MHC class II expression through a hitherto unknown pathway supports T helper cell dependent immune responses: implications for MHC class II deficiency

by

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Short title: Th cell dependent immunity in BLS models
Scientific heading: Immunobiology

This work was supported by the Deutsche Forschungsgemeinschaft through SFB 456 and SFB576 (to I.F.), AW1600/1-1 (to A.W) and BU1410/1-1, by the Volkswagen Foundation (to I.F.), by the Croatian Ministry of Science and Technology through grants 0062004 and 0062005, and by Fonds der Chemischen Industrie and the Land Nordrhein-Westfalen through fellowships to T.B.
Abstract

MHC class II (MHCII) deficiency or Bare Lymphocyte Syndrome (BLS) is a severe immunodeficiency characterized by deficient T helper (Th) cell dependent immunity. The disease is caused by defects of the MHCII promoter complex resulting in low or absent MHCII expression. We demonstrate in a murine model of MHCII deficiency (RFX5- or CIITA-deficient mice) that residual MHCII expression by professional APC is sufficient to support activation of adoptively transferred Th cells. Furthermore, upon transplantation of WT thymic epithelium we observed development of endogenous Th cells with restoration of Th cell dependent antibody responses and immunity to cytomegalovirus infection, thus opening the possibility of an alternative treatment regimen for BLS. Residual MHCII expression was further induced by the presence of Th cells and also other stimuli. Analysis of CIITA/RFX5 double deficient animals revealed that this inducible MHCII expression is genetically independent of the known promoter complex and thus constitutes an alternative MHCII expression pathway. In these experiments, we also detected a novel repressive function of the RFX complex in the absence of CIITA.
Introduction

MHC class II (MHCII) molecules play a pivotal role in the development of protective T cell immunity by displaying antigenic peptides from the endocytic pathway to CD4⁺ Th cells.¹ MHCII gene expression is tightly regulated and mostly restricted to thymic epithelium²,³ and professional APC⁴-⁶, but inducible also in other cell types. Coordinated expression of all MHCII genes is controlled by a conserved promoter region.⁷,⁸ This promoter contains the so-called X, X₂, and Y boxes, which bind the heterotrimeric RFX complex⁹ consisting of RFX5, RFXAP, and RFXANK¹⁰-¹³, CREB (cAMP responsive element binding protein)¹⁴-¹⁶, and NF-Y¹⁷. Transcriptional activity of MHCII genes is, however, not only dependent on these DNA-binding molecules but requires the cell type-specific¹⁸,¹⁹ or inducible presence²⁰,²¹ of the transcriptional coactivator CIITA.²²

Defects in one of the genes of the RFX proteins or CIITA lead to almost complete absence of MHCII expression. This phenomenon was first observed in a human hereditary immunodeficiency, called MHCII deficiency or Bare Lymphocyte Syndrome (BLS).⁵,²³,²⁴ BLS patients have reduced Th cell numbers and are extremely susceptible to infections with bacterial, viral, and fungal pathogens.²⁵ The development of Th cell dependent class switched immunoglobulin responses is impaired in these patients, despite reports of residual MHCII expression on B cell lines from some patients and the presence of CD4⁺ T cells.²⁶,²⁷ It seems unlikely that the Th cells of BLS patients develop in the thymus since the thymic cortex, which is normally responsible for positive selection, showed no MHCII expression in BLS patients.²⁸-³⁰ To date the only available treatment for this fatal disease is allogeneic BM transplantation (BMT), which in the case of BLS has a low success rate.²⁷,³¹

To further investigate the mechanisms underlying this immunodeficiency and to test novel treatment strategies we and others have generated mouse models of BLS by inactivating the genes coding for CIITA and for RFX5.³²-³⁵ Similar to the human disease, CIITA⁻/⁻ and RFX5⁻
mice lack MHCII expression on the majority of peripheral APC. Unexpectedly, residual MHCII expression was found in the thymic medulla, on a subset of DC in peripheral lymphoid organs and on in-vitro activated RFX5−/− B cells. Due to the absence of MHCII in thymic cortex both mutants are unable to generate Th cells and thus fail to mount Th cell dependent immune responses.32-34

To test whether the residual MHCII expression in RFX5−/− and CIITA−/− mice (“BLS mice”), and potentially also in patients, could support Th dependent immune responses we reconstituted BLS mice with peripheral Th cells by implantation of WT embryonic thymi. The reconstituted mice were found to mount Th cell dependent humoral responses and were able to show Th cell mediated control of murine cytomegalovirus (MCMV) infection. In addition, reconstitution with Th cells led to an increase in MHCII expression on professional APC. This “alternative” MHCII expression was found to be inducible and independent of RFX5 and CIITA. Furthermore, CIITA/RFX5 double deficient (CR−/−) mice revealed a novel repressive function of RFX5.

Materials and Methods

Mouse Maintenance and Typing

RFX5−/− mice33 and CIITA−/− mice32 were on a mixed 129/Ola/C57BL/6 or 129/Sv/C57BL/6 background, respectively, and were intercrossed to generate CR−/− mice. The double knockout was confirmed by PCR and Southern Blot. For the RFX5 deficiency the primers RFX5-N8B 5´ACATAATGACCGTTCTCGAGG3´, RFX5-N4B 5´AGCAGACTTGGCTCTGAGCTG3´ and RFX5-N3 5´TCTACCTTCAGCTCCCATCGG3´ (WT 500 bp, KO 860 bp) and for the CIITA deficiency the primers JaxCIITA-1 5´GATCGGAGACAAAGGTTGTGT3´, JaxCIITA-2 5´GTCAGGGAGCAGGATCTTTG3´, and Neo-reverse
5′GACTAGTGAGACGTGCTACT4′ (WT 550 bp, KO 400 bp) were used. These PCR primers were also used to detect the presence of contaminating WT cells after transplantation. The Southern Blot analysis for the detection of the CIITA alleles was described previously.\textsuperscript{32} Aα\textsuperscript{−/−} mice\textsuperscript{36} were on a pure C57BL/6 background and were provided by H. Bluethmann (Basel, Switzerland). C57BL/6\textsuperscript{nu/nu} mice were purchased from Bomholtgard (Ry, Denmark). The animals were housed in specific pathogen–free conditions.

