichment observed when genes associated with CIITA BIs (combined set of CE marks and CIITA BIs without RFX5) were segregated into known CIITA targets (Additional file 1: Table S1) and novel CIITA targets identified in this study (Figure 2C).

At the same time, we also observed that there were other instances whereby differential gene expression between naïve and treated monocytes we previously defined in a large cohort [16] were not associated with a change in CIITA occupancy (Additional file 2: Figure S3).

Resolving proximal and distal sites of CIITA recruitment in the MHC class II region

CIITA is known to be recruited to the enhanceosome complex at proximal promoter SXY sequences of classical MHC class II genes with a small number of more distal enhancer elements recruiting CIITA identified based on sequence homology with the SXY module [9]. Within the MHC class II region we identified a total of 30 CIITA BIs across freshly isolated B cells and monocytes. The majority (73%) of these CIITA BIs were located within 10 kb of the TSS including classical genes such as HLA-DP, HLA-DR and HLA-DQ (Figure 3). Furthermore, for all of these genes there was a CIITA BI located proximally within 2 kb of the promoter (Additional file 1: Table S1). We observed that IFNγ treatment in monocytes resulted in nine additional CIITA BIs not present in naïve cells (8 proximal; 1 distal) that were associated with BRD2, PSMB8, HLA-DRA, -DQA, -DOB and -DOA (Figure 3).

We resolved proximal and distal CIITA recruitment within the MHC class II region as illustrated by the following examples. For HLA-DRA, we found evidence of a proximal and a more distal CIITA BI (Figure 4A) with the latter corresponding to a known site for CIITA occupancy [9]. In this instance, both CIITA BIs were associated with RFX5 binding (CE marks), located within transcriptionally accessible regions (DNase1 DHS) and associated with other features of the MHC enhanceosome including binding by CREB1, ATF1, NFYA and -YB.
Figure 2. CIITA BIs in relation to gene location, CIITA module and gene function. (A) Distribution of CIITA BIs showing relative distances from the TSS of genes. (B) Enrichment of genes that were differentially expressed in treated monocytes within two groupings of CIITA target genes, one associated with CE marks (CIITA BIs overlapping RFX5) and the other CIITA BIs without RFX5. The empirical cumulative distribution functions (eCDFs) for each grouping is shown with a 95% confidence interval. A group of CIITA targets containing a larger than expected number of differentially expressed genes will produce an eCDF that lies above the line corresponding to absence of enrichment. (C) Enrichment for all CIITA BIs (overlapping and non-overlapping with RFX5) associated with known and novel CIITA target genes in treated monocytes. (D) Box plot showing averaged rate of MATCH-PWM binding site scores for a CIITA module of RFX5, CREB and NF-Y motifs (co-factors of the classical CIITA enhanceosome) in promoters of known CIITA target genes and those identified in this study (denoted as novel targets where CIITA BIs localised within 10 kb 5’ of the TSS). For known and novel target genes, 150 bp intervals centred on CIITA peak summits were analysed. A control set comprised of 1,000 randomly selected segments is included for comparison. Distributions of the three sets were compared using Mann-Whitney-Wilcoxon test, showing that these are significantly different, between the control set and known CIITA targets ($P = 2.2 \times 10^{-16}$) or novel targets ($P = 1.5 \times 10^{-11}$), and between known and novel targets ($P = 2.7 \times 10^{-7}$).
We mapped CIITA recruitment to the proximal promoter regions of HLA-DRB1 and HLA-DQA1 and identified a further four CIITA BIs between these genes (Additional file 2: Figure S4). These included a CIITA BI localising to an isoform specific promoter for HLA-DQA1 (chr6: 32590730-32591333); a CIITA BI localising to the XL9 intergenic regulatory element previously reported to bind CIITA while also recruiting CTCF to enable chromatin looping and modulation of HLA-DRB1 and HLA-DQA1 gene expression (chr6: 32595564-32596093) [17]; a previously reported CIITA BI (chr6: 32577249-32578269) [9]; and a novel CIITA BI (chr6: 32571777-32572798) that has not been previously described. All were CE marks in at least a single cell type and condition.

