CML cells actively evade host immune surveillance through cytokine-mediated downregulation of MHC-II expression

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Abstract

Targeting the fusion oncoprotein BCR-ABL with tyrosine kinase inhibitors has significantly impacted on chronic myeloid leukemia (CML) treatment, transforming the life expectancy of patients; however the risk of relapse remains, due to persistence of leukemic stem cells (LSCs). Therefore it is imperative to explore the mechanisms that result in LSC survival and develop new therapeutic approaches. We now show that MHC-II and its master regulator class II transactivator (*CIITA)* are downregulated in CML compared to non-CML stem/progenitor cells in a BCR-ABL kinase independent manner. IFNγ stimulation resulted in an upregulation of *CIITA* and MHC-II in CML stem/progenitor cells, however the extent of IFNγ-induced MHC-II upregulation was significantly lower than when compared with non-CML CD34$^+$ cells. Interestingly, the expression levels of *CIITA* and MHC-II significantly increased when CML stem/progenitor cells were treated with the JAK1/2 inhibitor ruxolitinib (RUX). Moreover, mixed lymphocyte reactions (MLRs) revealed that exposure of CD34$^+$ CML cells to IFNγ or RUX significantly enhanced proliferation of the responder CD4$^+$CD69$^+$ T cells. Taken together, these data suggest that cytokine-driven JAK-mediated signals, provided by CML cells and/or the microenvironment, antagonize MHC-II expression, highlighting the potential for developing novel immunomodulatory-based therapies to enable host-mediated immunity to assist in the detection and eradication of CML stem/progenitor cells.

Key Points

- MHC-II and its master regulator *CIITA* are downregulated in CML stem/progenitor cells in a BCR-ABL kinase independent manner.
- JAK1/2 inhibition increased MHC-II expression, suggesting elevation of CML immunogenicity may provide a way to reduce CML persistence.

Introduction

The development of tyrosine kinase inhibitors (TKIs) to target BCR-ABL kinase has revolutionized the management of chronic phase CML with many patients now predicted to have a normal life expectancy.[1, 2] Remission is maintained by continuous administration of TKI and assessed by quantification of *BCR-ABL* transcripts in the blood. For the 10-20% of patients who achieve deep and durable molecular responses, discontinuation studies have been conducted. [3, 4] Approximately 60% of patients maintain a major molecular response over time.[5]

Prior to TKI introduction, CML was a common indication for allogeneic stem cell transplantation (alloSCT). In this setting disease remission was achieved by the combination of anti-leukemic chemo-radiotherapy and active graft versus leukemia (GVL) effect. The degree of immune recognition of leukemic cells by the donor immune system was such that disease relapse, if it occurred, could be managed successfully by administration of donor lymphocytes.[6] Whilst it is well recognised that the effect of alloSCT and GVL is mainly an allo-immune effect mediated through non-disease-specific minor histocompatibility antigens, it is likely that CML cells express disease-specific antigens recognizable by the donor immune system. The role of the patient's own immune system in recognizing BCR-ABL-expressing cells, and whether this can be boosted for beneficial effect, is currently under investigation in vaccination studies, although no convincing results have been reported.[7, 8] Similarly, it is not known whether immune recognition by the patient's immune system is playing a part in maintaining remission of non-relapsing patients in whom TKI treatment is discontinued.

While CD8^{+} cytotoxic T lymphocytes (CTL) are considered to play a major role in tumor immunity, CD4^{+} T helper cells are also important for mediating anti-tumor associated immune responses, possibly through optimal induction and maintenance of CTL responses, interactions with effector cells and production of anti-tumor-associated cytokines such as IL2 and IFNγ.[9, 10] As such, solid tumors (e.g. non small cell lung cancer, mammary adenocarcinoma, colorectal and gastric) and hematological cancers (B cell lymphomas) display MHC class II (MHC-II) downregulation, reducing the host immune response towards the tumor; correlations have been found between higher MHC-II expression and better prognosis.[11, 12] Our microarray datasets comparing the expression of genes between normal and CML stem/progenitor revealed a significant

downregulation in the antigen presentation (exogenous antigen) pathway in quiescent and dividing CD34^{+} CML cells.[13] Here, we investigate the biological relevance of this finding, determining the mechanisms that underlie MHC-II downregulation in CML stem/progenitor cells and examining whether its induction could render these cells more immunogenic.

Materials and Methods

Primary samples cell culture: CD34^{+} cells were enriched, after informed consent, from either CML patient chronic-phase samples at diagnosis (fresh or cryopreserved; Table 1) or allograft donors/lymphoma patients without bone marrow (BM) involvement as "non-CML" controls. The studies were approved by the West of Scotland Research Ethics Committee 4, NHS Greater Glasgow and Clyde (UK). Primary CML cells were cultured in serum-free medium, supplemented with Flt-3 ligand and SCF (each 100 ng/mL), IL3 and IL6 (each 20 ng/mL; StemCell Technologies, Cambridge, UK) and G-CSF (Chugai Pharma, London, UK) overnight. Thereafter for experimental conditions, CD34^{+}-enriched CML cells were cultured in SCF, GM-CSF, and MIP-α (all 0.2 ng/mL), G-CSF and IL6 (both 1.0 ng/mL) and 0.05 ng/mL leukemia inhibitory factor (StemCell Technologies). IFNγ and TGFβ were purchased from Peprotech EC Ltd., (London, UK), nilotinib (NIL) from Stratech Scientific Ltd., (Newmarket, UK) and imatinib mesylate (IM), dasatinib (DAS), SB-505124 (SB) and ruxolitinib (RUX) from Selleckchem (Houston, TX). Pan-MHC-II antibody (Ab; purified, clone Tü39) used for blocking experiments was purchased from BD Biosciences (Oxford, UK).