**Thymus Transplantation**

Thymic lobes were prepared from C57BL/6 embryos at gestational day 14.5. In some experiments these lobes were irradiated with 3000 rad and cultured for 5 days, or cultured in medium containing 1.35mM 2-deoxyguanosine (Sigma) for 5 days before transplantation. After anesthesizing 4-6 week old mice of the respective strains with Ketanest/Rompun, 6 thymic lobes were transplanted under the kidney capsule.\textsuperscript{37}

**Cell Preparation and Culture**

To enrich for DC from splenic or thymic cell preparations, the organ was digested with collagenase D (Roche Diagnostics, Mannheim, Germany) prior to preparation of a single-cell suspension.\textsuperscript{38} Anti-CD11c (N418) magnetic microbeads were used to positively enrich for DC from total splenocytes (Miltenyi Biotec, Germany). For analysis of MHCII expression on B cells splenocytes were cultured O/N in the presence of 10µg/ml anti-CD40 (Pharmingen), CpG-Oligodeoxynucleotide (ODN) 1668 (TCC-ATG-ACG-TTC-CTG-ATG-CT; TIB MOLBIOL, Berlin, Germany), or LPS (Sigma) and analyzed by flow cytometry the next day. To enrich Vα3 T cells from LN and spleen of 2D2 mice biotinylated anti-Vα3 (Pharmingen) and streptavidin coupled magnetic microbeads (Miltenyi Biotec) were used. To enrich B cells for adoptive transfer from CD45.1 congenic C57BL/6 mice (Charles River Laboratories) anti-CD19 magnetic microbeads (Miltenyi Biotec) were used.
Human peripheral blood lymphocytes were enriched on a Pancoll gradient (PanBiotech, Germany) and cultured in RPMI 1860 (Gibco). For activation either LPS (10 µg/ml final conc.), CpG 2006 (TIB MOLBIOL, Germany, 0.5 µM final conc.), polyI/C (Sigma, 50 µg/ml final conc.), or CD40L (Alexis, Switzerland) were added to the cultures. SJO and RO cells were gifts from B. Grosphierre (Paris, France) and M. Müschen (Düsseldorf, Germany) and cultured in DMEM (Gibco) supplemented with 15% FCS, glutamine, non-essential amino-acids, sodium pyruvate, β-mercaptoethanol, and penicillin/streptomycin.

**Cytofluorometric Analysis**

Fluorescence staining was performed as previously described. The following antibodies were purchased from Pharmingen: RM4-5 for CD4, 53-6.7 for CD8, 25-9-17 for I-A/β2, HL3 for CD11c (N418), H57 for TCRβ, M1/70 for CD11b. M5/114 for MHCII staining, anti-mouse-B220 (RA3-6B2), and anti-nitrophenyl (N1G9, isotype control for HLA-DQ) were purified from hybridoma supernatants. FITC-coupled anti TCRβ, CD11b and CD11c antibodies were combined as non-B cell marker. Anti-HLA-DQ and anti-HLA-DP (Pharmingen) were revealed with anti-mouse IgG1 (Pharmingen). Anti-human-CD19 and anti-human-CD3 were a gift of ImmunoTools (Germany). Dead cells were excluded by Topro-3, Propidium Iodide or Cytox Green staining (Molecular Probes). Analysis was performed on a FACScalibur (Becton Dickinson).

**Immunohistochemistry**

Three-micrometer frozen tissue sections were air dried, fixed in ice-cold acetone and blocked in phosphate-buffered saline containing 0.6% H2O2, 5% goat serum, and 0.1% NaN3 followed by 4% fetal calf serum in Tris-buffered saline; they were stained with digoxygenin-conjugated M5/114 and biotinylated RA3-6B2. The MHCII antibody was detected with anti-digoxygenin
peroxidase, followed by 3-amino-9-ethyl carbazole (Sigma) and the B220 antibody was detected with streptavidin-alkaline phosphatase and alkaline phosphatase substrate kit III/blue (Vector). For analysis by confocal microscopy cryosections of thymi were stained with UEA-1-FITC (Vector) biotin–anti-mouse I-A\(^b\) (BD) and anti-mouse-CD11c (BD). The antibodies were revealed with biotin-PE (BD) and anti-armenian Hamster-Cy5 antibody (Dianova). Slides were mounted in Fluoromount G (Southern Biotechnology) and analyzed with a Zeiss LSM 510 microscope (Thornwood, NY).

**Adoptive transfers**

For transfer of Th cells V\(\alpha\)3\(^+\) T cells were purified by magnetic sorting from 2D2 TCR transgenic mice and labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE). 5\(\times\)10\(^6\) of these cells were injected into RFX5\(^{-/-}\), CIITA\(^{-/-}\), A\(\alpha\)\(^{-/-}\) and C57BL/6 mice. Two days later the recipients were immunized at the tail base with 50 \(\mu\)g of MOG p35-55 in CFA. Four days after the immunization LN cells were stained with antibodies for CD4 and V\(\alpha\)3 and analyzed on a FACScalibur.