We also identified a distal CIITA BI in an intergenic region 50.9 kb 5′ of HLA-DQA2 (chr6: 32657986-32658366). This site has previously been reported as a target of CIITA and RFX5 [9] and we found evidence of a CE mark in B cells with inducibility in monocytes treated with IFNγ. Finally, we resolved in the region spanning HLA-DOA and HLA-DPA1 that there were two proximal promoter CIITA BIs and a further two more distal CIITA BIs (chr6: 32986547-32987178 and chr6: 32996961-32997920), both of which were also CE marks. The former is novel while the latter has been reported to be a target of CIITA and RFX5 [9].

Within the MHC class I region (chr6: 29640000-31325000), where CIITA plays an ancillary role [10], we identified 11 CIITA BIs of which 10 are CE marks (Additional file 1: Table S1). These included the proximal promoter regions of classical class I genes including HLA-A, HLA-B, HLA-C, HLA-E and HLA-F. In addition we found evidence of CE marks at TRIM26 in B cells and monocytes, consistent with earlier reports [8], and novel recruitment at the proximal promoter of PPP1R10 in B cells (Additional file 2: Figure S5). PPP1R10 encodes the protein phosphatase I regulator PNUTS, recently shown to play a key role in DNA damage repair [18].

Non-MHC CIITA recruitment

We observed that of 843 CIITA BIs identified across B cells and monocytes, the majority were located outside of the MHC (90% of CIITA BIs in B cells, 79% in naïve monocytes, 93% in treated monocytes). For these CIITA BIs, 40% were associated with RFX5 binding and classified as CE marks. As observed within the MHC, the majority of CIITA BIs associated with a gene (<10 kb from TSS) were similarly localised in terms of proximity to the TSS (70% are within 2 kb of the TSS compared to 82% within the MHC class II region; Figure 2A and Additional file 2: Figure S6) and within transcriptionally accessible regions of the genome (Additional file 2: Figure S1B and Additional file 1: Table S1).

Our global analysis of genes associated with CIITA BIs highlighted gene networks related to immunity and inflammation (Additional file 1: Table S1). Considering all CIITA BIs we identified outside the MHC, we found greater diversity in the associated pathways with EIF2...
Figure 4 CIITA binding intervals for a classical gene within the MHC class II region and in a histone cluster 1. (A) Identification of two CIITA BIs, one proximal and other distal to the TSS of HLA-DRA. Both are CE marks and binding is associated with other constituents of the CIITA Enhanceosome (NFYA/-YB; ATF1 and CREB1) and are positioned within DNase I hypersensitive sites. (B) Multiple CIITA BIs within a major histone cluster at chr 6p21.3.
signalling \( (P = 4.5 \times 10^{-10}) \) most significant while IL17A and JAK/STAT signalling were also enriched. Cell proliferation \( (P = 7.3 \times 10^{-12}) \) and RNA expression \( (P = 4 \times 10^{11}) \) were significantly enriched in terms of biological process. Striking enrichment was also seen for viral infection \( (P = 4.5 \times 10^{-12}) \) involving 82 genes together with a number of disease phenotypes involving arthritis (Additional file 1: Table S1).

CIITA binding has been previously reported for regions outside the MHC, notably CD74 and B2M which both encode key components found in MHC class II and class I molecules, respectively [8,19]. Our ChIP-seq maps resolved a CE mark located proximally to the TSS of CD74 on chromosome 5 (Additional file 1: Table S1 and Additional file 2: Figure S7). CIITA occupancy at this location has been previously described by Krawczyk and co-workers [8]. There was also a CE-mark located at the proximal B2M promoter (Additional file 1: Table S1 and Additional file 2: Figure S8). Our datasets also confirmed previously reported CIITA targets [8] with CIITA BIs including CE marks identified at RAB4B, ZNF672, HAUS5 and MYBPC2/SPIB. These reported targets had lower \( P \) values for the confidence of calling peak summits (Additional file 2: Figure S9).

