blood

Prepublished online February 13, 2013;
doi:10.1182/blood-2012-08-452995

IL-21 stimulated human plasmacytoid dendritic cells secrete Granzyme B, which impairs their capacity to induce T cell proliferation

Julien J. Karrich, Loes C.M. Jachimowski, Maho Nagasawa, Angela Kamp, Melania Balzarolo, Monika C. Wolkers, Christel H. Uittenbogaart, S. Marieke van Ham and Bianca Blom

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.

IL-21 stimulated human plasmacytoid dendritic cells secrete Granzyme B, which impairs their capacity to induce T cell proliferation

Julien J. Karrich[1], Loes C.M. Jachimowski[1], Maho Nagasawa[1], Angela Kamp[2], Melania Balzarolo[3], Monika C. Wolkers[3], Christel H. Uittenbogaart[4], S. Marieke van Ham[2], Bianca Blom[1]

[1]Department of Cell Biology and Histology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

[2]Department of Immunopathology, Sanquin Blood Supply Research, University of Amsterdam, Plesmanlaan 125, 1066 CX, Amsterdam, The Netherlands

[3]Sanquin Research/Landsteiner laboratory AMC, Department of Hematopoiesis, Amsterdam, The Netherlands

[4]Department of Microbiology, Immunology, and Molecular Genetics, David E. Geffen School of Medicine, University of California Los Angeles, Los Angeles, California, United States of America

Correspondence to: B. Blom, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands. Tel. 31-20-5664966, Fax 31-20-6974156. E-mail: b.blom@amc.uva.nl

Running title: IL-21 controls pDC function by induction of GrB

Keywords: plasmacytoid dendritic cell, IL-21, Granzyme B, T cell proliferation

Key Points

- pDCs functionally express the IL-21 receptor and produce Granzyme B in response to IL-21
- IL-21 induced Granzyme B in pDC impairs their capacity to induce T cell proliferation

Abstract

Plasmacytoid dendritic cells (pDCs) not only play a crucial role during innate immunity by secreting bulk amounts of type I Interferons (IFNs) in response to Toll-like-receptor (TLR)-mediated pathogen recognition, but can also contribute to adaptive immunity by activation of antigen-specific T cells. Furthermore, it is well-established that pDCs contribute to the pathogenesis of autoimmune diseases, including lupus. IL-21 is a cytokine produced by activated $CD4^+$ T and NKT cells and has a pleiotropic role in immunity by controlling myeloid DC, NK(T), T and B cell functions. It has remained elusive whether IL-21 affects pDCs. Here we investigated the role of IL-21 in human pDC activation and function and observed that IL-21 activated STAT3 in line with the finding that pDCs express the IL-21 receptor. While IL-21 did not affect TLR-induced type I IFNs, IL-6 and TNF-α nor expression of major-histocompatibilty-complex-class-II (MHC-II) or co-stimulatory molecules, IL-21 markedly increased expression of the serine protease Granzyme B (GrB). We demonstrate that GrB induction was in part responsible for IL-21-mediated downmodulation of $CD4^+$ T cell proliferation induced by TLR pre-activated pDCs. Collectively, our data provide evidence that pDCs are important cells to consider when investigating the role of IL-21 in immunity or pathogenesis.

Introduction

Plasmacytoid dendritic cells (pDCs) constitute a separate subset within the DC lineage and have been shown to exert both immunostimulatory and immunosuppressive functions. PDCs express the Toll like receptors (TLR)-7 and TLR9,[1] which upon sensing viral RNA or bacterial DNA, respectively, are able to produce large amounts of the type I Interferons (IFN)-α and IFN-β.[2] These are pleiotropic cytokines that can activate multiple arms of the immune system, including T cells, B cells, NK cells, and conventional (c)-DCs,[3] and also have a direct anti-replicative effect on the virus.[4] Further, TLR triggering induces secretion of additional cytokines such as IL-6 and TNF-α that mediate maturation of pDCs into antigen presenting cells (APCs) that can prime both CD4 and CD8 T cell responses.[5] Conversely, pDCs have been implicated in dampening of immune responses. TLR7/9 engagement induces expression of the immunosuppressive enzyme indoleamine-2,3-dioxygenase (IDO), which degrades the essential amino acid tryptophan, thereby suppressing T cell responses.[6] In addition, pDCs constitutively express the serine protease granzyme B (GrB),[7,8] which is upregulated and secreted in response to IL-3, either alone or in combination with IL-10.[9] PDC-derived GrB is active, as it was shown in cytotoxicity experiments using the erythroleukemic cell line K562.[7] In addition, it yields suppression of T-cell proliferation in a perforin-independent manner, although the mechanism underlying this effect remained elusive.[9]

Following activation and polarization of T cells by APCs, T cells produce cytokines that impact on the immune response. In a classical division, T helper (Th)-1 cells produce IFN-γ, while IL-4, IL-5 and IL-13 are the signature cytokines produced by Th2 cells.[10] In addition, other Th subsets have been defined, including Th17 cells, which predominantly

make IL-17.[11] Another cytokine that more recently attracted attention is IL-21, which is a member of the common γ-chain family of cytokines, to which IL-2, IL-4, IL-7, IL-9, and IL-15 belong as well.[12,13] Production of IL-21 was originally documented to be restricted to $CD4^+$ T cells in particular to T-follicular helper cells found in or near the B-cell areas of secondary lymphoid tissue. In mice it is clear that IL-21 is produced also by other types of T cells, including Th17 cells and natural killer T (NKT) cells.[14] The functional receptor for IL-21 exists as a heterodimer that comprises the IL-21R and the common γ chain (γc; CD132).[15] In the absence of the γc, IL-21 can bind the IL-21R, but does not transduce intracellular signaling. Expression of the IL-21R complex is detected in lymphoid tissues, including spleen, thymus, and peripheral blood cells, indicating that IL-21 has regulatory functions on many cell types. While the IL-21R is shown to be expressed on resting and activated B cells, T cells, NK cells, dendritic cells (DCs), macrophages and keratinocytes,[16,17] it has remained elusive whether pDCs express this cytokine receptor.

Here we have investigated the role of IL-21 on the phenotype and function of human pDCs. We observed that pDCs expressed a functional IL-21R as STAT3 was rapidly phosphorylated in response to IL-21. IL-21 did not impact on TLR-induced production of type I IFNs, IL-6 or TNF-α by pDCs. IL-21 also did not interfere with TLR induced maturation of pDCs, since expression of co-stimulatory molecules, such as CD40, CD80 and CD86, and MHC molecules were upregulated to a similar level either in the absence or presence of IL-21. Notably, we observed that IL-21 induced the expression and secretion of GrB in pDCs. Moreover, GrB secreted from TLR/IL-21 pre-activated pDCs inhibited proliferation of T cells. Our findings demonstrate a novel role for IL-21 to

control pDCs. We hypothesize that activated T cells in a negative feedback loop may be controlled by pDCs through production of GrB, which impairs the expansion of the T cell pool.