For transfer of B cells 2\(\times\)10\(^7\) CD19\(^+\) B cells from CD45.1 congenic C57BL/6 mice were injected into A\(\alpha\)\(^{-/-}\) and RFX5\(^{-/-}\) animals. Three weeks later splenocytes from these mice were analyzed by FACS for expression of CD45.1, I-A\(^b\), B220, and non-B cell marker. NP-CG Immunizations

Mice of the different strains were injected i.p. with 100 \(\mu\)g of alumn-precipitated and \(\gamma\)-irradiated NP15- chicken gamma globulin (CG) and boosted with 50 \(\mu\)g of NP-CG after 3 weeks. Serum was collected as described and NP-specific IgG1 or IgM titers were measured by ELISA.\(^{40}\)
MCMV infection and virus plaque assay

2 x 10⁵ PFU of the tissue culture grown Smith strain of MCMV (ATCC, VR-194) were injected into the footpads of the different mouse strains. Three weeks after infection salivary glands and lungs were collected under sterile conditions and stored at −70 °C. The virus titers in organ homogenates were determined by in vitro plaque assay after centrifugal enhancement of infectivity. To prevent secondary plaque formation we used methylcellulose (Merck) and the plaques were counted after 3-5 days of incubation at 37 °C. Detection limit of the assay was 100 PFU of MCMV per organ. MCMV-specific antibodies in sera were determined by ELISA.

Patients

The patients with MHCII deficiency were treated at the University Children’s Hospital, Ulm, and were one and four years of age, respectively. The molecular defect of both patients was located in the RFX-ANK gene and resulted in a amino acid exchange at position 121 (D->V). Analysis of PBL was performed after obtaining written consent from the parents.

RT-PCR

10⁵ SJO cells, RO cells, or blood lymphocytes were lysed in TRIzol (Gibco/BRL) reagent. RNA was prepared according to instructions of the manufacturer and DNaseI (Promega) digested. After reverse transcription (Gibco/BRL) 1 μl of cDNA was used for PCRs with the following primers: CGTCTTCCCCCTCCATCGTG and GTCATCTTCTCGCGGTTGGCC for β-actin (273 bp), ATGGCCATAAGTGGAGTCCCTGTGC and CCCTGCGTTCTGCTGCATTGC for HLA-DRα (754 bp), GTCATTTCTCAATGGGACGGAGC and CCGTAGTTGTGCTGCAGTGGTGTG for HLA-DRβ (208 bp). Magnetically purified mouse B cells and DC were lysed in Trizol (Gibco), RNA prepared, and reverse transcribed by Superscript II (Invitrogen). The following
probes from a probe library (Exiqon/Roche) and primers were used for the amplification: I- 
Aβ: probe mouse 16, cacaggactcagaagaggacact and gtcaaaactctgtcagactgc; I-α: probe 
mouse 90, tctgattctgggggtctc and accataggtgcctacgtggt; βActin: probe mouse 82, 
tgacaggatgcagaaggaga and cgctcaggaggagcaatg. The data was acquired on a ABI7500 PCR 
machine.

**Results**

*Residual MHCII expression in RFX5 and CIITA deficient mice supports Th dependent immune responses*

Since RFX5−/− mice and, to a lesser extent, CIITA−/− mice express MHCII on a proportion of 
peripheral APC, we wanted to explore the possibility if these APC can support MHCII- 
dependent immune responses when appropriately selected Th cells are present. CFSE labeled 
Vα3+ Th cells from 2D2 TCR transgenic mice, whose TCR is specific for the MOG-peptide 
p35-55 in the context of I-Aβ,44 were adoptively transferred into RFX5−/−, CIITA−/−, Aα−/− and 
C57BL/6 mice. The hosts were immunized with p35-55 and four days later the proliferation 
of the Vα3+CFSE labeled LN and spleen cells of graft origin was determined by FACS 
analysis. We used the Aα−/− strain,36 which lacks the MHCII structural genes, as a negative 
control to rule out that WT APC potentially introduced with the grafted cells restored immune 
function independent of host APC. C57BL/6 mice served as controls with WT I-Aβ 
expression allowing optimal activation of the transferred Th cells. We found that the 
transferred T cells proliferated only in C57BL/6, RFX5−/− and CIITA−/− but not Aα−/− hosts 
(Fig.1A), indicating that residual MHCII expression on endogenous APC in the mouse 
models of BLS is sufficient to support Th cell activation. The lower T cell proliferation in 
RFX5−/− and CIITA−/− mice is likely explained by the fact that not all APC express MHCII in
the mice. To validate the Aα−/− control we ascertained that MHCII+ APC were not rejected in Aα−/− mice in which MHCII expression is completely absent. For this purpose, WT B cells carrying the CD45.1 surface marker were adoptively transferred into Aα−/− mice and RFX5−/− mice, which are likely to be tolerant to MHCII. In both recipients, MHCII+ B cells survived equally for at least three weeks (Fig. 1B), demonstrating that no allo-rejection of MHCII+ cells takes place in Aα−/− mice.

Reconstitution with Th cells and normal humoral immune response in CIITA−/− and RFX5−/− mice after implantation of WT thymi

To analyze the ability of RFX5−/− and CIITA−/− mice to support MHCII dependent immune responses in the presence of Th cells in a more physiological setting, we allowed development of Th cells by transplanting embryonic thymi from C57BL/6 mice under the kidney capsule of BLS mice. We controlled for successful restoration of Th cell dependent immune responses by transplantation of WT thymi into athymic MHCII-expressing nude mice. We also included the Aα−/− mice mentioned above as controls for unintentionally co-transferred donor APC. Nine weeks after implantation of the thymi we found the compartment of CD4 T cells in inguinal LN to be restored to almost WT levels in all mutants (Fig. 2).