In both B cells and treated monocytes, we observed the presence of multiple histone genes within the enriched gene networks (Additional file 1: Table S1). These included the histone H3.3B gene H3F3B for which a CE mark can be found at the proximal promoter (chr17: 73775313-73776112). Within the large histone gene cluster at 6p21.3-6p22, we identified a further 11 CIITA BIs encompassing four CE marks corresponding to proximal promoter regions of HIST1 genes including HIST1H1C, HIST1H2BC, HIST1H2BD, HIST1H4D, HIST1H2AD, HIST1H4E, HIST1H2BG, HIST1H2BI and HIST1H4H (Figure 4B). We found another grouping of CIITA BIs including CE marks at a second major histone gene cluster with HIST2H2AB and HIST2H2BE at chr1q21.2. In B cells, we identified a CE mark at the proximal promoter of HIST4H4 (Additional file 1: Table S1).

Given that 60% of CIITA BIs identified outside of the MHC were not overlapping RFX5 BIs, we sought to further characterise CIITA recruitment and possible associated co-factors. We first investigated the occurrence of the tri-motif CIITA module comprised of RFX5, CREB and NF-Y [20] within 150 bp of sequence around peak summits for CIITA BIs localised to within 10 kb upstream of a TSS. The module was most prevalent for CIITA BIs localised at known CIITA targets that include genes within the MHC Class II region (Figure 2D and Additional file 1: Table S1). The module was also enriched within the novel CIITA BIs identified in this study but significantly less than observed for CIITA BI involving known loci.

We then investigated occurrence of known DNA binding motifs for other transcription factors where CIITA recruitment was occurring in the absence of RFX5 binding. We analysed the 150 bp sequences spanning CIITA peak summits for enriched consensus sequences using the MEME-ChIP suite [21,22]. This showed significant enrichment of several consensus motifs notably STAT3 and SPI1 in IFN-treated monocytes (Additional file 1: Table S1).

To further investigate this, we analysed upstream regulators of genes associated with CIITA BIs not overlapping RFX5 BIs using IPA (Additional file 1: Table S1). Analysis of all genes associated with CIITA BIs revealed that while CE marks were most significantly associated with RFX5 \( (P = 4.5 \times 10^{-10}) \) and NLRC5 \( (P = 1.4 \times 10^{-8}) \), CIITA BI not overlapping RFX5 were significantly enriched for transcription factors STAT1 \( (P = 2.2 \times 10^{-7}) \), JUN \( (P = 8.7 \times 10^{-7}) \), MYC \( (P = 8.9 \times 10^{-7}) \), STAT3 \( (P = 5.4 \times 10^{-6}) \) and NFkB complex \( (7.5 \times 10^{-6}) \). A similar pattern was seen when genes associated with CIITA BI in the MHC were compared with non-MHC genes. Considering specific cell types, this effect was most clearly seen on analysis of induced monocytes (Additional file 1: Table S1) with STAT1 \( (P = 2.3 \times 10^{-6}) \), NFkB complex \( (P = 2.8 \times 10^{-7}) \) and STAT3 \( (P = 3.8 \times 10^{-7}) \) the most significantly enriched transcriptional regulators in CIITA BI not overlapping RFX5.

The motif analysis and upstream regulator data raise the hypothesis that members of the STAT family may be involved in CIITA recruitment in the absence of RFX5 but further work is required to test this. In terms of diseases and functions, the overall gene set we identified as associated with CIITA BIs not overlapping RFX5 was most significantly enriched for infectious disease (viral infection) \( (P = 1.6 \times 10^{-9}) \) followed by a variety of autoimmune diseases with effects most clearly seen in treated monocytes. In B cells and naïve monocytes enrichment for cancer phenotypes was noted, with death receptor signalling identified on pathway analysis in B cells (Additional file 1: Table S1).