Microarray analysis: The microarray analysis leading to the initial identification of the MHC-II gene family (Figure 1A) was described previously.[13] The experimental details for the second, larger quiescent/dividing microarray dataset (Figure 1B&C) and the CML primitive/progenitor microarray dataset (Figure 1C, Supplementary Figure 1A&B) are described previously.[14, 15] The processing and normalization procedures for all datasets was carried out as described.[16] All microarray datasets are summarized with respect to sample size, sorting strategy and the relevant figure in Supplemental Table 1; by combining these datasets, transcriptional profiles of 19 independent CML samples and 10 independent normal samples were analyzed. Where genes were represented by multiple probes, expression was summarized using the median value. Any genes not represented on all microarray chips were removed.

Gene set enrichment analysis (GSEA): Gene set enrichment was performed using GSEA[17] (gsea2-2.2.2.jar) as obtained from the Broad Institute (http://software.broadinstitute.org/gsea/index.jsp); q values were calculated using

10,000 permutations of the phenotype label. The MHC-II geneset analysed by GSEA is shown in Figure 1C and is comprised of the Ingenuity pathway shown in Figure 1A and "MHC class II" molecules identified by searching Metacore KB (https://portal.genego.com).

Flow cytometry: Cells were harvested and resuspended in 1 x 10^6 cells/100 µL FACS Buffer (PBS with 2% FCS and 0.02% sodium azide). All antibodies (Abs) were purchased from BD Biosciences unless otherwise stated. Cell staining was performed using 0.5 µg of each flurochrome-conjugated Ab for 30 min, 4°C in dark (anti-CD34, -CD38, -HLA-DP, -DQ, -DR, -CD4, -CD69 Ab). Anti-CD8 and -CD107a Ab were purchased from eBioscience (Hatfield, UK). Events were acquired using a FACSCanto™ II cytometer and FACSDiva software (BD Biosciences) and data was analyzed using FlowJo software (Treestar Inc, Ashland, OR). To isolate CD34$^+$CD38$^+$ (bulk) and CD34$^+$CD38$^-$ (leukemic stem cell (LSC)) populations, cells were sorted using a FACSAria™ cytometer (BD Biosciences).

RT-PCR and Fluidigm analysis: Total RNA was prepared using RNAeasy Plus extraction kit (Qiagen, Valencia, CA). RNA (1 µg) was reverse-transcribed using SuperScript reverse transcriptase and oligo dT primers (Invitrogen Life Technologies, Paisley, UK); or where mentioned, cDNA was prepared from 300 cells using the SuperScript™ III One-Step RT-PCR System with Platinum$^®$ Taq High Fidelity (Invitrogen). PCR analysis was performed on a 48.48 dynamic array using the BioMark™HD System (Fluidigm, San Francisco, CA) or Applied Biosystems Prism 7900HT system (Applied Biosystems, Paisley, UK) as per manufacturers' instructions. Relative gene expression was calculated using the 2$^{-\Delta\Delta CT}$ method.

Mixed lymphocyte reactions (MLRs) and CD107a expression assay: Peripheral blood mononuclear cells (PBMCs; responder cells) were isolated from healthy donors by density centrifugation using Histopaque (Sigma-Aldrich, Poole, UK). The PBMCs were incubated with Dynabeads® Human T-Activator CD3/CD28 (Life Technologies) as per manufacturer's instructions for 48h in RPMI-1640 medium supplemented with 10% heat inactivated FCS, penicillin (100 U/mL), streptomycin (100 U/mL) and L-glutamine (2 mM) (Life Technologies). Activated PBMCs were sequestered from the beads using a magnet and labelled with Cell Trace Violet (CTV) (Life Technologies) prior to co-culture

with stimulator/target (bulk CD34$^+$ CML) cells. For MLRs, activated PBMCs were co-cultured with stimulator cells for up to 6 days at a ratio of 3:1 responder:stimulator. Proliferation was measured as a loss of CTV mean fluorescence intensity (MFI) upon analysis by flow cytometry. For CD107a expression, activated PBMCs were co-cultured with bulk CD34$^+$ CML target cells for up to 6 days at a ratio of 1.5:1 CD4$^+$ T cells:target.

Statistical analysis: Average responses from at least 3 individual CML donors are shown (mean ± SEM). Statistical analysis was performed using GraphPad Prism 4 (GraphPad Software Inc., CA), using students paired or unpaired *t*-test (*p<0.05; **p<0.005; ***p<0.001), as appropriate.

Results:

MHC-II expression is downregulated in CML CD34+ stem/progenitor cells, in a BCR-ABL kinase independent manner

Microarray datasets analyzing the expression of genes in either normal or CML CD34+ quiescent (Hoechst^{lo}Pyronin Y^{lo}) or dividing (Hst$^+$Py$^+$) cells revealed that the antigen presentation pathway was the top deregulated pathway in the CML versus normal comparison (p=5.93x10^{-9}; Figure 1A; significantly downregulated components highlighted in green).[13] To validate this observation *in silico*, and assess more broadly whether the MHC-II gene family is suppressed in CML cells, we extended this genelist to include other relevant MHC-II molecules and identified a second, complementary dataset[14] in which we could perform Gene Set Enrichment Analysis (GSEA)[17] of this MHC-II gene family. Limited numbers of normal samples prevented such an analysis in the original dataset. This analysis confirmed significant downregulation of the MHC-II family in both quiescent and dividing CML cells (FDR≤0.15; Figure 1B). The same analysis was then performed in another dataset transcriptionally profiling primitive (Lin$^-$CD34$^+$CD38$^-$CD90$^+$) CML cells against three progressively more mature cell populations all from a single cohort of CML patients and non-CML controls.[15] While some genes are not downregulated individually, there is a trend for downregulation of the MHC-II genes in the primitive (Lin$^-$CD34$^+$CD38$^-$CD90$^+$) and the three more mature (Lin$^-$CD34$^+$CD38$^+$CD123$^+$CD45RA$^-$; Lin$^-$CD34$^+$CD38$^+$CD123$^-$CD45RA$^-$; Lin$^-$CD34$^+$CD38$^+$CD123$^+$CD45RA^{lo}) cell populations (Figure 1C and Supplementary Figure 1A respectively). This represents a significant downregulation of the MHC-II gene family overall in all four cell populations, as confirmed by GSEA (Supplementary Figure 1B). Overall, this *in silico* validation process considered 19 independent CML samples and 10 independent normal samples (Supplementary Table 1). Analysis of co-stimulatory molecules indicated that while there was some variation in their expression across the populations, TNFSF4 (OX-40L) and CD40 appeared to be downregulated in LSC-enriched populations compared with non-CML populations (Supplementary Figure 1C).