Subsequently we tested whether after restoration of a peripheral Th cell compartment RFX5−/− or CIITA−/− mice generated a Th cell dependent IgG1 response to the hapten nitrophenyl coupled to chicken globulin (NP-CG). Ten and 13 weeks after transplantation the mice were immunized with NP-CG and bled three weeks after the second immunization. Four of 5 transplanted RFX5−/−, 4/4 CIITA−/− and 3/3 nude mice responded with high serum IgG1 titers (Fig. 3A), showing the ability of functional T–B interaction in reconstituted CIITA−/− and RFX5−/− mice. Since the transplanted Aα−/− mice developed no NP-specific IgG1 responses despite the presence of Th cells (Fig.3A and 2), it is unlikely that donor APC, which may have been transferred along with the thymi, were responsible for the recovery of Th cell
dependent antibody responses in RFX5\(^{-/-}\) or CIITA\(^{-/-}\) mice. Nevertheless, we performed an additional experiment in which irradiated or 2-deoxyguanosine-treated thymi consisting only of radiation resistant thymic stroma were used for transplantation. Although the efficiency of Th cell reconstitution was transient and relatively low due to the treatment (data not shown), 4/9 RFX5\(^{-/-}\) mice and 4/6 CIITA\(^{-/-}\) mice showed increased serum levels of NP-specific IgG1 (>10 µg/ml) in contrast to non-transplanted RFX5\(^{-/-}\) (0/3) and CIITA\(^{-/-}\) mice (0/3) (Fig. 3B). Transplanted nude mice showed a similar efficiency of reconstitution as RFX5\(^{-/-}\) and CIITA\(^{-/-}\) mice after transplantation of treated thymi (2/6 mice; Fig. 3B). Since NP-CG represents a non-pathogenic antigen we challenged reconstituted RFX5\(^{-/-}\) animals also with the viral pathogen MCMV six months after transplantation. We determined serum levels of MCMV-specific antibodies of the IgM and IgG isotypes at day 0 and day 21 after MCMV infection. Th independent MCMV-specific IgM titers were present in all mice analyzed, including non-transplanted A\(\alpha\)\(^{-/-}\) mice, and did not differ between the groups (data not shown). All of the 5 transplanted RFX5\(^{-/-}\) mice produced MCMV-specific antibodies of the IgG type on day 21 at levels comparable to WT controls (Fig. 3C). In contrast, non-transplanted RFX5\(^{-/-}\) controls as well as transplanted and non-transplanted A\(\alpha\)\(^{-/-}\) mice did not generate MCMV-specific IgG antibodies (Fig 3C).

**Increased CD4 T cell dependent clearance of MCMV after transplantation with WT thymi**

Th cells are not only able to provide help to other cells of the immune system, but also exhibit effector functions themselves. Clearance of MCMV infection in salivary glands but not other organs is Th cell dependent\(^{45-48}\) and mediated by IFN\(\gamma\)\(^{49}\) and TNF\(\alpha\).\(^{50}\) To test whether the Th cells in transplanted RFX5\(^{-/-}\) mice were also able to control MCMV infection, virus titers were determined in salivary glands and lungs three weeks after infection with MCMV. Non-
transplanted RFX5−/− mice could not clear the virus from the salivary gland and the virus titers there were ca. 1000 fold higher than in C57BL/6 controls. The viral titers in the salivary glands of transplanted RFX5−/− mice, however, were 10 fold lower than in non-transplanted RFX5−/− controls (p< 0.01) (Fig. 3D) but yet higher than in WT controls. In contrast, we detected identical virus titers in the salivary glands of transplanted and non-transplanted Aα−/− mice (data not shown), indicating that the effect observed in transplanted RFX5−/− mice is specific and requires residual MHCII expression. In agreement with previous reports that Th cells are not required to resolve MCMV infection of the lung, we found that the infection was cleared from this organ by all animals without significant differences (Fig 3D, data not shown).

Residual MHCII expression is independent of both RFX5 and CIITA

As shown above, reconstitution of the Th cell compartment by transplantation of WT thymi into BLS mice allows the generation of Th dependent immune responses because of residual MHCII expression in the peripheral immune system. Although it was reported that a) the transcription of MHCII is solely regulated by association of CIITA with the constitutively assembled promoter complex22 and b) absence of RFX5 results in disassembly of this promoter complex,15,51 the data available from knockout mice implied that RFX5 deficiency permits higher “residual” MHCII expression than CIITA deficiency 32-34. To directly compare the RFX5−/− and CIITA−/− strains and to investigate whether “residual” MHCII expression in these mice is completely independent of CIITA and RFX5 we assessed MHCII expression in various lymphoid organs of CR−/− mice in comparison to RFX5−/− and CIITA−/− mice. If CIITA was the sole regulator for the induction of MHCII transcription we expected these mice to exhibit a phenotype similar to single CIITA deficient mice with residual MHCII expression present on few cells only. When we analyzed MHCII expression on thymic and splenic
sections by immunohistochemistry we made the surprising observation that CR<sup>/−</sup> mice showed stronger residual MHCII expression than CIITA<sup>/−</sup> mice, phenotypically resembling RFX5<sup>/−</sup> mice (Fig. 4). We further investigated the localization and phenotype of MHCII<sup>+</sup> cells in the thymus by confocal microscopy. Sections were stained for MHCII, CD11c and UEA-1<sup>/−</sup> to reveal MHCII expression on DC and medullary epithelial cells. We observed expression of MHCII on a fraction of both cell types in RFX5<sup>/−</sup> and CR<sup>/−</sup> mice and, in particular, on thymic DC which were located in the vicinity of UEA-1<sup>+</sup> epithelial cells (Fig. 4A). In agreement with a previous report CIITA<sup>/−</sup> mice expressed high levels of MHCII only on few medullary cells<sup>/34</sup> (Fig. 4A). FACS analysis was then performed to quantify and further characterize the MHCII expressing cell types. We found a sizable fraction of thymic MHCII<sup>+</sup>CD11c<sup>+</sup> DC in RFX5<sup>/−</sup> and CR<sup>/−</sup> mice (mean values of 20% and 14% of total CD11c<sup>+</sup> cells, respectively) and only a few of these cells in CIITA<sup>/−</sup> mice (3%) as well as CIITA<sup>/−</sup>/RFX5<sup>/−</sup> mice (4%) (Fig. 5A and 5B). In the spleen, MHCII expression was found on 2–3 % of DC in RFX5<sup>/−</sup> and CR<sup>+</sup> mice but not in CIITA<sup>/−</sup> mice (Fig. 5A). These findings were also confirmed by quantitative RT-PCR analysis (Fig. 5D). In contrast to spleen and thymus, a high proportion of MHCII<sup>+</sup> DC was detectable in inguinal and brachial LN of all three mutant strains (Fig. 5C). All LN MHCII<sup>+</sup> DC expressed the DEC205 marker<sup>/53</sup> (Fig. 5C) in agreement with findings reported previously for CIITA<sup>/−</sup> mice.<sup>/34,54</sup> B cells did not express MHCII in spleen or Peyer’s Patches of all three BLS mouse strains analyzed (data not shown).