**Insights from the genetical genomics of CIITA**

Mapping gene expression as a quantitative trait has the potential to define master regulatory networks through resolution of *trans* associations in which differential gene expression for a gene set are seen to be associated with a specific genetic marker [23]. We have reported genome-wide expression quantitative trait (eQTL) analysis in different primary human cell populations [24]. This global analysis included evidence of a local, cis-eQTL involving *CIITA* that was specific to B cells. Given the role of CIITA as a master regulator we sought to further resolve this association and to investigate whether there was evidence of associated *trans*-eQTL networks that could inform CIITA function and modulated genes.
We used a linear model which incorporated major principal components of the expression data as covariates [25] to further define association within 1 Mb of CIITA for the 281 healthy volunteers using a genome-wide panel of 651,210 SNP markers [24]. This resolved that the strongest association involved a single nucleotide polymorphism (SNP) rs11074938 in the 13th intron of CIITA (P = 6.9×10^{-20}, FDR = 3.2×10^{-15}) (Figure 5A to C). Analysis of trans associations involving this SNP (Figure 5D) revealed multiple associations with MHC Class II genes, notably HLA-DMA, HLA-DOA and HLA-DRA consistent with the known role of CIITA recruitment in regulation of these genes. Non-MHC trans associations included genes that are known to show evidence of CIITA recruitment such as RFX5 and TPP1 while for others including OLFML2A and PARVG we find ChIP-seq mapping supports a role for CIITA.

We sought to determine whether the trans association with transcript expression seen for rs11074938 with MHC Class II genes was also observed at the protein level. We assayed MHC class II surface expression on B cells in whole blood from healthy volunteers by multilevel. We assayed MHC class II surface expression on B cells in whole blood from healthy volunteers by multilevel. We assayed MHC class II surface expression on B cells in whole blood from healthy volunteers by multilevel. We assayed MHC class II surface expression on B cells in whole blood from healthy volunteers by multilevel.

In light of the cis and trans associations for rs11074938 in B cells, we sought to investigate the functional basis for this. We first studied the regulatory functional genomic landscape of the sequence variant. Analysis of publically available ENCODE data revealed that SNP rs11074938 was located within a DHS in human primary B cells and lymphoblastoid cell lines (LCLs) but not monocytes (Figure 6A). The position in a region of open chromatin was consistent with the hypothesis that the variant is located in a regulatory element. This was further supported by the presence of specific histone marks including H3K4Me1 and evidence that this is a phylogenically conserved region (vertebrate conserved element, LOD score 31).

Analysis of ChIP-seq data [26] revealed that the SNP was located within an NF-KB target site in LCLs (Figure 6A) and contained an NF-KB consensus binding motif. We investigated whether the A to G substitution could modulate predicted binding affinity using a publically available database identifying binding sites of NF-KB by a combination of protein-binding microarrays and surface plasmon resonance [27]. The ancestral A allele was predicted to show higher binding affinity for NFKB1/RELA (z-score human 3.25 with A allele vs. 2.83 with G allele) and NFKB2/RELβ (z-score 3.16 A vs. <2 G) but the situation is complex with the G allele also predicted to show higher affinity for the NFKB2 homodimer (z-score 4.38 G vs. 3.86 A).

To investigate this further we characterised NF-KB binding by electrophoretic mobility shift assay (EMSA). EMSA using recombinant NFKB1 and RELA proteins demonstrated that with the NFKB1/RELA heterodimer there was greater binding to the A allele compared to the G allele (Figure 6B). Weak binding was observed for NFKB1 alone with no evidence of RELA binding as a homodimer.