Material and methods

Human pDC, T and B cell isolations

Peripheral blood of healthy volunteers was used for isolation of pDCs upon donor consent in accordance with the Declaration of Helsinki (Sanquin Bloodbank, Amsterdam, The Netherlands). Postnatal thymus tissue was obtained from surgical specimens removed from children up to 3 years of age undergoing open heart surgery (LUMC, Leiden, The Netherlands). Tonsils were obtained from routine tonsillectomies (department of Otolaryngology, AMC, Amsterdam, The Netherlands). Use of these tissues was approved by the medical ethical committee of the AMC. Thymocytes and lymphocytes from peripheral blood and from tonsils were isolated from a Ficoll-Hypaque density gradient (Lymphoprep; Nycomed Pharma, Oslo, Norway). Subsequently, $BDCA4^+$ cells were enriched by immunomagnetic bead selection, using the BDCA4 cell separation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD123^+CD45RA^+$ pDCs were sorted by flow cytometry on a FACSAria (BD Biosciences). Peripheral blood T cells and B cells were enriched using the CD3 and CD19 cell selection kits (Miltenyi Biotec), respectively, and sorted using anti-CD3 and anti-CD19 antibodies. Purity was ≥ 99% and confirmed by reanalysis of sorted cells.

Reagents for functional assay

To test activation and maturation of pDCs, cells were cultured in Yssel's medium,[18] supplemented with 2% human serum (Invitrogen). Oligodeoxynucleotides CpG-A (ODN2216) and CpG-B (ODN2006), and R848 were purchased from Invivogen. Mouse Recombinant IL-21 was obtained from R&D Systems, and is cross-reactive between mouse and human. The Z-AAD-CMK specific inhibitor of GrB was purchased from Enzo Life Sciences (NY, USA). Recombinant GrB (Sigma-Aldrich (St Louis, MO, USA) was used at 10 or 100 ng/mL. Recombinant IFN-α (Roferon-A; Hoffmann La Roche, Basel, Switzerland) was used at 1000 U/mL.

Flow cytometry

For flow cytometric analysis, single cell suspensions were stained with fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE-Cy7, allophycocyanin (APC), APC-Cy7, or Peridinin chlorophyll (PerCP) coupled anti-human monoclonal antibodies (Abs) targeting the following cell surface markers: CCR7, CD40, CD45RA, CD80, CD86, CD123, HLA-DR, CD3, CD11c, CD14, CD19, CD56, or isotype controls (BD Bioscience), and BDCA2 (Miltenyi Biotech). Human IL-21R was detected on the cell surface using an APC-mouse-anti-human-IL-21R Ab (R&D systems). For detection of phosphorylated STAT (pSTAT1, pSTAT3, pSTAT5) proteins, cells were fixed using cytofix/cytoperm buffer, permeabilized in ice-cold methanol and washed with

Perm/Wash buffer (BD Pharmingen) before incubation with APC-conjugated pSTAT3 Ab, Alexa-Fluor-647 conjugated pSTAT3 or pSTAT5 Abs or Alexa-Fluor-488 pSTAT1 Ab (all BD Pharmingen). For intracellular detection of IFN-α protein and GrB protein cells were cultured 4 hours with GolgiPlug prior to fixation and permeabilization using the Fixation/Permeabilization kit from BD, according to manufacturer's protocol. Fixed cells were stained either with a PE-conjugated IFN-α[2b] Ab (BD Pharmingen), or with a PE- or APC-conjugated GrB Ab (Sanquin, Amsterdam, The Netherlands). In some experiments Alexa-Fluor-647-conjugated GrB Ab was used and compared to a matching isotype control Ab (Biolegend). For apoptosis staining we used either Annexin-PE or -FITC (BD Pharmingen) and 7-AAD viability staining solution (Ebioscience). Samples were analyzed on a LSRII fluorescence-activated cell sorter (FACS) analyzer (BD Bioscience) and analyzed using FlowJo software (TreeStar).

ELISA and CBA assays

PDCs were activated overnight with CpG-A (10 µg/mL), CpG-B (10 µg/mL) or R848 (10 µg/mL), in the presence or absence of recombinant mouse IL-21 (25 ng/mL). Cell-free culture supernatants were collected and analyzed for the presence of cytokines IL-6 and TNF-α using ELISA (IL-6, U-CyTech biosciences, Utrecht, The Netherlands; TNF-α, eBioseciences, CA, USA), or using the Cytometric Bead Array (CBA), according to the manufacturer's protocol (CBA Human Inflammation kit, BD Biosciences). GrB present

in the culture medium was quantified using the PeliKine Compact human Granzyme B ELISA kit (M1936, Sanquin), according to the manufacturers protocol.

PCR

For PCRs, total RNA was extracted using Trizol reagent (Invitrogen). RNA concentration and quality was determined using the Nanodrop spectrophotometer (Thermo Fisher Scientific). Equal amounts of total RNA were reverse transcribed into cDNA using the RNA-to-cDNA kit (Roche) according to the manufacturer's instructions. cDNA was amplified using a PCR machine for conventional RT-PCR and separated on a 1,5% agarose gel, or amplified using an iCycler and SYBR green supermix (BioRad) for quantitative PCR (QPCR) using specific primer sets (supplementary table 1). Each sample was analyzed in triplicates and expression levels were normalized to the three housekeeping genes β-Actin, GAPDH and HPRT.

Allogeneic T cell stimulation

Lymphocytes from peripheral blood were isolated by Ficoll gradient centrifugation as described in the "human pDC isolation" section. $CD4^+$ T cells were sorted as lineage ($CD11c^-CD14^-CD19^-CD56^-BDCA2^-$) negative cells using a FACS ARIA (BD Biosciences). Sorted pDCs from peripheral blood were incubated with or without the TLR7 ligand R848 for 48h in the absence or presence of IL-21 (25ng/mL). Subsequently, pDCs were co-cultured with T cells at a 1:5 ratio for 6 days in Yssel's medium[18]

supplemented with 2% human serum. Cell proliferation was assessed using the CellTrace-violet proliferation kit (Invitrogen) according to the manufacturer's instructions. T cells activated with human T-expander CD3/CD28 beads (Dynabeads, Dynal, Invitrogen) were used as a positive control.

Statistical analyses

Data were subjected to two-tailed paired Student's t-test analysis using Graphpad Prism 5 for Windows (Graphpad software, San Diego, USA) and considered significant when at least $P < 0.05$.

Results

pDCs express a functional IL-21R

The IL-21R was shown to be expressed by several immune cell types, including B cells and T cells.[19] Evidence that pDCs express this receptor is lacking. We therefore analyzed human pDCs from 2 independent donors for expression of the IL-21R transcript by RT-PCR (Figure 1A) and compared expression to that in primary B cells (CD19 MACS-enriched) and T cells (CD3 MACS-enriched or sorted total $CD4^+$ T cells). We observed that pDCs, B cells, and T cells all expressed the *IL-21RA* chain. Furthermore, protein expression of the IL-21Rα chain was detected by flow cytometry at the cell surface of freshly isolated pDCs from peripheral blood, thymus and tonsil (Figure 1B and data not shown). The expression level of the IL-21R on peripheral blood pDCs was comparable to

the levels detected on T and B cells and other (non-pDC/T/B) cells from the same donor (Figure 1B). To further characterize whether the IL-21R expressed on pDCs was functional, we stimulated human pDCs from blood, thymus and tonsils with IL-21 and evaluated the phosphorylation of STAT3, which is a key component of the IL-21R downstream signalling pathway.[19] As shown by flow cytometric analysis, IL-21 upregulated pSTAT3 levels as compared to unstimulated cells independent of their source of origin (Figure 1C). pSTAT3 levels in unstimulated tonsil pDCs appear increased compared to thymus and blood pDCs, which may suggest that STAT3 is constitutively activated in tonsil pDCs. While this may reflect the inflamed condition of tonsil, it may well be independent of IL-21. IL-21 did not phosphorylate STAT1 in pDCs (Suppl. Figure 1A) in contrast to IFN-α (Suppl. Figure 1B). Increased levels of pSTAT5 in response to IL-21 could only be observed in pDCs isolated from blood, but not thymus or tonsil, although the results may suggest that STAT5 may be constitutively activated in thymus and tonsil pDCs (Suppl. Figure 1). Taken together, these data show that human pDCs not only express the IL-21R chain, but in addition that the receptor is functional when engaged by its physiological ligand.