At the protein level we demonstrated a significant reduction in MHC-II surface expression on CML stem/progenitor cells, both in the bulk CD34+ population and in the CD34$^+$CD38$^-$ LSC population, compared with normal hematopoietic stem cell (HSC) populations (Figures 1D&E; Supplementary Figure 2). IFNγ, an established activator of

MHC-II expression[18] which signals through the JAK/STAT pathway, upregulated MHC-II expression in CML stem/progenitor cells (Figures 1D&E; Supplementary Figure 2). However, the level of IFNγ-induced MHC-II upregulation in CML cells was significantly reduced compared with non-CML HSCs (Figures 1D&E; Supplementary Figure 2). Analysis of the master regulator of MHC-II expression, class II transactivator (CIITA),[19] revealed a downregulation of this gene in CML stem/progenitor cells compared to non-CML cells (Figure 1F). Supporting the MHC-II data, IFNγ increased *CIITA* transcription in CML stem/progenitor cells, but not to the level observed in IFNγ-treated non-CML cells (Figure 1F). These data indicate that CML stem/progenitor cells exhibit MHC-II downregulation, which may assist in their evasion from host immunity.

Treatment of bulk CD34$^+$ and CD34$^+$CD38$^-$ CML cells for 48h with TKIs (NIL, DAS, IM) had no effect on either MHC-II or *CIITA* expression, demonstrating that BCR-ABL activity did not significantly influence *CIITA* or MHC-II expression on CML stem/progenitor cells, in the absence or presence of IFNγ (Figure 2; Supplementary Figure 3). This suggests that MHC-II regulation in CML is BCR-ABL kinase independent.

To determine whether MHC-II expression remains downregulated upon extended treatment with TKI, bulk CD34$^+$ CML stem/progenitor cells were cultured in the presence or absence of IM for 7 days prior to analysis of the residual cells *in vitro*. Over this time, IM may be expected to act either by inducing reversible changes in expression of genes that are kinase dependent, or by selecting for a population of cells expressing higher or lower levels of certain genes as the result of killing more mature CD34$^+$ cells that are sensitive to apoptosis. Surface protein and gene expression of MHC-II in d7 cultured CML cells was normalized to untreated (UT) d0 expression levels. Although no significant differences in MHC-II expression were observed between d7 IM treated and UT CML cells, either on the surface of CD34$^+$CD38$^+$ or CD34$^+$CD38$^-$ LSC populations, or in the expression of MHC-II genes in bulk CD34$^+$ CML cells (*HLA-DR*, *HLA-DP*, *HLA-DQ*; Figure 3A-C; Supplementary Figure 4), there was a trend towards further downregulation of MHC-II expression in 7 day IM treated CML cells. This was supported by a downregulation in *CIITA* in IM treated cells when comparing to UT on d7 (Figure 3D). When IM treated cells at d7 were compared to UT cells at d0, the observed trends reached significance for surface expression of MHC-II on CD34$^+$CD38$^-$ LSC CML cells, and *CIITA* expression (Figure 3A, B&D). These data suggest that the regulation of MHC-II expression in CD34$^+$CD38$^+$ or CD34$^+$CD38$^-$ LSC

CML populations is BCR-ABL kinase independent, as it is not normalized after 48h (Figure 2) or 7 day of IM treatment (Figure 3). These data also suggest that by killing more mature cells within the CD34 compartment IM enriches for cells with lower expression of MHC-II and *CIITA*, inferring that these surviving cells would be even less susceptible to immune attack.

MHC-II/CIITA expression is not modulated by aberrant methylation or TGFβ-mediated signaling in CML stem/progenitor cells

Aberrant DNA hypermethylation of the *CIITA* promoter has been identified as a mechanism for diminished IFNγ-induced CIITA and MHC-II expression in both hematopoietic and solid tumors.[20, 21] Analysis of *CIITA* promoter methylation did not reveal any statistical differences between CML and normal stem/progenitor cells (Supplementary Figure 5). Moreover, treatment of CML cells with methyltransferase inhibitor 5-azacytidine, did not lead to increased MHC-II expression (Supplementary Figure 6).

TGFβ has previously been shown to antagonize CIITA[18, 22, 23] and MHC-II expression in the presence and absence of IFNγ.[19] Indeed, previous work has shown that TGFβ-mediated signals are activated in CML cells,[24] and CML has been demonstrated to drive deregulated cytokine expression in mouse models.[25] Initially, to analyse the impact of TGFβ on CIITA/MHC-II, we treated CML and non-CML cells with SB (TGFβRI, ALK4 and ALK7 inhibitor[26]). While CML stem/progenitor cells were slightly more responsive to TGFβ-mediated downregulation of MHC-II levels than non-CML cells, SB treatment did not effect MHC-II/*CIITA* expression on CML stem/progenitor cells (Supplementary Figure 7).

Inhibition of JAK elevates MHC-II expression in CML stem/progenitor cells and increases their immunogenicity.