Taken together, it appears that CIITA is not essential for residual expression of MHCII in RFX5<sup>/−</sup> mice and that in the absence of CIITA RFX5 represses rather than induces MHCII expression in some cell types.

**Alternative MHCII expression is inducible in vitro and in vivo**

When we analyzed MHCII expression in transplanted RFX5<sup>/−</sup> or CIITA<sup>/−</sup> mice we found that the fraction of MHCII expressing B cells increased from background values of less than 2% in
non-transplanted mice to values in the range of 5–15% (Fig. 6A and B, data not shown), suggesting that alternative MHCII expression was further inducible. To exclude that these MHCII⁺ cells were derived from donor cells emigrating from the transplanted WT thymus we amplified their RFX5- and CIITA-alleles by PCR. Only in some samples a weak band for the WT allele could be detected following sensitive Southern Blot hybridization (Fig. 6C, top) but not in the ethidium bromide stained gel (Fig. 6C, bottom), indicating that only very few contaminating donor cells were present within the sorted MHCII⁺ B cells and DC (Fig. 6C, and data not shown). In addition, we failed to detect MHCII⁺ B cells in transplanted Aα⁻/⁻ animals (Fig. 6A).

To analyze whether the residual MHCII expression in CIITA⁻/⁻ or RFX5⁻/⁻ mice could be upregulated we tested splenic B cells from non-transplanted mice for MHCII expression after over-night culture in the presence of various activating agents. We observed that stimulation with anti-CD40 antibody, CpG-oligodeoxynucleotide, and LPS but not pI/pC resulted in strong induction of MHCII expression in RFX5⁻/⁻ and CR⁻/⁻ B cells (Fig. 6D and data not shown). In contrast, only few B cells lacking expression of CIITA were induced to moderately increase MHCII expression after activation (Fig. 6D). For both, WT and mutant mice, the increased surface expression of MHCII on activated B cells was not accompanied by increased mRNA expression levels, indicating that posttranslational modifications of MHCII expression were responsible for this effect (Fig. 6F). DC isolated from the spleen of BLS mutant mice also responded to in vitro maturation on plastic cell culture dishes by up-regulation of MHCII and B7.2 expression (Fig. 6E and data not shown). Remarkably, more than 80% of splenic DC from RFX5⁻/⁻ and CR⁻/⁻ mice but also 40% of DC from CIITA⁻/⁻ mice expressed MHCII after the culture period (Fig. 6E).
Residual MHCII expression in human MHCII deficiency

To assess whether residual MHCII expression was also detectable in human MHCII deficiency, in particular following activation through TLR ligands, we first analyzed the RFX5 deficient B cell lines RO and SJO\textsuperscript{10,55} for MHCII expression in the absence or presence of CpG or LPS. Solely on a fraction of SJO cells we found HLA-DR but not HLA-DP and HLA-DQ expression irrespective of the stimulation (Fig. 7A, B), indicating that also in humans MHCII expression was indeed possible in the absence of RFX5. We confirmed this observation by RT-PCR for HLA-DR $\alpha$- and $\beta$-chain transcripts and found that the HLA-DR$^+$ cell population also contained more HLA-DR transcripts, especially for the $\alpha$-chain.

We also had the possibility to analyze PBL derived from two patients with MHCII deficiency, both with mutations in the RFX-ANK gene. We stimulated PBL from these patients and a healthy control with LPS, CpG, polyI/C, or CD40L O/N. The next day the cells were analyzed for HLA-DR, -DP, and -DQ expression. In none of the cultures we could detect MHCII expression (Fig. 7D-G and data not shown). Thus, in one RFX5 deficient cell line but not in RFX-ANK-deficient patients residual MHCII expression could observed.

Discussion

MHCII deficiency (BLS) represents a severe immunodeficiency syndrome in humans caused by mutations of parts of the MHCII promoter complex.\textsuperscript{24} The underlying defects in immune function are twofold: 1) inability in MHCII-restricted positive selection of Th cells due to lack of MHCII on thymic cortical epithelium and 2) absence of MHCII-dependent antigen presentation due to impaired MHCII expression on peripheral APC. We report here that the presence of properly selected Th cells in mouse models of MHCII deficiency results in further upregulation of residual MHCII expression on hemopoietic cells, restores Th cell dependent humoral immunity, and significantly improves CD4 T cell dependent clearance of MCMV.
Beyond these clinically relevant results, our analysis of the genetic requirements for residual MHCII expression revealed a so far unknown mechanism of MHCII expression, which is independent of the known promoter complex and appears to be repressed by RFX5.

**RFX5- and CIITA-independent expression of MHCII**

Although CIITA\(^{-/-}\) and RFX5\(^{-/-}\) mice both possess some MHCII\(^{+}\) cells,\(^{32-34}\) MHCII expression is more prominent in RFX5\(^{-/-}\) mice. Surprisingly, CR\(^{-/-}\) mice were phenotypically indistinguishable from RFX5\(^{-/-}\) mice, with increased expression levels of MHCII on DEC205\(^{+}\) DC, activated B cells, and thymic UEA-1\(^{+}\) medullary epithelial cells. Although this result appears paradoxical at first, it could be explained if the RFX complex serves as a transcriptional suppressor in the absence of CIITA. Thus, the role of the RFX complex may be a dual one: a) suppression of MHCII transcription in the absence of CIITA and b) activation of MHCII transcription by binding of CIITA.\(^{22}\) This might apply to professional APC in particular, since somatic cells were not found to be MHCII positive in the CR\(^{-/-}\) mice. A suppressive activity of RFX5 has only been reported for a collagen promoter construct in rat fibroblasts,\(^{56}\) but the mechanism of suppression is different in this case because the RFX binding site in the collagen promoter overlaps with the transcriptional start site.