IL-21 does not affect pDC survival and cytokine production

To unravel the putative role of the IL-21R on pDCs, we first investigated the effect of IL-21 on pDC survival, both in the presence and absence of TLR stimulation. We observed no significant differences of IL-21 on cell survival after 4 days, neither in TLR unstimulated cells, nor after stimulation with the TLR7 ligand R848, as shown by

apoptosis staining using AnnexinV and 7-AAD (Figure 2A). To determine the role of IL-21 in combination with TLR9 engagement, pDCs were activated using the TLR9 agonist CpG-A in the presence or absence of IL-21 (Figure 2B). Equal percentages of IFN-α expressing cells were found in response to CpG-A either with or without IL-21, as shown by intracellular cytokine staining. Furthermore, the presence of IL-21 during TLR triggering did not influence the ability of pDC to secrete the pro-inflammatory cytokines IL-6 and TNF-α in response to stimulation with CpG-B or R848 as detected by cytokine bead array analysis (Figure 2C-D) or ELISA (data not shown).

IL-21 does not interfere with TLR-induced maturation of pDCs

Engagement of TLR induces differentiation of pDCs into mature DCs that have upregulated expression levels of MHC-II, chemokine receptor CCR7, and co-stimulatory molecules, including CD40, CD80, and CD86.[3] We were interested to analyze whether IL-21 affects this process. We observed that the maturation of pDCs from blood, tonsil or thymus upon CpG-B or R848 stimulation was not changed by IL-21, as equal levels of the co-stimulatory molecules CD40, CD80, CD86 were detected by flow cytometry both after 1 and 2 days of stimulation (Figure 3, Suppl. Fig. 2 and 3, and data not shown). Similarly, no effect of IL-21 on expression of HLA-DR and CCR7 was observed. Taken together, these results indicate that IL-21 does not interfere with the TLR induced functionality of pDCs in terms of activation and maturation.

IL-21 induces GrB expression in pDCs and is modulated by TLR stimulation

PDCs, unlike conventional DCs, constitutively express GrB, although conflicting data have been reported in literature.[7-9] To further elucidate this and to address whether IL-21 affects GrB expression, we cultured pDCs in the presence or absence of IL-21. We confirmed earlier observations[7,8] that freshly isolated pDCs constitutively express GrB independent of their source of origin (tonsil, thymus, peripheral blood) (Figure 4A). Notably, IL-21 had a major impact on GrB levels in pDCs as IL-21 induced GrB expression both at the transcriptional (Figure 4B) and at the protein (Figure 4C) level. Already 4h after stimulation of pDCs, IL-21 induced the level of GrB mRNA 3.2-fold as measured by QPCR analysis, while after 6h this was even higher 7.5-fold as compared to medium cultured pDCs (Figure 4B). Secretion of active GrB from pDCs (tonsil, n = 5; blood, n = 2) was measured in the supernatant after culture in the presence or absence of IL-21 in serum-free medium for 16h by ELISA (Figure 4C). In all donors, IL-21 induced higher levels of GrB in pDCs as compared to medium cultured cells. Tonsil pDCs secreted 2.5-fold more GrB in response to IL-21 (515+/-195 pg/mL; $p < 0,05$) compared to medium (209+/-134 pg/mL). Blood pDCs secreted 2.3-fold higher GrB levels in response to IL-21 (566+/-261 pg/mL) compared to medium (245+/-205 pg/mL). Notably, low levels of GrB could be detected when cells were cultured in the absence of IL-21, and is in line with our findings that pDCs constitutively express GrB (Figure 4C).

To investigate the effect of IL-21 on GrB production upon pDC activation, *ex vivo* pDCs from tonsil or blood were stimulated with the TLR9 agonist CpG-B or with the TLR7 agonist R848 in the presence or absence of IL-21. After overnight or 2 days of stimulation, GrB expression was assessed by flow cytometry after intracellular staining

(Figure 4D-E). We observed that stimulation of pDCs with either CpG-B or R848 reduced GrB levels as compared to medium cultured pDCs. This is in contrast to pDCs incubated with IL-21 alone in the absence of TLR ligands, where GrB expression increased as compared to the medium control cultured pDCs. Notably, TLR-induced GrB inhibition in the concomitant presence of IL-21 was only partial. In this condition, GrB levels were comparable to the levels that were expressed in medium cultured cells (Figure 4D-E). The effect of IL-21 on the GrB levels in R848 stimulated blood pDCs for 2 days was comparable to overnight stimulation, although less pronounced (Figure 4E). Analysis of the secreted levels of GrB in the supernatant of overnight cultured pDCs (Suppl. Fig. 4) reflected our findings when analysing the intracellular GrB levels (Figure 4C). Collectively these results show that pDCs constitutively express and secrete GrB and that IL-21 potently induces GrB expression, which can be partially antagonized by TLR co-ligation.

IL-21-induced GrB production by pDCs inhibits T cell proliferation

During a viral or bacterial infection, $CD4^+$ T cells and NKT cells are the main producers of IL-21.[12] To investigate the role of IL-21 in the interactions between pDCs and T cells during an immune response, we performed *in vitro* allogeneic T cell stimulation assays using pre-activated pDCs. First, pDCs were activated with the TLR7 ligand R848, or with the TLR9 ligand CpG-B, in the presence or absence of IL-21 for 48h (Figure 5 and data not shown). After extensive washing, pre-activated pDCs were co-cultured with resting allogenenic $CD4^+$ T cells for 6 days. T cell proliferation was assessed by measuring the loss of the dye CellTrace-violet upon cell division using flow cytometry. In addition, we

included 7-AAD in our analyses to measure cell death. Both TLR7 as well as TLR9 activated pDCs were able to induce T cell proliferation (Figure 5A and data not shown). Allogenenic T cell proliferation induced by activated pDCs was lower than that induced by polyclonal stimulation using anti-CD3/CD28 beads. IL-21 pre-activated pDCs did not induce the proliferation of T cells as compared to medium cultured pDCs. Notably, when pDCs were pre-activated by the TLR agonists in the presence of IL-21 they were less capable to induce T cell proliferation as compared to TLR-activated pDCs in the absence of IL-21 (R848: 40% versus R848+IL-21: 15%). This decrease in the percentage of expanded T cells was not due to increased cell death, since the percentage of 7-AAD$^+$ T cells were similar in both conditions (Figure 5A). To demonstrate that GrB could be involved in blocking T cell proliferation we added a GrB inhibitor during the pDC-T cell co-culture. Indeed, T cell proliferation was restored at least in part by impairing the activity of GrB. In all conditions statistically significant differences were observed when analyzing multiple donors (n = 4) (Figure 5B). Culture of CD4+ T cells in the presence of recombinant GrB did not affect proliferation (Suppl. Figure 5). Hence, our data support the notion that GrB, which is increased in pDCs upon stimulation with IL-21, affects the level of T cell proliferation induced by TLR-activated pDCs in a cell contact dependent manner.

Discussion

To our knowledge, this study is the first to show constitutive expression of the IL-21R on human pDCs. We show that the IL-21R is functionally expressed, since IL-21 stimulation

of pDCs resulted in activation of the downstream signaling molecule STAT3. IL-21 by itself did not affect pDC activation nor maturation or cytokine production in response to TLR ligation. However, IL-21 induced GrB expression in pDCs. Moreover, we demonstrate that IL-21-induced GrB was secreted by pDCs, which contributes to impairing $CD4^+$ T cell proliferation in an allogeneic setting.