We then proceeded to determine whether allelic differences in NF-KB binding were present ex vivo using ChIP-seq data for a panel of five LCLs heterozygous for rs11074938 [26]. This revealed that there was significantly more RELA binding to the A allele compared to the G, with on average 66.1% (SD 8.1) of reads mapping to the A allele (P =0.008) (Figure 6C).

In order to investigate whether the intronic sequence spanning the SNP rs11074938 had regulatory activity that could be modulated in an allele-specific manner, we cloned the region into a reporter gene construct. Given the low transient transfection efficiency of human B cell lines, we performed reporter gene assays in a lymphoblast cell line of T cell origin (Jurkat). This demonstrated evidence of increased enhancer activity for the A versus the G allele of rs11074938 with allele-specific differences in protein-DNA interactions also seen in this cell line (Additional file 2: Figure S10).

A cis-eQTL for CIITA involving allele-specific recruitment of NF-KB

The biology of CIITA regulation for MHC class II genes is well established [2,7,8]. Our data support evidence for distal as well as classical proximal sites of CIITA recruitment [9], including novel BIs which, as elsewhere in the class II region, appear highly stereotyped in terms of enhanceosome composition and partner DNA binding proteins notably RFX5. Similarly, for the class I region the observed binding is consistent with previous findings at classical genes while supporting evidence for binding sites proximal to TRIM26 and PPP1R10 [10,28].

Genome-wide, the picture is more complex with 60% of observed CIITA BIs not associated with RFX5 binding based on ChIP-seq performed on the same input chromatin. As seen in the MHC, occupancy was
Figure 5 Local and distant eQTL associations for eSNP rs11074938 involving CIITA. (A) Manhattan plot showing association on chromosome 16p13. (B) Regional association plot for CIITA demonstrating peak association for rs11074938 located in intron 13 of CIITA. (C) Box-plot showing allelic association of rs11074938 with levels of CIITA expression across 281 individuals. (D) Circular plot illustrating genomic location of eQTLs for rs11074938 in B cells. From outside to inside: chromosome number; relative distance along chromosome; coloured dots are cis and trans-eQTLs (size relative -log10 P value as per legend); the outer grey dot is a cis-eQTL for CIITA while inner blue dots are trans-eQTLs; lines indicate genes regulated in trans rendering these as potential CIITA targets, only eQTL mapping to genes with P < 3 × 10^{-4} in the dataset (n = 281) have been shown. (E) Association of rs11074938 with levels of pan MHC class II protein across 37 individuals quantified using multi-parametric flow cytometry. Difference in measured protein levels between homozygous individuals with the A allele as compared to the G allele was significant (*denotes Kruskall-Wallis P = 0.02; MFI = Median fluorescence intensity; data shown have been normalised to control isotype staining).
associated with accessible transcriptionally active chromatin and was predominantly within 2 kb of a gene. The data confirmed some previously identified non-MHC genes such as CD74 and B2M [8,11,19] but also highlighted many additional loci notably after monocytes were treated with IFNγ in which hundreds of additional BIs were revealed compared to the naïve state consistent with previous observations that CIITA-mediated regulation is intimately linked to treatment with interferons [2]. Examples of genes associated with CIITA BIs include key mediators such as STAT1 and STAT3, observed specifically in monocytes treated with IFNγ and B cells, respectively, as well as regions of enrichment for CIITA BIs such as identified within the major histone gene clusters at chromosomes 6p21 and 1q21.

It has been proposed that in addition to its well-defined enhanceosome function in the MHC, CIITA may function to modulate transcription of a target gene through an alternative mode of action involving formation of a TFIID-like complex in conjunction with partners TAF6, TAF9 and TBP [19]. Within this complex, a TATA-box binding protein (TBP) is proposed to bind DNA and through the involvement of other chromatin modifying enzymes that bring about re-modelling of localised regions within the chromatin, CIITA within this TFIID-like complex may subsequently further dimerise with CIITA in a canonical RFX5-containing enhanceosome. The entire mechanism is however, currently not well understood.