The cytokine IL4 is also known to antagonise MHC-II and CIITA expression[18, 22] suggesting that aberrant alterations in cytokine production within the niche may be responsible for their deregulation. Moreover, IL4 has been shown to maintain survival of Ph+ cells upon TKI inhibition.[27] We demonstrate elevated transcript levels of IL4 in CML compared with non-CML stem/progenitor cells (Figure 4A). As IL4 and many cytokines signal via the JAK/STAT pathway we analyzed the impact of cytokine-mediated signaling on *CIITA*/MHC-II, treating CML cells with RUX (a broad spectrum JAK1/2

inhibitor[28]). RUX treatment of CML stem/progenitor cells significantly increased MHC-II and *CIITA* expression both on bulk CD34$^+$ cells (Figures 4B, D&E) and CD34$^+$CD38$^-$ LSC populations (Figures 4C,D&F). As IFNγ activates the JAK/STAT pathway and RUX inhibits the same pathway, it was anticipated that RUX treatment combined with IFNγ might reduce the level of MHC-II upregulation compared to that of IFNγ alone. However this reduction was not significant, suggesting that IFNγ signaling was not fully inhibited in the presence of a JAK inhibitor (Figure 4B-F). Supporting this, analyzing genes associated with the IFN response, only the *GBP1* response was partially modulated downwards in CML cells when IFNγ and RUX treatments were combined (Supplementary Figure 8). These data indicate that RUX-mediated elevation in MHC-II may result from an inhibition of JAKs downstream of IL4-receptors, thus arresting antagonistic signals and enhancing MHC-II expression.[22]

To determine whether the IFNγ- or RUX-mediated elevation in MHC-II expression on CML cells could induce an immune response from alloreactive CD4$^+$ T cells we co-cultured previously treated or control (UT) CML CD34$^+$ stem/progenitor cells with CD3/CD28 activated PBMCs. No increase in expression of the cytolytic marker CD107a (LAMP1) was observed on CD4$^+$ T cells after exposure to the CML targets, suggesting that there was no induction of cytotoxic CD4$^+$ T cells (Supplementary Figure 9). However, when MLRs were conducted, IFNγ and RUX treatments enhanced proliferation of responder CD4$^+$CD69$^+$ T cells, as indicated by decreased mean fluorescence intensity of CTV (Figure 5A). Importantly, the induction of proliferation was MHC-II dependent, as addition of anti-MHC-II blocking Ab blocked cellular proliferation in the cultures containing IFNγ and RUX treated CML cells (Figure 5A).

Discussion:

Eradicating CML LSCs represents a significant therapeutic challenge that will not be addressed with TKI therapy alone, and requires novel treatments to enable removal of the disease reservoir to pave the way to a cure. One potential mechanism utilized by CML stem/progenitor cells to maintain a reservoir of LSCs is to evade the immune system by downregulating MHC-II expression. Our studies demonstrate that modulation of MHC-II is associated with an increase in CML cell immunogenicity in a JAK-dependent manner, leading us to propose a cytokine-mediated pathway for MHC-II deregulation in CML cells (Figure 5B). MHC-II and *CIITA* expression was independent of BCR-ABL kinase activity, as TKI treatment of CML cells did not significantly alter expression levels. Indeed, during IM exposure over 7d the levels of MHC-II and *CIITA* were even further downregulated, rather than normalized. Of note, *in silico* analysis of co-stimulatory molecules revealed a mixed pattern of expression depending on the dataset, although it does appear that OX40L is consistently downregulated in more quiescent CML cell subsets, compared with non-CML cells. These findings suggest that in addition to downregulating MHC-II genes, CML stem/progenitor cells may downregulate additional co-stimulatory molecules, further enhancing immune evasion. Exploitation of therapies that enhance CML stem cell immunogenicity to host immune cells could intensify immune responses against CML LSCs, thereby assisting in the elimination of this population, and ultimately the disease.

While a number of mechanisms regulate MHC-II expression, one main cause for the loss of constitutive or inducible MHC-II expression, in both hematopoietic and non-hematopoietic tumor cells, is epigenetic silencing of the gene encoding CIITA.[29, 30] CIITA acts as a scaffold, interacting with DNA-binding transcription factors, regulatory factor X family, NF-Y and CREB, which interact with and induce MHC-II expression.[31, 32] In addition to recruiting transcription factors, CIITA orchestrates the recruitment of histone acetyltransferases and histone methyltransferases to MHC-II gene promoters to regulate MHC-II expression.[33] While aberrant DNA hypermethylation of the CIITA promoter and breaks (non-random chromosomal translocations) can aid in the downregulation of MHC-II in hematopoietic malignancies,[21, 29, 31] analyses of methylation marks on the CIITA promoter of CML LSCs revealed no difference in the CpG methylation profiles compared with non-CML stem/progenitor cells. Supporting this, 5-azacytidine treatment did not lead to increased MHC-II expression on CML cells.

These data indicate that MHC-II downregulation in CML does not occur as the result of CIITA promoter hypermethylation, suggesting other molecular mechanisms are responsible for regulating CIITA/MHC-II expression in CML.

MHC-II and CIITA expression can be modulated by a number of cytokines with TGFβ, TNFα, IL4 and IL10 known to antagonise their expression,[18, 22, 23] while IFNγ upregulates expression (Figure 5B). Therefore it is likely that the cytokine profile of the BM microenvironment and/or of the leukemic cells themselves would have a significant impact on surface MHC-II expression of CML stem/progenitor cells. Indeed CML LSCs have been demonstrated to produce higher levels of growth factors, via an autocrine loop, including elevated levels of TNFα[34] and *IL4* in CD34+ CML cells. Furthermore, analysis of the BM stroma in CML patients and CML mouse models revealed elevated IL1, IL6, GCSF and TNFα, which together confer a growth advantage of CML LSCs over normal HSCs.[25, 35] These findings indicate that modulation of the cytokine profile in the CML BM will have a significant impact not only on CML versus normal stem/progenitor cell survival, but also on CML LSC immunogenicity.