Despite the pleiotropic effect of IL-21 on a wide range of immune cells, its role on pDCs has previously not been investigated. In mice, bone marrow-derived conventional DCs (BMDCs) generated in the presence of IL-21 (IL-21-DCs) showed an impaired activation and maturation capacity.[20] Consistent with this, IL-21 pretreated BMDCs when adoptively transferred failed to induce T cell activation *in vivo*.[21] Human monocyte-derived DCs pretreated with IL-21 also failed to upregulate CD86 and MHCII expression in response to LPS, although the consequence of this with respect to T cell stimulatory capacity was not analyzed.[22] In our studies using human pDCs, we did not observe significant differences in expression of maturation markers, including CD40, CD80, CD86, MHCII, or CCR7, in response to TLR agonists either in the presence or absence of IL-21. Therefore, this cannot explain the impaired T cell proliferation we observed in the presence of TLR/IL-21 stimulated pDCs as compared to TLR only stimulated pDCs. Moreover, impaired ability of TLR/IL21-pDCs to activate T cells cannot be due to increased apoptosis induction of the pDCs themselves, since IL-21 did not affect pDCs survival. Increased apoptosis induction of T cells was not observed either, hence it is more likely that GrB secreted by IL-21 stimulated pDCs hampered T cell proliferation. This is underscored by our finding that addition of a GrB inhibitor partially restored T cell expansion.

It is generally accepted that differentiation, expansion and survival of T cells is enforced in response to cues delivered by DCs. While antigen presentation is key to DCs functioning, additional signals will polarize T cells to induce a tailored immune response against microbial infections. In return, T cell responses should be downmodulated in order to avoid overactivation of the immune system. In physiological conditions, activated T cells and pDCs may meet in the lymph node where follicular helper $CD4^+$ T (Tfh) cells produce its primary cytokine IL-21, which provide B cells with signals that are important for the generation of high-affinity antibodies and immunological memory.[23] It is reasonable to assume that IL-21 reversely may serve to dampen Tfh responses through induction of GrB in pDCs and thereby help to return to homeostasis at the stage where antibodies have been formed and there is resolution of infection.

Previously, GrB was shown to enter T cells and via an as yet unknown mechanism to impair proliferation.[9] A molecule known to steer T cell responses is Notch. The Notch pathway is evolutionarily conserved in multi-cellular eukaryotes playing a broad and important role during embryonic development and in adult tissue homeostasis.[24] Notch proteins coordinate cell-cell communication through receptor-ligand (i.e. Delta, Jagged) interactions. Activation cleaves the Notch intracellular domain, which translocates into the nucleus where it binds the transcription factor CSL (CBF1/suppressor of Hairless/Lag-1) in human or RBP-Jκ (recombination signal-binding protein 1 for Jκ) in mice, thereby activating Notch target genes.[25] Notch activity is tightly controlled by proteases that activate (i.e. γ–secretase) Notch receptor signaling. Within the $CD4^+$ T cell lineage Notch can skew T cell responses towards a Th2 phenotype by controlling expression of the lineage specific factor GATA-3.[26-28] More recently it was demonstrated

that Notch can also control T cell proliferation[29] and longevity.[30] Interestingly, NOTCH1 has been described as a substrate of GrB,[31] resulting in loss of its transcriptional activity.[32] Hence, impaired T cell proliferation induced by IL-21 pretreated pDCs may be the result of GrB-mediated NOTCH1 degradation. Taken together, a model is emerging that $CD4^+$ T cells after activation will inhibit their own expansion via production of IL-21, and induction of GrB by pDCs, forming a negative feedback loop in the regulation of an adaptive immune response.

In primary human B cells, IL-21 induced activation of STAT3.[33,34] Here, we show that IL-21 also activated STAT3 in human primary pDCs leading to increased levels of GrB. Other cytokines, including IL-3 and IL-10, were previously reported to induce expression of GrB in pDCs.[9] As these cytokines act through activation of the JAK/STAT3 as well, it is likely that this is a common pathway for induction of GrB. Interestingly in mice, targeting Stat3 in $CD11c^+$ myeloid cells by CpG-siRNA improved key effector functions and proliferation of adoptively transferred T cells.[35] While direct evidence is lacking, it is attractive to consider that this may partially be due to reduced levels of GrB expressed in $Stat3^{-/-}$ DCs, thereby allowing more extensive T cell proliferation.

PDCs form the first line of defence against viruses and bacteria by rapid production of IFN-α.[36] Conversely, uncontrolled or unwanted pDC-derived production of IFN-α plays a key role in human autoimmune pathogenesis, such as systemic lupus erythematosus (SLE),[37] Sjögren's syndrome (SS),[38] and psoriasis.[39] In addition to IFN-α, also elevated serum levels of IL-21 were detected in patients suffering from SS, SLE, and RA compared to healthy controls.[40,41] Furthermore, SLE is associated with two single nucleotide polymorphisms of the IL-21 gene.[42] In agreement with these data, elevated IL-

21 levels were found in SLE mouse models.[43] While IL-21 is known to enhance anti-CD3 induced proliferation of T cells,[44] it also controls the functional activity of effector T helper (Th) cells, the differentiation of Th17 cells, and counteracts the suppressive effects of regulatory T cells.[45] Moreover, IL-21 either alone or in synergy with BAFF is capable of promoting B cell expansion and plasma B cell differentiation.[44,46] Adding to this list, we show that IL-21 induced GrB production in pDCs. Interestingly, GrB plays a role in autoimmunity as GrB-mediated cleavages of intracellular auto-antigens may enhance their immunogenicity via the generation of new antigenic epitopes (reviewed in Darrah & Rosen[47]). Also $CD5^+$ SLE B cells constitutively express GrB,[48] and IL-21 was previously shown to induce GrB in B cells.[49] This, together with our findings that IL-21 induced the production of GrB in pDCs, enforces its crucial role in the pathogenesis of autoimmune diseases.

Our study highlights the dichotomic role of IL-21 in the activation of pDCs: on one hand, IL-21 induced GrB may drive or accelerate autoimmunity by cleavage of autoantigenic epitopes,[50] and on the other hand IL-21 induced GrB may be causal for the termination of immune responses by inhibiting T cell expansion. These findings open new perspectives in the development of therapies specifically targeting the IL-21/IL-21R pathway in autoimmunity and inflammation.

Acknowledgements

The authors wish to thank Berend Hooibrink and Toni van Capel for maintenance of the AMC FACS facility and help during cell sorting. We thank Prof.dr. Hazekamp and staff from the LUMC (Leiden, The Netherlands) for providing human thymus tissue, and Prof. dr. W. Fokkens (Department of Otolaryngology, AMC) for providing tonsil tissue. Rosa de Groot (Department of Cell Biology & Histology, AMC) is acknowledged for her technical help performing ELISA. JJK is financially supported through a personal VIDI grant to BB (Dutch Science Foundation, no. 917.66.310). LCMJ and CHU are supported by a grant from the National Institutes of Health (AI 080564, University of California Los Angeles, Center for AIDS Research).

Authorship

Contributions: J.J.K. designed research, performed experiments, analyzed data, and wrote the manuscript; L.C.M.J., M.N., A.K., M.B. performed experiments and analyzed data; M.C.W., C.H.U. and S.M. van H. analyzed data; B.B. designed research, analyzed data and wrote the manuscript.

Conflict of interest

The authors declare no conflict of interest.