We find that the well characterised, tri-motif CIITA module (RFX5, CREB and NF-Y) was less prevalent within novel CIITA BIs we identified but occurred more frequently than in a control set. Our analysis of peak summits within CIITA BIs not overlapping RFX5 highlighted

Figure 6 Functional characterisation of CIITA regulatory variant. (A) Genomic landscape of CIITA and the eSNP rs11074938. Data from ENCODE project for a lymphoblastoid cell line, GM12878 and primary CD20+ B cells and CD14+ monocytes. The SNP, rs11074938 is localised to a DHS specific to B cells that is further coincident with a NF-KB ChIP-seq peak. (B) EMSA demonstrating allele-specific binding for NF-KB involving rs11074938. Recombinant NF-KB proteins NFκB1 (lanes 1 and 4), RELA (lanes 2 and 5) or both (lanes 3 and 6) incubated with probes corresponding to the two different alleles for rs11074938, either A (lanes 1 to 3) or G (lanes 4 to 6). (C) Ratio of A and G alleles within sequenced reads spanning SNP rs11074938 from NF-KB ChIP-seq analyses of five heterozygous individuals.
enrichment of the STAT3 consensus DNA binding motif while STAT1 and STAT3 were significant predicted upstream transcriptional regulators for gene sets associated with such CIITA BIs. These data suggest a possible role for STAT family transcription factors in the recruitment of CIITA outside the MHC but further work is required to characterise this.

This work has highlighted the potential informativeness of integrating genomic profiling approaches such as ChIP-seq with trans-eQTL mapping to define modulated gene networks. We identified a strong local association with CIITA expression that we resolved to a specific cis acting expression-associated SNP (eSNP) involving allele-specific recruitment of NF-kB at an intronic regulatory element and differential gene expression. Further work is required to determine the detailed allele-specific transcriptional mechanisms involved in this cis-association. Strikingly, we find this eSNP was associated in trans with a gene network that included classical class II genes demonstrated at the transcript level and subsequently validated at the protein level. Moreover, the trans network also included non-MHC genes, both known targets such as proacmyb and novel genes where we also find evidence of CIITA BIs by ChIP-seq. Our study had limitations in terms of sample size for determining a more extensive modulated gene network and further analysis at different time points following activation and CIITA expression are likely to be required for in-depth profiling and complete integration with maps of CIITA occupancy, together with functional evidence linking recruitment with differences in gene regulation. However the data illustrate that an integrated approach may be highly informative.

Conclusions
Our study has demonstrated that CIITA recruitment commonly occurs outside the MHC, often not in the setting of a classical enhanceosome with RFX5, and involves genes enriched for immune function and infectious disease. We have functionally characterised a local regulatory variant of CIITA acting through allele-specific recruitment of NFkB which modulates MHC and non-MHC CIITA target genes in trans. The work provides a route-map for further studies to understand the role of CIITA in gene regulation genome-wide, defining complexity and cell type specificity as well as the potential utility of combining analysis of BIs through approaches such as ChIP-seq with experiments of a nature whereby gene networks may be identified through common variants modulating gene expression locally and at a distance through master regulators.

Materials and methods
Nuclear extracts, electrophoretic mobility shift assays (EMSA)
Recombinant NFKB1 (#31101) and RELA (#31102) proteins were obtained from Active Motif, UK. Nuclear extracts were prepared from Jurkat cells as previously described [29]. Oligonucleotide probes were radiolabelled with 32P-dCTP (Perkin Elmer, Beaconsfield, UK) and EMSA performed as previously described [30]. Probes were generated by annealing forward and reverse oligonucleotides spanning rs11074938 (A_for AGCTGA AAAGGGAAATGTCTGAAA, A_rev AGCTTTTCA GACATTTCCCCCTTTTC; G_for AGCTGAAAAGGG GGAATGTCTGAAA; G_rev AGCTTTTCAAGACATT CCCCTTTTTC).