It is of particular interest for the understanding of the immune defect in BLS that in the absence of the RFX complex a substantial proportion of splenic and LN DC express MHCII. Such cells are also present in LN but not spleen of CIITA\(^{-/-}\) mice as previously reported.\(^{33,34,54}\) Remarkably, the majority of splenic DC of all three BLS mutant strains analyzed in this study drastically upregulated MHCII expression upon maturation* in vitro* (Fig. 6E). This finding indicates that RFX5- and CIITA-independent expression of MHCII in DC is not restricted to a certain subset of DC but is rather dependent on the maturation status of the cells. A similar, but activation dependent upregulation of MHCII was observed for B cells of RFX5\(^{-/-}\) and CR\(^{-/-}\) mice and for a minor fraction of CIITA\(^{-/-}\) B cells (Fig. 6D). Interestingly, a CIITA independent mechanism of
MHCII mRNA stabilization has been described for B cells stimulated with CpG DNA. However, using quantitative RT-PCR analysis we were not able to detect increased levels of MHCII mRNA in activated B cells, implying that posttranslational modifications of MHCII expression also occur in activated B cells similar to DC. It seems possible that such mechanisms have evolved to allow MHCII expression to be continued in activated professional APC, even after inhibition of the classical MHCII transcription pathway through a pathogen. Also thymus transplantation led to an increase of MHCII+ B cells (Fig. 6AB), indicating that the mere presence of CD4 T cells in BLS mice increases the proportion of MHCII+ B cells. This effect may result from preferential expansion of B cells expressing MHCII or from upregulation of MHCII expression in response to signals provided by CD4 T cells.

**Residual MHCII expression in BLS mice supports Th cell mediated immune responses**

In an initial adoptive transfer experiment we observed that residual MHCII expression in RFX5−/− and CIITA−/− mice supports activation and expansion of specific Th cells in vivo (Fig 1A). This result seems to contradict previously published data which showed that Th cells from immunized CIITA−/− animals do not show an in vitro proliferative response against the same antigen. A possible explanation is that the endogenous CD4+ T cells present in CIITA−/− animals are mainly restricted to non-classical MHC class I molecules as has been described for Aβ−/− mice. To better study whether MHC+ APC in BLS mice could support Th dependent immune responses WT thymi were transplanted into RFX5−/− or CIITA−/− or Aα−/− mice and led to reconstitution of the peripheral CD4 T cell compartment, in accordance with most but not all previous experiments.

Following reconstitution with a functional CD4 T cell compartment derived from endogenous T cell precursors BLS mice were able to mount normal Th cell dependent antibody responses. This applied not only to immunization with the model antigen NP-CG but also to infection with
MCMV. The latter is of major importance since viral infections are the main cause of death in BLS patients before and after BMT.\textsuperscript{27,65} Furthermore, we also determined the CD4 T cell dependent clearance of MCMV in the salivary glands of infected mice.\textsuperscript{48} Although full clearance of the virus could not be achieved in transplanted RFX5\textsuperscript{+/−} mice, the viral load was reduced by a factor of 10 compared to non-transplanted RFX5\textsuperscript{+/−} mice. The lower viral clearance of transplanted RFX5\textsuperscript{+/−} mice compared to WT controls may be explained by residual MHCII expression in RFX5\textsuperscript{+/−} mice being restricted to professional APC. Thus, induced MHCII expression on infected epithelial cells, which is IFN\textsubscript{γ} and thus CIITA dependent, may be required for more efficient clearance of the virus. In addition, The RFX complex does not only regulate expression of the classical MHCII genes but also of accessory molecules like H2-M.\textsuperscript{66} Expression of the latter may still be impaired in the transplanted mice. Nevertheless, our findings that Th cell dependent immune responses were substantially improved in transplanted versus non-transplanted BLS mice demonstrate that the presence of a WT thymus in BLS mice is therapeutically efficient, without the need for BMT.

**Implications for treatment of Bare Lymphocyte Syndrome**

In the light of the present results that thymic transplantation represents a potent therapy in mouse models of BLS, it appears important to reexamine residual MHCII expression in patients with MHCII deficiency and to consider reconstitution of thymic selection as a treatment strategy. Due to the low number of patients and their young age at diagnosis, a comprehensive analysis of residual MHCII expression is difficult. We could detect MHCII expression only by one RFX5-deficient B cell line, but not by unstimulated or stimulated PBL of two patients with RFX-ANK deficiency (Fig. 7). Since RFX-ANK deficient mice have not been generated so far, it is unclear whether or not this deficiency would allow residual MHC II expression in the mouse. Residual MHCII expression was, however, reported on subsets of Langerhans cells, monocytes and B lymphocytes from BLS patients after long-term culture.\textsuperscript{26,67} In particular,
significant levels of HLA-DR expression could be detected on monocyte-derived DC and epidermal Langerhans cells but not B cells or monocytes of twin brothers with RFX5 deficiency. However, compared to other patients with RFX5-deficiency these twin brothers presented with a rather mild disease phenotype, indicating that differences in the type of RFX5 mutation may influence residual MHCII expression. Patient-derived B cell lines failed to upregulate MHCII upon IFNγ stimulation, but since IFNγ-induced MHCII expression depends on the action of CIITA and indirectly the RFX-complex other stimuli should be tested in addition.