References

1. Kadowaki N, Ho S, Antonenko S, et al. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J Exp Med*. 2001;194(6):863-869.
2. Izaguirre A, Barnes BJ, Amrute S, et al. Comparative analysis of IRF and IFN-alpha expression in human plasmacytoid and monocyte-derived dendritic cells. *J Leukoc Biol*. 2003;74(6):1125-1138.
3. Kadowaki N, Antonenko S, Lau JY, Liu YJ. Natural interferon alpha/beta-producing cells link innate and adaptive immunity. *J Exp Med*. 2000;192(2):219-226.
4. Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD. How cells respond to interferons. *Annu Rev Biochem*. 1998;67:227-264.
5. Fonteneau JF, Gilliet M, Larsson M, et al. Activation of influenza virus-specific CD4+ and CD8+ T cells: a new role for plasmacytoid dendritic cells in adaptive immunity. *Blood*. 2003;101(9):3520-3526.
6. Munn DH, Sharma MD, Hou D, et al. Expression of indoleamine 2,3-dioxygenase by plasmacytoid dendritic cells in tumor-draining lymph nodes. *J Clin Invest*. 2004;114(2):280-290.
7. Bratke K, Nielsen J, Manig F, et al. Functional expression of granzyme B in human plasmacytoid dendritic cells: a role in allergic inflammation. *Clin Exp Allergy*. 2010;40(7):1015-1024.
8. Rissoan MC, Duhen T, Bridon JM, et al. Subtractive hybridization reveals the expression of immunoglobulin-like transcript 7, Eph-B1, granzyme B, and 3 novel transcripts in human plasmacytoid dendritic cells. *Blood*. 2002;100(9):3295-3303.
9. Jahrsdorfer B, Vollmer A, Blackwell SE, et al. Granzyme B produced by human plasmacytoid dendritic cells suppresses T-cell expansion. *Blood*. 2010;115(6):1156-1165.
10. Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. *Nature*. 1996;383(6603):787-793.
11. Harrington LE, Hatton RD, Mangan PR, et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol*. 2005;6(11):1123-1132.
12. Mehta DS, Wurster AL, Grusby MJ. Biology of IL-21 and the IL-21 receptor. *Immunol Rev*. 2004;202:84-95.
13. Spolski R, Leonard WJ. Interleukin-21: basic biology and implications for cancer and autoimmunity. *Annu Rev Immunol*. 2008;26:57-79.
14. Leonard WJ, Zeng R, Spolski R. Interleukin 21: a cytokine/cytokine receptor system that has come of age. *J Leukoc Biol*. 2008;84(2):348-356.
15. Asao H, Okuyama C, Kumaki S, et al. Cutting edge: the common gamma-chain is an indispensable subunit of the IL-21 receptor complex. *J Immunol*. 2001;167(1):1-5.
16. Ozaki K, Kikly K, Michalovich D, Young PR, Leonard WJ. Cloning of a type I cytokine receptor most related to the IL-2 receptor beta chain. *Proc Natl Acad Sci U S A*. 2000;97(21):11439-11444.
17. Parrish-Novak J, Dillon SR, Nelson A, et al. Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. *Nature*. 2000;408(6808):57-63.
18. Yssel H, De Vries JE, Koken M, Van Blitterswijk W, Spits H. Serum-free medium for generation and propagation of functional human cytotoxic and helper T cell clones. *J Immunol Methods*. 1984;72(1):219-227.

19. Leonard WJ, Spolski R. Interleukin-21: a modulator of lymphoid proliferation, apoptosis and differentiation. *Nat Rev Immunol*. 2005;5(9):688-698.
20. Brandt K, Bulfone-Paus S, Foster DC, Ruckert R. Interleukin-21 inhibits dendritic cell activation and maturation. *Blood*. 2003;102(12):4090-4098.
21. Brandt K, Bulfone-Paus S, Jenckel A, Foster DC, Paus R, Ruckert R. Interleukin-21 inhibits dendritic cell-mediated T cell activation and induction of contact hypersensitivity in vivo. *J Invest Dermatol*. 2003;121(6):1379-1382.
22. Strengell M, Lehtonen A, Matikainen S, Julkunen I. IL-21 enhances SOCS gene expression and inhibits LPS-induced cytokine production in human monocyte-derived dendritic cells. *J Leukoc Biol*. 2006;79(6):1279-1285.
23. Crotty S. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol*. 2011;29:621-663.
24. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. *Science*. 1999;284(5415):770-776.
25. Bray SJ. Notch signalling: a simple pathway becomes complex. *Nat Rev Mol Cell Biol*. 2006;7(9):678-689.
26. Amsen D, Antov A, Jankovic D, et al. Direct regulation of Gata3 expression determines the T helper differentiation potential of Notch. *Immunity*. 2007;27(1):89-99.
27. Fang TC, Yashiro-Ohtani Y, Del Bianco C, Knoblock DM, Blacklow SC, Pear WS. Notch directly regulates Gata3 expression during T helper 2 cell differentiation. *Immunity*. 2007;27(1):100-110.
28. Tu L, Fang TC, Artis D, et al. Notch signaling is an important regulator of type 2 immunity. *J Exp Med*. 2005;202(8):1037-1042.
29. Palaga T, Miele L, Golde TE, Osborne BA. TCR-mediated Notch signaling regulates proliferation and IFN-gamma production in peripheral T cells. *J Immunol*. 2003;171(6):3019-3024.
30. Helbig C, Gentek R, Backer RA, et al. Notch controls the magnitude of T helper cell responses by promoting cellular longevity. *Proc Natl Acad Sci U S A*. 2012;109(23):9041-9046.
31. Loeb CR, Harris JL, Craik CS. Granzyme B proteolyzes receptors important to proliferation and survival, tipping the balance toward apoptosis. *J Biol Chem*. 2006;281(38):28326-28335.
32. van Tetering G, Bovenschen N, Meeldijk J, van Diest PJ, Vooijs M. Cleavage of Notch1 by granzyme B disables its transcriptional activity. *Biochem J*. 2011;437(2):313-322.
33. Diehl SA, Schmidlin H, Nagasawa M, et al. STAT3-mediated up-regulation of BLIMP1 Is coordinated with BCL6 down-regulation to control human plasma cell differentiation. *J Immunol*. 2008;180(7):4805-4815.
34. Avery DT, Deenick EK, Ma CS, et al. B cell-intrinsic signaling through IL-21 receptor and STAT3 is required for establishing long-lived antibody responses in humans. *J Exp Med*. 2010;207(1):155-171.
35. Herrmann A, Kortylewski M, Kujawski M, et al. Targeting Stat3 in the myeloid compartment drastically improves the in vivo antitumor functions of adoptively transferred T cells. *Cancer Res*. 2010;70(19):7455-7464.
36. Liu YJ. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu Rev Immunol*. 2005;23:275-306.
37. Bennett L, Palucka AK, Arce E, et al. Interferon and granulopoiesis signatures in systemic lupus erythematosus blood. *J Exp Med*. 2003;197(6):711-723.
38. Gottenberg JE, Cagnard N, Lucchesi C, et al. Activation of IFN pathways and plasmacytoid dendritic cell recruitment in target organs of primary Sjogren's syndrome. *Proc Natl Acad Sci U S A*. 2006;103(8):2770-2775.