One family of protein kinases responsible for relaying signals downstream of cytokine receptors are the JAKs. BCR-ABL+ cells exhibit constitutively active JAK/STAT signaling, with JAK2 playing a central role in BCR-ABL-induced leukemogenesis.[36] However STAT5 activation is not dependent on JAK2 activity,[37] and JAK2 can also act independently of STAT5 by activating Myc,[38] β-catenin,[39] or acting directly as a histone modifier,[40] indicating that JAK2 may have pleiotropic roles in CML cells. Moreover one of the main roles of JAK signalling is in immune cell activation/regulation.[41] Here, we showed that treatment of cells with the JAK1/2 inhibitor RUX enhanced the expression of MHC-II on CML stem/progenitor cells, enhancing CD4+ T cell proliferation. While we demonstrated the induction of a helper T cell response through RUX-mediated induction of MHC-II expression, CD4+ T cells have also been demonstrated to function as cytotoxic effectors against leukemic cells.[42, 43] Upon assessment of the cytolytic marker CD107a *in vitro*, we were unable to detect an elevation in expression, indicating that under our experimental conditions cytotoxic CD4+ T cells were not induced. These results may reflect the limitations of the assay regarding the polyclonality of the CD4+ T cell population, or a requirement for optimization at the level of effector/target ratio or co-culture duration prior to assessing for the presence of cytotoxic CD4+ T cells.[42, 43]

Recently RUX has been shown to modulate immune responses in mouse models of graft versus host disease (GVHD) and hemophagocytic lymphohistiocytosis

by dampening the hyperactive immune responses typical of these conditions.[44, 45] However, despite its anti-inflammatory properties, RUX was able to preserve the GVL effect in GVHD mouse models comprising *in vivo* tumour inoculation.[46, 47] Some of these selective effects were explained by differential responses of distinct T cell subsets to RUX particularly when used at different concentrations.[45] It is therefore possible to speculate that the modulation of the immune system by RUX is cell context dependent and potentially affected by the intensity of the inflammatory responses. Moreover using different doses of RUX might enable titration of its effects, thus maximising its ability to enhance immunogenicity of CML cells while sparing T cell immune function.

In conclusion our studies suggest a key role for cytokines, such as IL4, in reducing CML immunogenicity, and offer a novel therapeutic route to aid in the elimination of CML LSCs. Due to the BCR-ABL-independent nature of MHC-II downregulation, combination therapies of JAK inhibitors with TKI could target the disease bulk and the LSCs.[39, 48] Indeed recent studies demonstrate synergy between RUX and NIL, inducing apoptosis in CML LSCs.[49] Our studies could suggest that LSCs remaining after dual RUX/TKI treatment may have a reduced ability to evade host immunity, due to re-expression of MHC-II. Analysis of MHC-II surface expression on LSCs of patients treated with RUX in combination with NIL in current clinical trials (clinicaltrials.gov NCT 01751425; 02253277; 01702064; 01914484) would be of great interest to test this *in vivo*. Boosting MHC-II expression on CML stem/progenitor cells may represent a valid therapeutic approach to reduce disease persistence and our findings should promote the development of novel immunomodulatory-based therapies for CML patients. These therapeutic strategies could enable CML stem cells to be more readily detected and eradicated by the host immune response, which in turn will deliver long-term remission or cure for patients.

Authorship Contributions:

AT designed/performed the majority of experiments, analyzed and interpreted the data, carried out statistical analysis and drafted the manuscript; PG, KK and FP performed some experiments and carried out data analysis; LEMH carried out microarray and pathway analyses; JC carried out the cell sorting for all the experiments and AH processed all of the primary samples; HGJ supervised the studies and performed some of the experiments; DV, TLH, AMM obtained funding for the study, designed the research, supervised the studies, analyzed and interpreted the data and wrote the manuscript. All authors reviewed the manuscript.

Disclosure of Conflicts of Interest:

The authors have no disclosures to declare.

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Sample No.	Sample ID	Source	Gender	Stage	FISH/ t(9:22)	Figures
1	Spirit CML 0762	PB	M	CP	ND	1, 2, 5
2	CML 339	Leukapheresis	F	CP	+	2, 4, 5
3	CML 340	Leukapheresis	M	CP	+*	4, 5
4	CML 341 (Fresh)	Leukapheresis	M	CP	+*	1, 2
5	CML 378	Leukapheresis	F	CP	+	1, 2
6	CML 381	Leukapheresis	F	CP	+	1, 2
7	CML 385	Leukapheresis	M	CP	+*	1, 2
8	CML 388	Leukapheresis	F	CP	+*	1, 2
9	CML 411 (Fresh)	PB	F	CP	ND	4, 5
10	CML 412 (Fresh)	Leukapheresis	M	CP	ND	2, 4, 5
11	CML 441	Leukapheresis	M	CP	+	2
12	CML 442	PB	F	CP	+	4, 5
13	CML 450	PB	M	CP	+	5
14	CML 452	Leukapheresis	M	CP	+	2
15	CML 454 (Fresh)	Leukapheresis	M	CP	ND	3
16	CML 456	Leukapheresis	F	CP	ND	3
17	CML 457 (Fresh)	PB	M	CP	ND	3,4
18	CML 459	Leukapheresis	M	CP	ND	3
19	CML 460 (Fresh)	Leukapheresis	F	CP	ND	1, 3, 4
20	CML 461 (Fresh)	Leukapheresis	F	CP	ND	3, 5

Table 1: Source of clinical samples

PB, peripheral blood; CP, chronic phase; * indicates that the $CD34^+CD38^-$ CML LSCs were also confirmed to be Ph^+ by FISH after sorting; ND, not determined.

Figure Legends:

Figure 1. MHC-II and CIITA are selectively downregulated in CML stem/progenitor cells. (**A**) Ingenuity's Antigen Presentation Pathway overlaid with deregulation in CML versus normal G_0 CD34^{+} cells (n=5 CML, n=2 non-CML). Deregulated members for MHC II-α and II-β (including HLA-DP, DQ, DR) are shown expanded in the bottom left. Significantly downregulated components highlighted in green; (**B**) GSEA analysis demonstrating significant (FDR≤0.15) downregulation of the extended MHC-II gene family in a larger, complementary microarray dataset of G_0/quiescent (left) and dividing (right) CML and normal cells;[14] (**C**) Heat map showing deregulation (as represented by logFC, see colour scale below heatmap) of the MHC-II gene family across multiple CML populations as compared to corresponding non-CML cells from distinct microarray datasets as indicated.[14,15] The data derived from the original dataset[13] is highlighted by the orange bar above the corresponding columns. Average normalized MFI of surface MHC-II expression in primary (**D**) CD34^{+}CD38^{+} CML and non-CML stem/progenitor cells or (**E**) CD34^{+}CD38^{-} CML LSCs and non-CML HSCs cultured for 48h in the presence or absence (untreated (UT)) of IFNγ (100U/mL; n=6 for CD34^{+}CD38^{+}, n=4 for LSC; ± SEM). CD34^{+}CD38^{-} cells, when stringently gated, represent ~1-5% of bulk CD34^{+} cells and overlap considerably with either Hst^{lo}/Py^{lo} or CD34^{+} CFSE[max] popoulations described previously.[13] (**F**) Average gene expression of *CIITA* in bulk CD34^{+} CML and non-CML stem/progenitor cells cultured for 48h ± IFNγ (mean fold change; n=6; ± SEM). Statistical significance was calculated between UT CML sample and all other samples, and if significant, it is indicated by asterisks above the bars. Additional comparisons between samples are indicated by lines.