An obvious difference between the mouse models and findings in BLS patients is the relatively high frequency of peripheral CD4 T cells in patients. These T cells were shown to express a polyclonal TCR repertoire which could mean that thymic CD4 T cell selection is at least partially functional in BLS patients. However, structural abnormalities were described for TCRs from BLS patients within the complementarity determining region and it still remains to be clarified whether the CD4 T cells present in the patients are MHCII restricted. At least in one case, restriction of CD4 T cells to MHC class I rather than MHCII was reported. From the analysis of CD4 T cells of MHCII deficient (Aβ−/−) mice it is known that these cells are restricted to non-classical MHC class I molecules. In addition, the persisting immunodeficiency in many BLS patients after BMT, despite the presence of sufficient numbers of MHC-matched or haplo-identical APC, supports the notion that the available CD4 T cells are either not selected on MHCII, are functionally incompetent or have a skewed repertoire. Since in the published cases of successfully cured BLS patients the grafted bone marrow was only partially or not depleted of T cells, expanded donor T cells but not de novo thymic T cell generation may play a major role in the reconstitution of the immune system after BMT in BLS patients. It is striking in this context that the CD4 T cell population of three BMT patients which exhibited a high chimerism in the B cell and monocyte population was almost entirely donor derived. This is most likely explained by peripheral expansion of mature T cells.
transplanted with the BM graft and a failure of thymic CD4 T cell development from stem cell precursors. Alternatively, successful T cell reconstitution in cases of MHC-matched BMT may be explained by positive selection of recipient T cells on donor-derived DC as has been described in the mouse.  

The application of thymic transplantation in human therapy has recently been shown to be technically feasible in patients with complete DiGeorge syndrome and did not require MHC matching. Difficulties for the treatment of human MHCII deficiency by thymus transplantation would arise, however, from the presence of T cells in BLS patients, probably making MHC matching necessary. As an alternative, local gene therapy, which reconstitutes expression of the mutated transcription factor in the thymic epithelium, could be combined with BMT for the generation of MHCII expressing APC. In principle, such an approach has been shown feasible for MHCII structural genes in Aα−/− mice using adenoviral vectors.

Acknowledgments

We are grateful to J. Kirberg for advice on thymic transplantation procedures; H. Bluethmann for providing Aα−/− mice; V. Kuchroo and F. Frommer for providing 2D2 mice; S. Bauer and ImmunoTools for providing antibodies; C. Uthoff-Hachenberg for technical help; and F. Rieux-Laucat, M. Alimzhanow, and C. Tertilt for comments on the manuscript.

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Figure Legends:

**Figure 1:** Adoptive Transfer of T and B cells into mouse models of BLS. (A) Proliferation of CFSE-labeled Vα3+ T cells from 2D2 TCR transgenic mice after adoptive transfer into RFX5−/−, CIITA−/−, Aα−/− and C57BL/6 mice and immunization with MOG p35-55. The histogram analysis of CFSE fluorescence of CD4 Vα3+PI- lymphocytes is shown. (B) Survival of CD45.1+ B cells after transfer into Aα−/− or RFX5−/− hosts. The dot plots show B220 and CD45.1 expression of non-B cell marker negative splenocytes three weeks after transfer. The histogram analysis depicts the I-Ab expression of the indicated populations.

**Figure 2:** Th cell reconstitution in MHCII deficient mice after thymic transplantation. Thymic lobes from e14 C57BL/6 embryos were transplanted under the kidney capsule of Aα−/−, RFX5−/−, and CIITA−/− mice. Nine weeks after transplantation inguinal LN cells of transplanted and control mice were analyzed for CD4 and CD8 expression by flow cytometry. The percentage of cells in the indicated quadrants is given in the upper right corner of each dot plot. The CD4/CD8 ratio was calculated for each mouse and is shown at the lower right. The results are representative of several experiments performed.

**Figure 3:** Th cell dependent immune responses in thymus transplanted mice. Aα−/−, RFX5−/−, CIITA−/− or C57BL/6 nu/nu mice received e14 thymi (TTX) either without further treatment (A, C, D) or after irradiation/2′-dideoxyguanosine treatment (B). (A,B): Ten weeks after transplantation the mice were immunized with NP-CG and boosted three weeks later. Blood was taken three weeks after the boost and analyzed for NP-specific IgG1. (C,D): MCMV-specific immune responses. Groups of 5 C57BL/6, RFX5−/− and thymus transplanted RFX5−/− mice were infected with MCMV 6 months after transplantation and viral titers were
determined in salivary glands three weeks after infection. (C) IgG serum titers of MCMV-specific antibodies in non-transplanted RFX5\(^{+/c}\), A\(\alpha^{-/-}\) and C57BL/6 mice and thymus transplanted (TTX) RFX5\(^{+/c}\) or A\(\alpha^{-/-}\) mice. (D) Clearance of MCMV in salivary glands and lungs.

**Figure 4: MHCII expression in thymus and spleen of MHCII-deficient mouse strains.**

(A) Thymic sections from the indicated mouse strains were analyzed by three-color immunofluorescence staining and confocal microscopy. Medullary regions of the sections are shown. The sections were stained for I-A\(^b\) (red, upper left quadrant), CD11c (blue, upper right quadrant), and UEA-1 (green, lower left quadrant); lower right quadrant: overlay of all three stains (UEA\(^-\)I-A\(^b\)\(^+\) cells = yellow; CD11c\(^-\)I-A\(^b\)\(^+\) cells = pink; triple-positive cells = white). (B) Immunohistochemical analysis of I-A\(^b\) (red) and B220 (blue) expression on splenic sections of the different mouse strains. Staining for I-A\(^b\) (red) in splenic T cell zones was consistently stronger in RFX5\(^{+/c}\) and CR\(^{-/-}\) mice than in the C57BL/6 control (see also Clausen\(^{33}\)).