39. Nestle FO, Conrad C, Tun-Kyi A, et al. Plasmacytoid predendritic cells initiate psoriasis through interferon-alpha production. *J Exp Med*. 2005;202(1):135-143.
40. Kang KY, Kim HO, Kwok SK, et al. Impact of interleukin-21 in the pathogenesis of primary Sjogren's syndrome: increased serum levels of interleukin-21 and its expression in the labial salivary glands. *Arthritis Res Ther*. 2011;13(5):R179.
41. Wang XF, Yuan SL, Jiang L, et al. [Changes of serum BAFF and IL-21 levels in patients with systemic lupus erythematosus and their clinical significance]. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi*. 2007;23(11):1041-1042.
42. Sawalha AH, Kaufman KM, Kelly JA, et al. Genetic association of interleukin-21 polymorphisms with systemic lupus erythematosus. *Ann Rheum Dis*. 2008;67(4):458-461.
43. Herber D, Brown TP, Liang S, Young DA, Collins M, Dunussi-Joannopoulos K. IL-21 has a pathogenic role in a lupus-prone mouse model and its blockade with IL-21R.Fc reduces disease progression. *J Immunol*. 2007;178(6):3822-3830.
44. Ozaki K, Spolski R, Feng CG, et al. A critical role for IL-21 in regulating immunoglobulin production. *Science*. 2002;298(5598):1630-1634.
45. Monteleone G, Pallone F, Macdonald TT. Interleukin-21 (IL-21)-mediated pathways in T cell-mediated disease. *Cytokine Growth Factor Rev*. 2009;20(2):185-191.
46. Ettinger R, Sims GP, Robbins R, et al. IL-21 and BAFF/BLyS synergize in stimulating plasma cell differentiation from a unique population of human splenic memory B cells. *J Immunol*. 2007;178(5):2872-2882.
47. Darrah E, Rosen A. Granzyme B cleavage of autoantigens in autoimmunity. *Cell Death Differ*. 2010;17(4):624-632.
48. Hagn M, Ebel V, Sontheimer K, et al. CD5+ B cells from individuals with systemic lupus erythematosus express granzyme B. *Eur J Immunol*. 2010;40(7):2060-2069.
49. Hagn M, Schwesinger E, Ebel V, et al. Human B cells secrete granzyme B when recognizing viral antigens in the context of the acute phase cytokine IL-21. *J Immunol*. 2009;183(3):1838-1845.
50. Goldbach-Mansky R, Suson S, Wesley R, Hack CE, El-Gabalawy HS, Tak PP. Raised granzyme B levels are associated with erosions in patients with early rheumatoid factor positive rheumatoid arthritis. *Ann Rheum Dis*. 2005;64(5):715-721.

Figure Legends

Figure 1: IL-21R is expressed by human pDCs and is functional. (A) Expression of IL-21 receptor was measured by RT-PCR on RNA extracted from human sorted pDCs from thymus (2 different donors) and compared to peripheral blood human T cells (sorted or MACS-enriched for CD3) and human B cells (sorted or MACS-enriched for CD19). Amplification of actin was done as a loading control. (B) Total human PBMCs were analysed for expression of IL-21R by flow cytometry. IL-21R surface expression levels on pDCs, T cells ($CD4^+$ MACS), B cells ($CD19^+$ MACS) and other cells (other) are shown (black lines) (isotype control stainings are shown as grey filled histograms). IL-21R expression on total B cells is shown as positive control. (C) Flow cytometric analysis of phosphorylated (p) STAT3 protein in primary pDCs from peripheral blood, thymus, and tonsils stimulated in the presence (black lines) or absence (grey filled histograms) of IL-21 (25 ng/mL) for 20 min. RCN, relative cell number. One representative experiment out of 3 is depicted.

Figure 2: IL-21 stimulation does not affect survival nor alter cytokine production and secretion in pDCs upon TLR activation. (A) Freshly isolated human thymic pDCs were cultured with or without TLR7 agonist R848 (10 μg/mL), in the presence or absence of IL-21 (25 ng/mL) for 4 days, and subsequently stained with an AnnexinV conjugated APC antibody and 7-AAD. The percentages of $AnnexinV^+7\text{-}AAD^{-/+}$ early and late apoptotic cells were analyzed by flow cytometry. Numbers represent percentages of cells in the indicated gates. (B) Freshly isolated pDCs were cultured overnight with or without

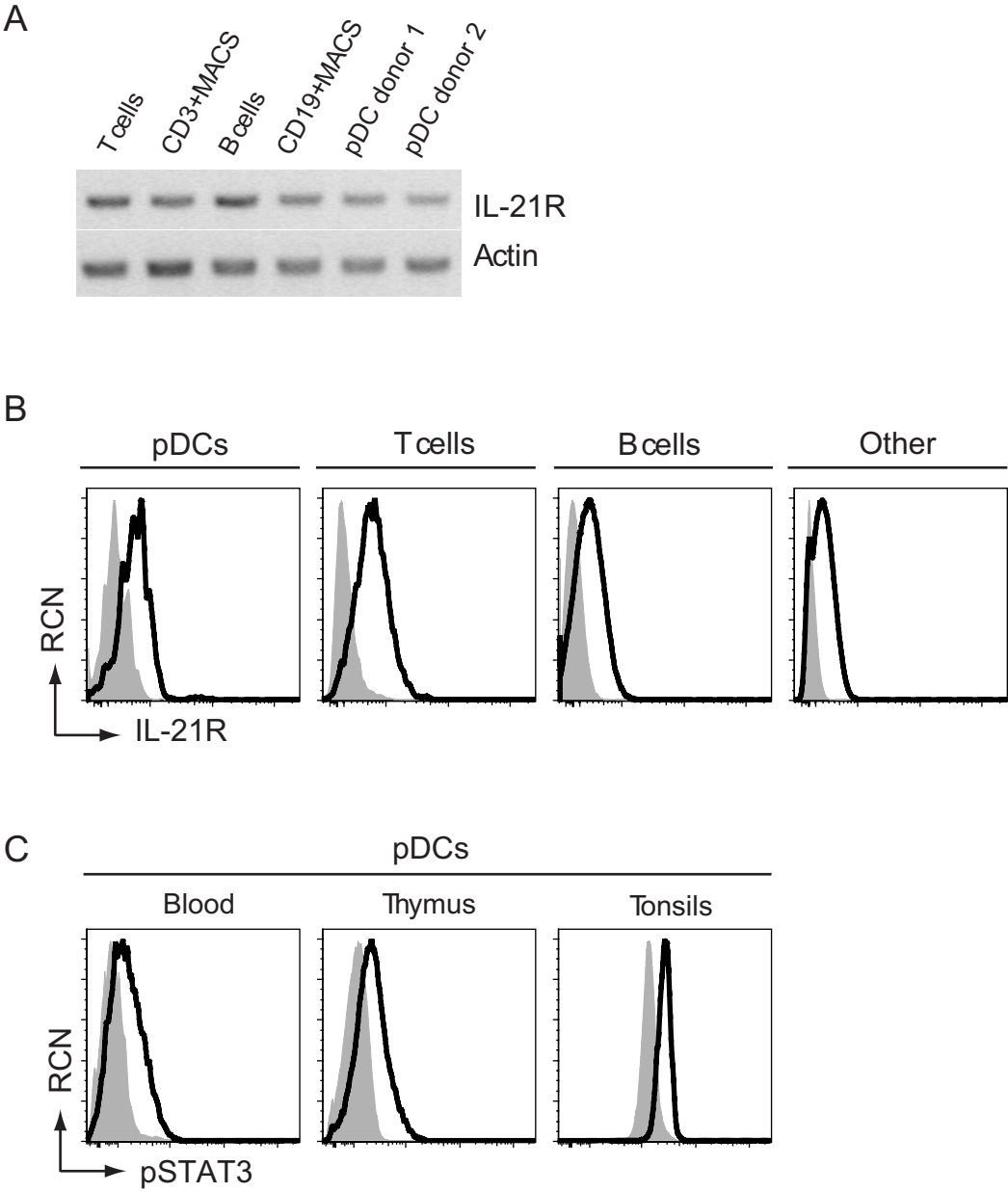
CpG–A (10 μg/mL), in the presence or absence of IL-21 as indicated. Cells were incubated with GolgiPlug for the last 4h, and analyzed by flow cytometry after intracellular staining using a PE-conjugated antibody directed against IFN-α[2b] protein. CD45RA expression was analyzed to confirm the presence of pDCs. Numbers represent percentages of cells in the indicated gates, which were set on basis of an IgG-PE isotype control antibody. (C-D) Freshly isolated pDCs were cultured in the presence of (C) CpG-B (10 μg/mL) or (D) R848 (10 μg/mL) with IL-21 (black bars) or without IL-21 (white bars) for 20 hours. Culture supernatants were analyzed for the presence of IL-6 and TNF-α by cytokine bead array using flow cytometry. One representative experiment out of 2 is depicted.