Volunteer recruitment
The study was approved by the Oxfordshire Research Ethics Committee (COREC reference 06/Q1605/55) and volunteers of European ancestry were recruited in the Oxfordshire area following written informed consent through NIHR Oxford BioBank.

Flow cytometry for analysis of HLA Class II expression
We assayed HLA class II expression by multi-parametric flow cytometry for whole blood from 37 healthy volunteers using a three-step procedure adapted from a protocol kindly shared by R. Apps from the National Cancer Institute [31]. Human IgG (Sigma-Aldrich, Dorset, UK) was added to each tube, followed by mouse anti-human antibodies directed against all class II alleles (clone Tu39 and SK10/SPV-L3 combined) or an isotype control (murine IgG, Sigma-Aldrich). After 20 min, cells were washed with Dulbecco's phosphate buffered saline (DPBS, Sigma-Aldrich) and sheep IgG was added to each tube followed by sheep-anti-mouse PE (Sigma-Aldrich). After a further 20-min incubation at 4°C, cells were washed, secondary antibody sites were blocked with mouse IgG (Sigma-Aldrich) and mouse-anti-human CD19-FITC (clone J3-119, Beckman Coulter) was added. This suspension was incubated for 20 min at 4°C, cells were subsequently washed and red blood cells lysed with VersaLyse (Beckman Coulter, UK) over 20 min, then washed and fixed with Reagent A (Life Technologies, Paisley, UK). Data were acquired immediately on a single FACS Canto (BD) machine using standardized settings verified for each data collection batch using BD seven-colour setup beads (BD). A minimum of 10,000 gated B cells were acquired for each sample (gating strategy illustrated in Additional file 2: Figure S11). The genotype of samples was anonymised during flow cytometry. Fluorescence-minus-one controls were used to set gate thresholds. Data were analysed in FlowJo (v10.0.6) and Graphpad Prism (v6). Data were normalised for isotype staining by simple subtraction of the isotype MFI in the PE channel from the HLA-II signal.

Isolation of primary immune cells and ChIP-seq
Leukocyte cones derived from different anonymous individuals were obtained from the NHS Blood and Transplant
Unit. These were processed using standard techniques as described by Neron and co-workers [32]. Briefly, a Ficoll gradient coupled with successive washes were used to isolate PBMCs from the cones [24]. Automated magnetic activating cell sorting, or autoMACS method (Miltenyi) was used to positively separate CD14+ and CD19+ cell populations starting from 500 million PBMCs following manufacturer’s instructions [33]. The technique routinely yields cell purity levels greater than 90% when cross-validated using a FACS-based assay. CD19+ cells were cross-linked for 10 min in 1% formaldehyde, while CD14+ cells were treated with 10 ng/mL IFNγ (R&D Systems) for 24 h alongside an untreated control before cross-linkage. Chromatin isolation, sonication using a BioRuptor (Diagenode) and immunoprecipitation using CIITA (Diagenode) and RFX5 (Santa Cruz) ChIP-grade antibodies were performed. Quality control of ChIP-ped DNA was achieved by quantification on a Qubit 2.0 fluorometer (Invitrogen) using the Quant-iT dsDNA HS Assay Kit (Invitrogen). In addition, real-time quantitative PCR was employed to determine enrichment at various genomic locations prior to sequencing (Additional file 2: Supplemental Methods). Approximately 5 to 10 ng of ChIP-ped DNA from each sample was processed into libraries, amplified and sequenced using 51-bp paired-end runs on the Illumina HiSeq (Additional file 2: Supplemental Methods). Approximately 1.6 billion reads were generated from the genome with sequence extracted 10 kb 5′ and 150 bp 3′ of the TSS from which 150 bp segments randomly selected. The tool Biostrings 2.32.1 from the BioConductor Package was used to scan for presence of RFX5, CREB and NF-Y motifs (source for PWM-Position Weight Matrices used: JASPAR [35], Transfac [36] and Human Protein-DNA Interactome (hPDI) [37]) and score similarity of any motif identified within a segment against the PWM in question. This was carried out iteratively within a segment to obtain a CIITA module with motifs identified in the correct order and with the best score (highest similarity to PWM for all components). An average of scores for the components was computed and this is used as a measure of the prevalence of this known CIITA module within a CIITA BI.