Figure 2. MHC-II and CIITA downregulation occurs independently of BCR-ABL kinase activity in CML stem/progenitor cells. Primary (**A**) CD34^{+}CD38^{+} CML cells and (**B**) CD34^{+}CD38^{-} CML LSCs were cultured for 48h with TKIs (5 µM NIL, 150 nM DAS, 5 µM IM) or no drug control (NDC) in the presence or absence of IFNγ. Average normalized MFI of MHC-II expression was determined using flow cytometry (n=5; ± SEM); (**C**) *CIITA* expression levels in CD34^{+}CD38^{+} CML cells were analyzed by qRT-PCR (n=5; ± SEM; calibrated to UT NDC sample). Statistical significance was calculated between UT CML sample and all other samples, and if significant, it is indicated by asterisks above the bars.

Figure 3. Extended treatment of CML cells with IM does not normalise MHC-II or CIITA expression. Primary CD34$^+$ CML were treated with 5 µM IM for 7d. Average surface MHC-II expression levels were determined by flow cytometry, normalized to CML UT (d0) sample for (**A**) CD34$^+$CD38$^+$ CML cells and (**B**) CD34$^+$CD38$^-$ CML LSCs; (**C**) The average expression of MHC-II encoding genes *HLA- DR, HLA-DP* and *HLA-DQ* was determined in CD34$^+$ CML cells by qRT-PCR (n=7, calibrated to UT (d0) CML sample). (**D**) The average gene expression of *CIITA* was determined in CD34$^+$ CML cells by qRT-PCR. Statistical significance was calculated between d0 UT CML sample and all other samples, and if significant, it is indicated by asterisks above the bars. Additional comparisons between samples are indicated by lines. NS – not significant.

Figure 4. JAK inhibition elevates MHC-II expression in CML stem/progenitor cells. (**A**) qRT-PCR of RNA/cDNA generated from either non-CML or CML stem/progenitor samples for IL4 transcripts revealed higher expression levels of IL4 in CML cells. Data is expressed relative to the reference gene HPRT1; Primary (**B**) CD34$^+$CD38$^+$ CML cells and (**C**) CD34$^+$CD38$^-$ CML LSCs were treated with IFNγ and/or JAK inhibitor (RUX (200 nM)), as indicated. The average MHC-II expression levels were determined by flow cytometry, normalized to CML UT sample. NS – not significant; (**D**) Representative histograms showing MHC-II expression on CD34$^+$CD38$^+$ and CD34$^+$CD38$^-$ CML cells treated with IFNγ and/or RUX as indicated; Primary CD34$^+$ CML stem/progenitor cells were treated with IFNγ and/or RUX, as indicated above. Thereafter, 300 cells were sorted for either (**E**) CD34$^+$CD38$^+$ CML and (**F**) CD34$^+$CD38$^-$ CML LSC populations and the average gene expression of *CIITA* was determined by qRT-PCR (n=6 for CD34$^+$CD38$^+$, n=3 for LSC; calibrated to UT CML sample). Statistical significance was calculated between UT CML sample and all other samples, and if significant, it is indicated by asterisks above the bars.

Figure 5. Elevated MHC-II expression is associated with an increase in CD34$^+$ CML cell immunogenicity. (**A**) Activated and CTV labelled T cells (MNCs from healthy donors) were co-cultured with bulk CD34$^+$ CML cells for 72h (± treatment as indicated; Block – 10 μg/mL anti-pan HLA-class II blocking Ab). Proliferation of the responder cells was measured as a reduction in the MFI of CTV-labelled CD4$^+$CD69$^+$ T cells (n=6 ± SEM; n=3 for MHC-II blocking assays); (**B**) The autocrine or paracrine growth

factor/cytokine signaling pathways that regulate MHC-II expression. Statistical significance was calculated between UT CML sample and all other samples, and if significant, it is indicated by asterisks above the bars.

Figure 1 - Tarafdar et al.