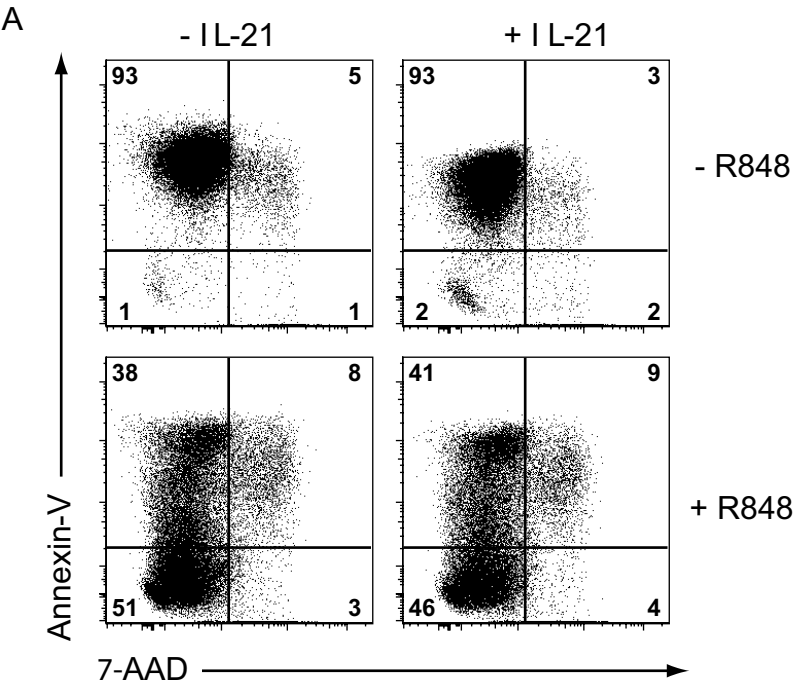
Figure 3: IL-21 stimulation does not affect TLR-induced pDC maturation. Surface expression of the co-stimulatory molecules CD80, CD86 and CD40, and expression of HLA-DR and CCR7 were measured by flow cytometry on blood pDCs that were cultured in medium, or IL-21 (25 ng/mL), or R848 (10 μg/mL), or R848 plus IL-21 for 1 or 2 days. (A) Shown are histograms representing the level of protein expression as indicated after 2 days of culture. One representative experiment out of 2 is depicted. Medium, dark grey shaded histogram; IL-21, light grey shaded histogram; R848, black line open histogram; R848 plus IL-21, grey line open histogram. (B) Mean fluorescence intensities (MFIs) were calculated based on the flow cytometry data as described in A after 1 and 2 days. Shown are the mean MFIs of two experiments. Error bars indicate SD values.

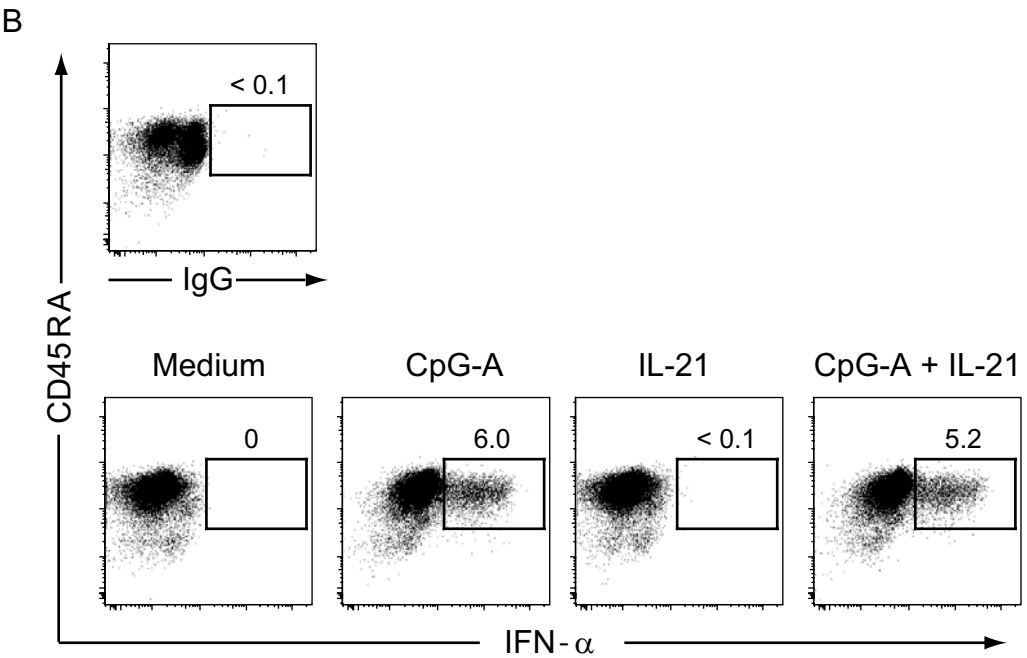
Figure 4: GrB is endogenously expressed in pDCs and is strongly induced upon IL-21 stimulation. (A) Human pDCs purified from sources as indicated were stained for intracellular GrB expression (black lines) and analyzed by flow cytometry, using a GrB-PE conjugated (thymus and tonsil) or a GrB–APC conjugated (peripheral blood) antibody. Grey filled histograms represent appropriate isotype control stainings. (B) GrB RNA levels were measured by quantitative PCR in pDCs after incubation in the presence (black bars) or absence (white bars) of IL-21 for 4 and 6h as indicated. Expression of the housekeeping genes (β-Actin, GAPDH and HPRT) were used to control for the amount of RNA used. Values were normalized to cells incubated without IL-21, which was set to 1. (C) Freshly isolated pDCs from tonsil (n = 5) and blood (n = 2) were cultured overnight in the presence or absence of IL-21 in serum-free medium. Culture supernatants were analyzed for the presence of GrB by ELISA. Shown are the mean GrB levels of the pDC donors tested +/- SD. * $p < 0,05$. (D-E) Freshly isolated pDCs from 2 different tonsil donors (D, upper and lower panels) or from blood (E) were cultured for 1 day (D-E) or for 2 days (E) in medium (dark grey shaded histograms) or stimulated (black lines) with either IL-21 alone, or with CpG-B (10 μg/mL) or R848 (10 μg/mL) in the presence or absence of IL-21 as indicated. Intracellular GrB expression was analyzed by flow cytometry. Isotype control stainings are shown as light grey filled histograms. The numbers in E indicate the differences in mean fluorescence intensity (ΔMFI), which were calculated by subtracting the MFI of medium cultured pDCs from the MFI of stimulated (IL-21, R848, or both) pDCs.