**Motif search**

Peak summits in BIs were used to identify enriched consensus sequences with known DNA-binding motifs using MEME-ChIP suite. The peak summits for each sample were extended to 75 bp-length sequences into both directions. Equivalent sized DHS in primary cells (ENCODE) were used for background estimation. A customised database containing 569 motifs was created using information from the JASPER and hPDI repositories. We estimated a new Markov model for background
frequencies. Discovered motifs with similar E-values of less than 1.0 (default option) and P value of less than 0.01 to the known motifs were used for further study. To get motifs similar to known motifs, we searched ‘tomtom.txt’ output files for each sample and selected motifs occurring in both individuals in the same condition.

Determining biological themes from genes that are regulated by CIITA
We annotated BIs within 10 kb from the TSS of the nearest gene using ChIPpeakAnno [38] and CisGenome [39]. Gene clusters formed using CIITA BIs from B cells, naïve and treated monocytes were analysed through the use of the DAVID Bioinformatics Suite and Ingenuity Pathway Analysis (IPA, Ingenuity Systems [40]). IPA was used to define gene networks, significant upstream regulators and relationships with canonical signalling pathways.

Enrichment of differentially expressed genes within
CE-marks and CIITA BIs
To investigate whether genes associated with CIITA BIs in treated monocytes are enriched for genes that are differentially expressed when compared to naïve monocytes we used our published gene expression data [16]. Differential gene expression analysis was performed using the limma package. All genes with expression data were ranked by gene expression analysis was performed using the limma package. All genes with expression data were ranked by differential expression (P values). We then considered the distribution of ranks within two different sets of genes; genes associated with a CE mark and genes associated with CIITA BIs without RFX5. We also examined genes associated with CIITA BIs (includes CE marks and CIITA BIs without RFX5) that are either known CIITA targets (Additional file 1: Table S1) or novel targets identified in this study.

Data access
ChIP-seq datasets have been deposited in the ArrayExpress database [41] under accession number E-MTAB-2424.

Additional files

| Additional file 1: Table S1. Worksheets S1.1-1.22. |
| Additional file 2: Supplemental Methods and Figures S1-12. |

Abbreviations
ATF1: activating transcription factor 1; BI: binding interval; CE mark: CIITA Enhancosome mark; ChIP: chromatin immunoprecipitation; ChIP-seq: analysis of ChIP by next generation sequencing; CREB1: cAMP responsive element binding protein; DAVID: database for annotation, visualization and integrated discovery; EMSA: electrophoretic mobility shift assay; eQTL: expression quantitative trait; eSNP: expression-associated SNP; IFN: interferon-gamma; LCL: lymphoblastoid cell line; MACS: Model-based analysis of ChIP; MEME: Motif-based sequence analysis tools; MFI: median fluorescence intensity; MHC: Major Histocompatibility Complex; NFI: nuclear factor Y; SNIP: single nucleotide polymorphism; SXY: S-X-Y boxes; TBP: TATA-box binding protein.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
DW executed ChIP-based experiments. Data from ChIP-seq was analysed by WL, PH, and PH contributed to design of the study. SM, EL, VN and KC performed EMA, flow-cytometry, reporter-construct experiments in addition to relevant data analysis. PH and BPF provided analysis and integration of eQTL data. WL and PH provided all relevant bioinformatics support. KP provided technical support for experiments within the laboratory. DW, WL and JCK drafted the manuscript. JCK conceived, supervised and coordinated the study. All authors have read and approved the manuscript for publication.

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