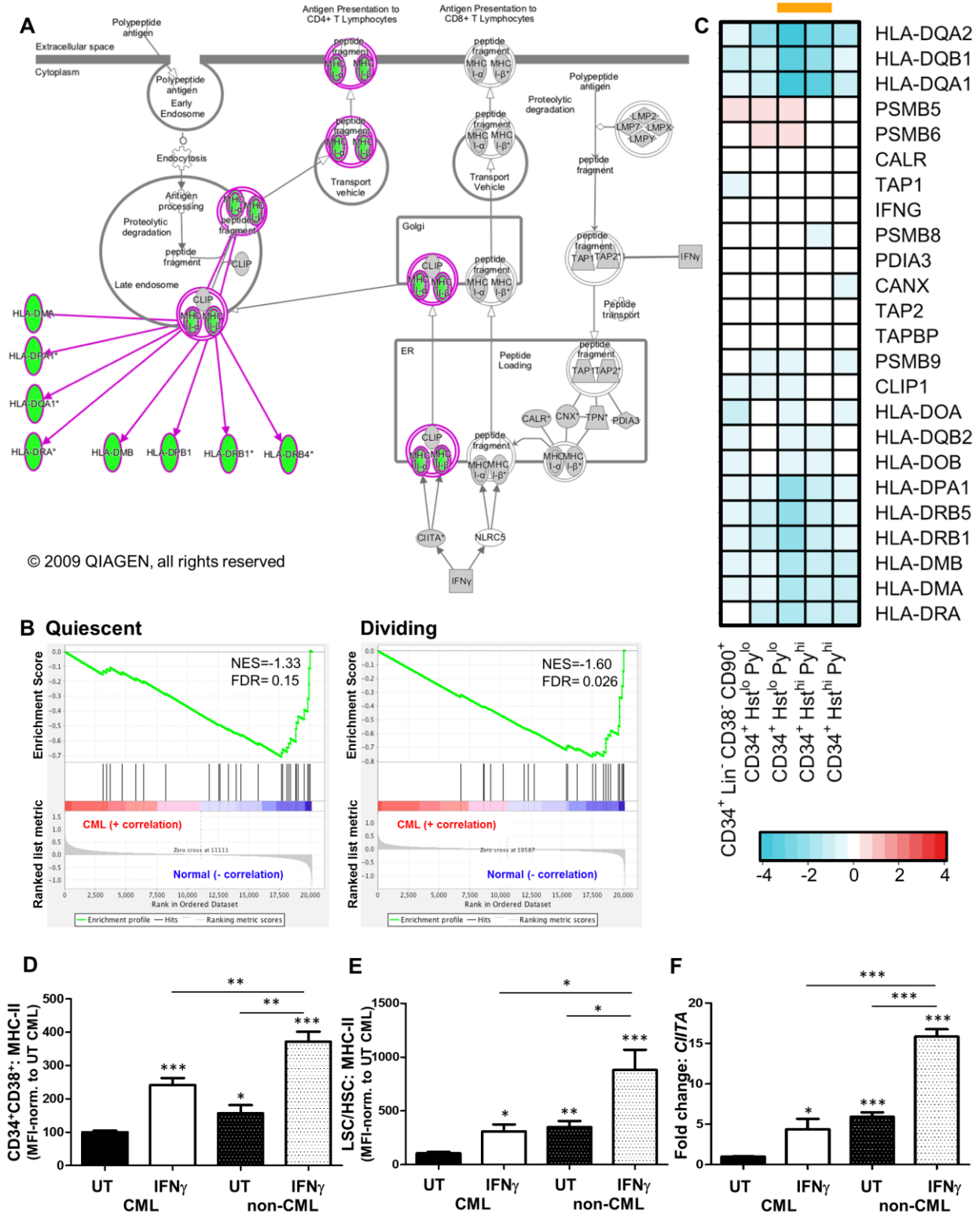© 2009 QIAGEN, all rights reserved

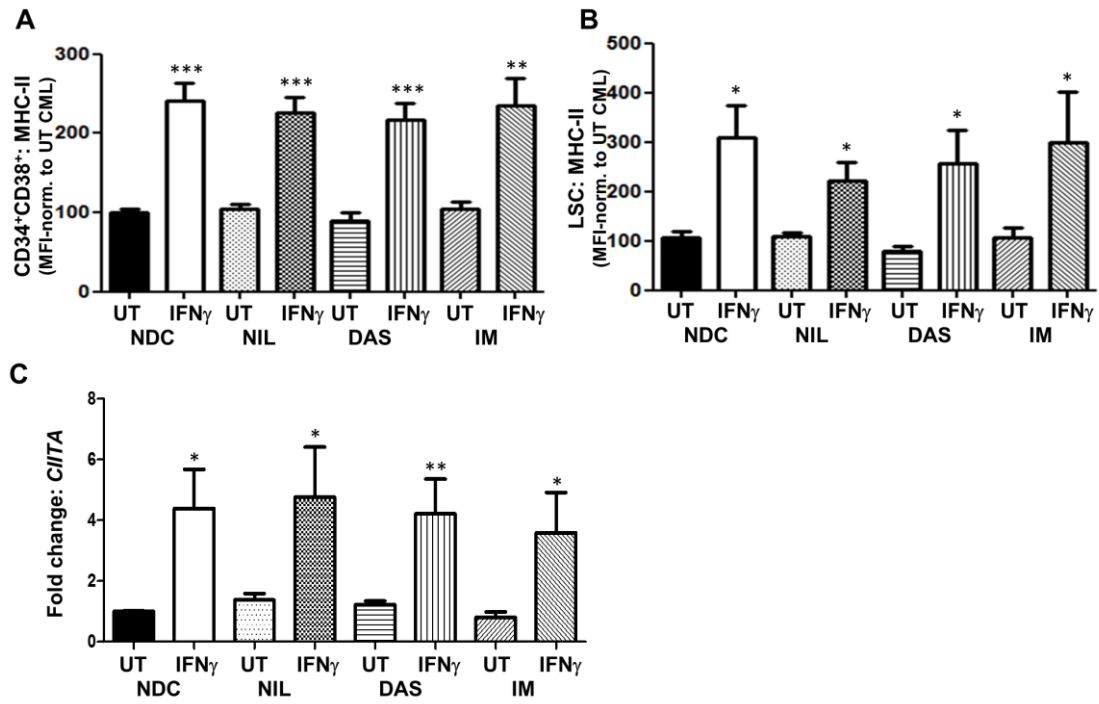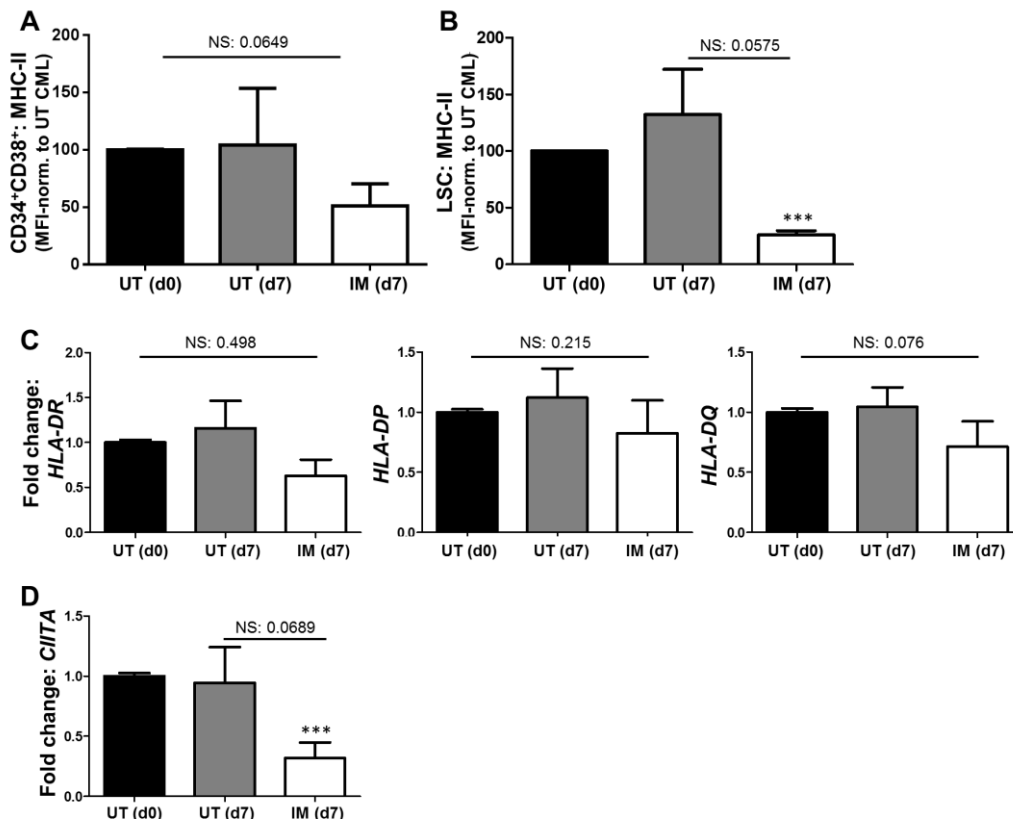