**Figure 5: Flow cytometric analysis of MHCII expression on thymic, splenic, and LN DC in MHCII-deficient mouse strains.** (A) Cells from collagenase digested thymi (upper row) and spleens (lower row) were stained for CD11c and I-A\(^b\). Shown are histograms of I-A\(^b\) expression on CD11c\(^+\) cells of the indicated mouse strains (bold line) or the A\(\alpha^{-/-}\) control (dotted line). The percentage of MHCII expressing cells in the indicated gates are given as bold numbers (those for the A\(\alpha^{-/-}\) controls are given in parenthesis in the diagram on the left). (B) Mean frequencies +/- SD of MHCII\(^+\) cells among thymic CD11c\(^+\) DC of three mice analyzed in each group. To control for background differences in the analysis of the RFX5/CIITA-double deficient (CR\(^{-/-}\)) mice we also examined CIITA\(^{-/-}\)RFX5\(^{+/c}\) mice which were derived from the same breeding stock as CR\(^{-/-}\) mice. (C) Cells from collagenase
digested brachial and inguinal LN were magnetically enriched for CD11c⁺ cells and subsequently stained for CD11c, DEC205, and I-Aβ⁺. Top: The histograms show I-Aβ expression of CD11c⁺ cells (bold line, bold number). The dotted line indicates I-Aβ staining on control cells from Aα⁻/- animals. Bottom: CD11c and DEC205 expression on I-Aβ expressing LN cells. (D) The expression of I-Aβ α- and β-chain in magnetically purified DC from collagenase digested spleens and thymi of the indicated mouse strains was analysed by qRT-PCR. Analysis of βActin expression was used for standardisation. Shown are expression levels relative to the ones in DC from C57BL/6 mice. The error bars indicate the standard deviation.

Figure 6: Induced MHCII expression in vivo and in vitro. (A,B) LN cells of TTX and non-transplanted Aα⁻/-, RFX5⁻/-, CIITA⁻/- mice and control C57BL/6 mice were analyzed for I-Aβ, B220, and CD11c expression. The dot plots show I-Aβ expression on gated B220⁺CD11c⁻ cells. The percentage of MHCII⁺ cells is indicated in the respective windows. Parts A and B are from independent experiments. (C) I-Aβ-expressing and I-Aβ-negative B cells and DC of transplanted CIITA⁻/- animals were sorted by FACS and the genotype of the cells assessed by a three point PCR for the WT allele (lower row, upper band) and KO allele (lower row, lower band). The products of the PCR reaction using primers for the WT fragment only were hybridized with a CIITA-specific probe (upper row). Shown are results from two independent sorting experiments (left). The PCR reactions were standardized based on cell number and the sensitivity of amplification determined by dilution of WT cells in cells from CIITA⁻/- animals at ratios of 1:1 to 1:10000 as indicated (right) (D) Splenocytes of non-transplanted Aα⁻/-, RFX5⁻/-, CIITA⁻/-, CR⁻/- and C57BL/6 mice were cultured O/N in the presence (bold line) or absence (thin line) of anti-CD40. The histograms show I-Aβ expression on CD19⁺ cells of the indicated mouse strains. The Aα⁻/- control is shown as a dotted line in each histogram. (E)
Collagenase-digested spleen cells of the same mouse strains as shown in (D) were incubated O/N in medium without additional stimuli. Cells were stained with anti-CD11c and anti-I-A<sup>b</sup> and gated on CD11c<sup>+</sup> DC. Percent of MHCII<sup>+</sup> cells in the indicated gates are given in the figure. The Aα<sup>−/−</sup> control is shown as dotted line. (F) The expression of I-A<sup>b</sup> α- and β-chain in magnetically purified splenic B cells of the indicated mouse strains was analysed by qRT-PCR after overnight culture in the presence or absence of 10µg/ml LPS. Analysis of βActin expression was used for standardisation. Shown are expression levels relative to the ones of untreated B cells from C57BL/6 mice. The error bars indicate the standard deviation.

**Figure 7: Residual MHCII expression in human MHCII deficiency.** The RFX5 deficient human B cell lines RO (A) and SJO (B) were cultured over-night with 10 µg/ml LPS, stained for CD19 and HLA-DR and analyzed by flow cytometry. PBL were used as healthy control cells. Only live cells are shown in all cases. RT-PCR analysis of HLA-DR expression by SJO cells (C). The cells were FACS sorted into HLA-DR<sup>+</sup> and HLA-DR<sup>−</sup> fractions. 10<sup>5</sup> cells were used for RT-PCR with primers for HLA-DRα and β. Primers were placed into highly homologous regions according to aligned HLA-DR alleles at the IMGT HLA database (http://www.ebi.ac.uk/imgt/hla/). The α-chain PCR was intron spanning, the β-chain PCR not. For the latter control reactions lacking reverse transcriptase (RT) were included. 10<sup>5</sup> PBL from a healthy donor were used as positive controls. A PCR for β-actin served as control for the RT reaction. (D, E, F, G) MHCII expression on PBLs derived from two patients previously diagnosed to be MHC II deficient was analyzed after over night culture in the presence of 10 µg/ml LPS and 0.5 µM CpG (patient 1) or 10 µg/ml LPS (patient 2). The patients were one (F, G) and four years (D, E) of age, respectively, and the molecular defects were located in the RFX-ANK gene. After culture the cells were stained for CD19 and HLA-DR (D, F) or CD3 and HLA-DQ (E, G) or CD3 and HLA-DP (data not shown) and analyzed.
by flow cytometry. Antibodies of irrelevant specificities were used as isotype controls for the HLA-DQ, HLA-DR, and HLA-DP stainings. Dot plots show cells in lymphocyte- and live-cell gates. In all experiments similar results were obtained without treatment or treatment with 0.5 µM CpG, 50 µg/ml pI/pC, or CD40L.
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Buch et al.
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Buch et al.
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Buch et al.
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Buch et al.
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Buch et al.
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Buch et al.
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Buch et al.
MHC class II expression through a hitherto unknown pathway supports T helper cell dependent immune responses: implications for MHC class II deficiency

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