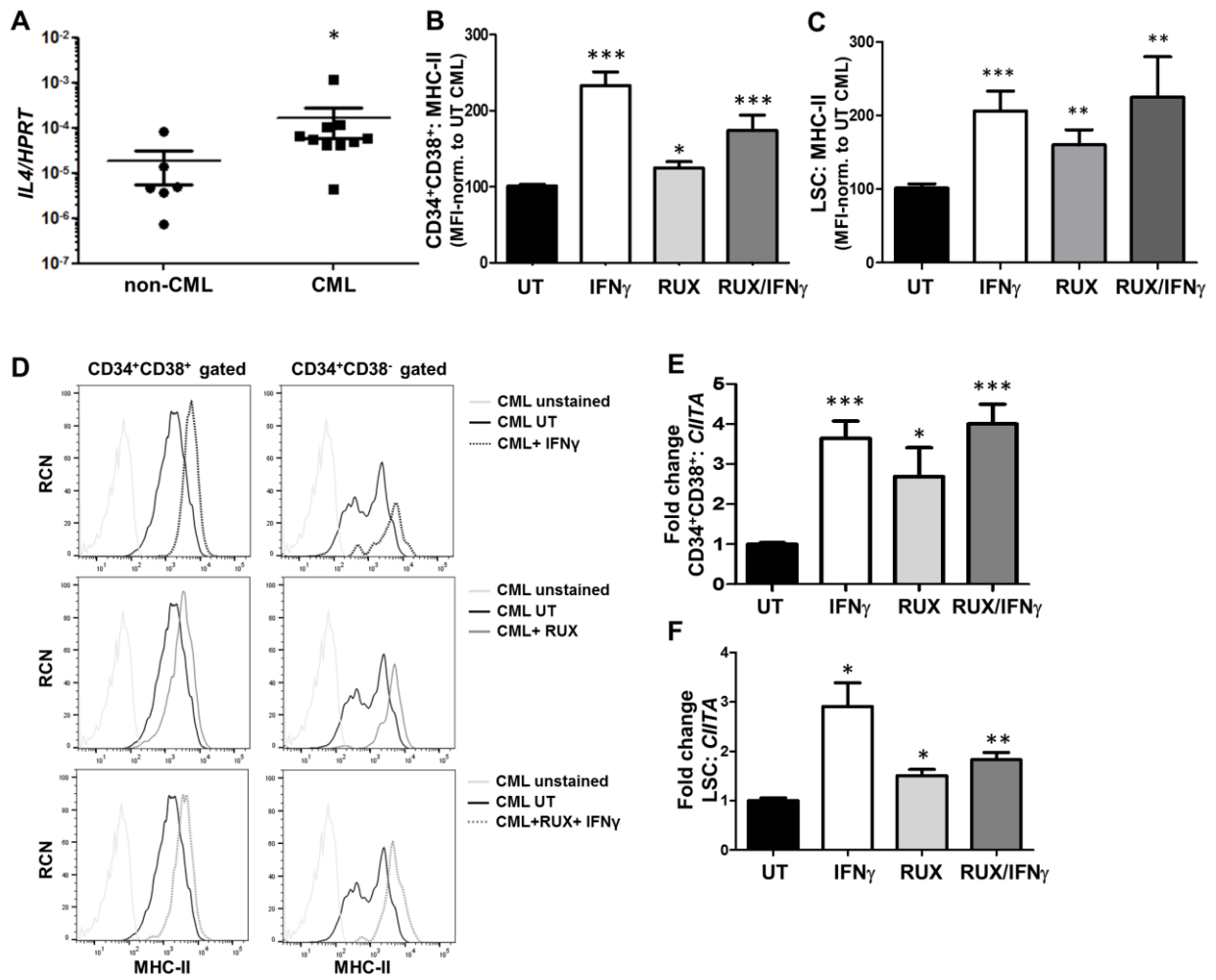Figure 2 - Tarafdar et al.

Figure 3 - Tarafdar et al.

Figure 4 - Tarafdar et al.

Figure 5 - Tarafdar et al.