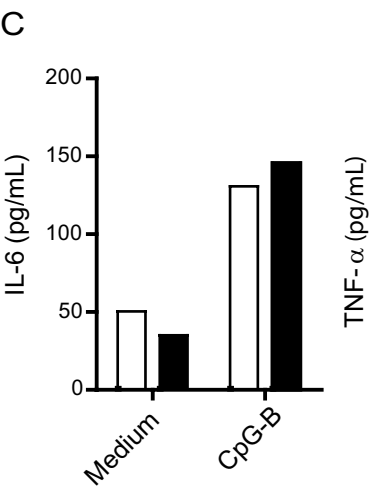
Figure 5: IL-21-induced GrB production in pDCs inhibits allogeneic CD4$^+$ T cell proliferation. Freshly isolated pDCs from blood were pre-activated for 2 days in medium with or without the TLR7 agonist R848 (10μg/mL), and in the presence or absence of IL-21 (25ng/mL) as indicated. After extensive washing, pDCs were co-cultured with freshly isolated allogeneic CD4$^+$ T cells (ratio pDC:T cell = 1:5) after labeling with the CellTrace violet dye. After 6 days, T cells were analyzed by flow cytometry for expression of CD3, the flurorescent CellTrace violet dye and 7-AAD. Dotplots shown are gated on CD3+ T cells. Numbers represent percentages of cells in the indicated quadrants. CellTrace-violet^{lo}7-AAD$^-$CD3$^+$ cells (lower left panel) represent living T cells that have proliferated. CD4$^+$ T cells activated with anti-CD3/CD28 beads are shown as a positive control for proliferation (black line histogram) in comparison with CD4$^+$ T cells cultured with medium only a (grey filled histogram). The GrB inhibitor Z-AAD-CMK (5 ug/mL) was added during the pDC/T cell co-culture. (B) CD4+ T cell expansion was measured as described in A. Shown are the mean percentages of CellTrace-violet^{lo}7-AAD$^-$CD3$^+$ cells from 4 different donors (* $P < 0.05$). Error bars indicate SD values.

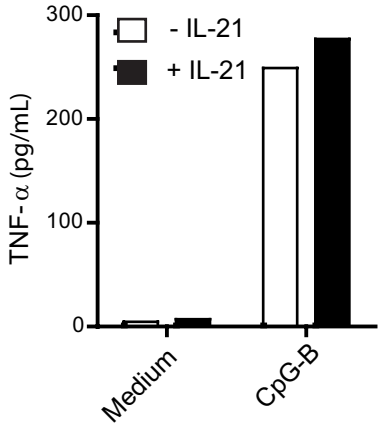
Karrich et al. Figure 1

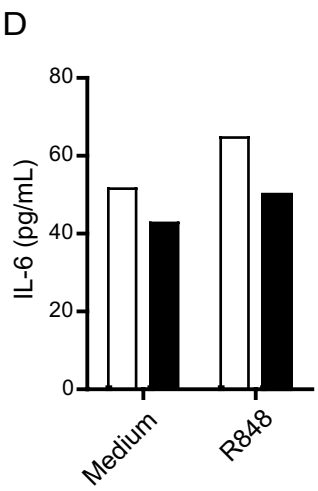


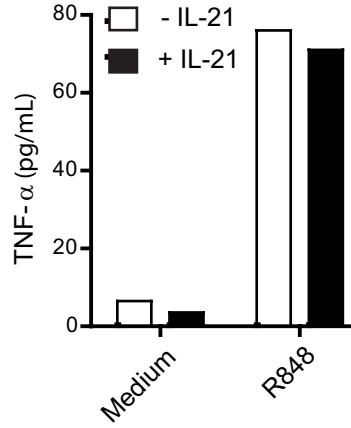




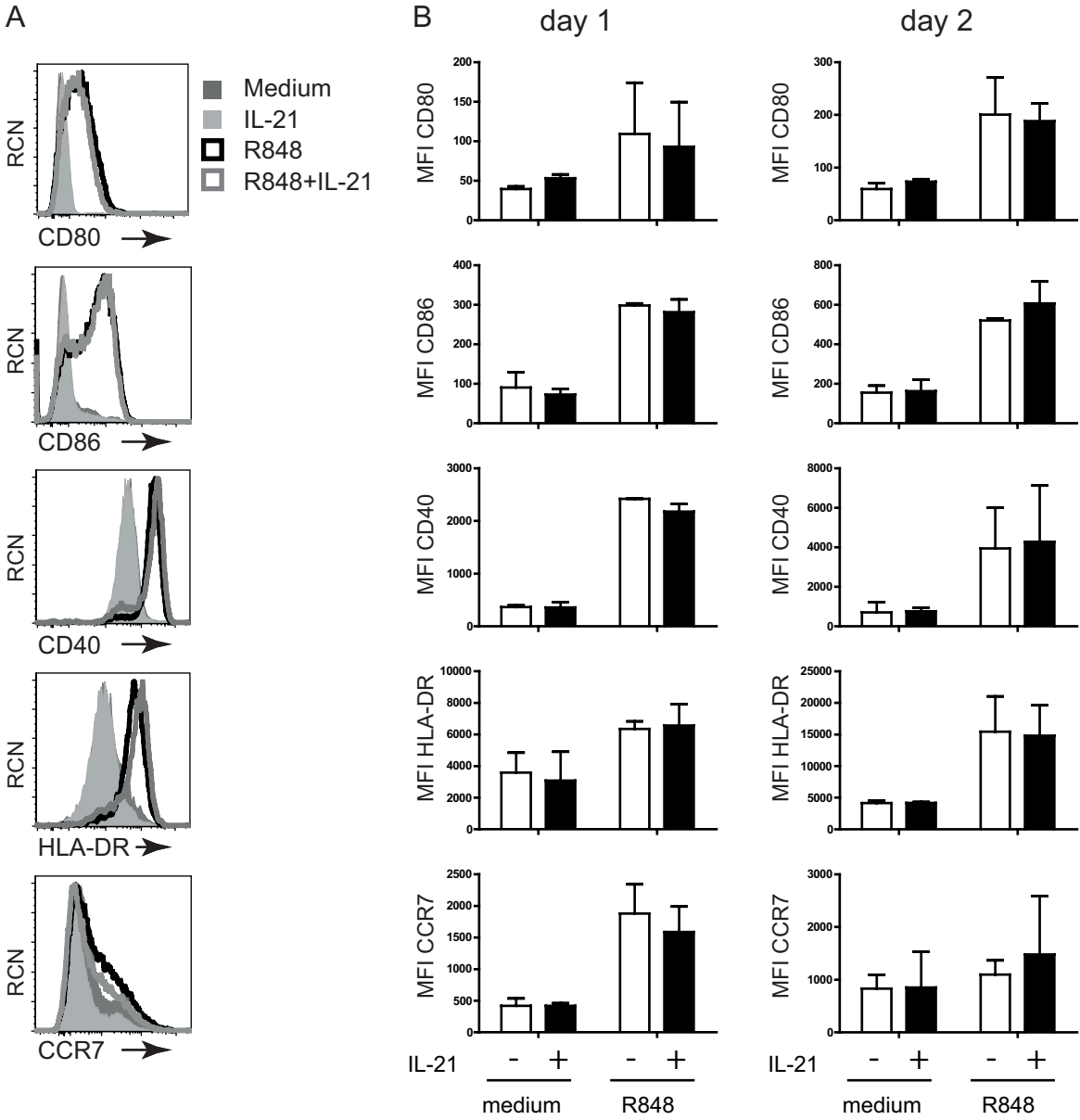


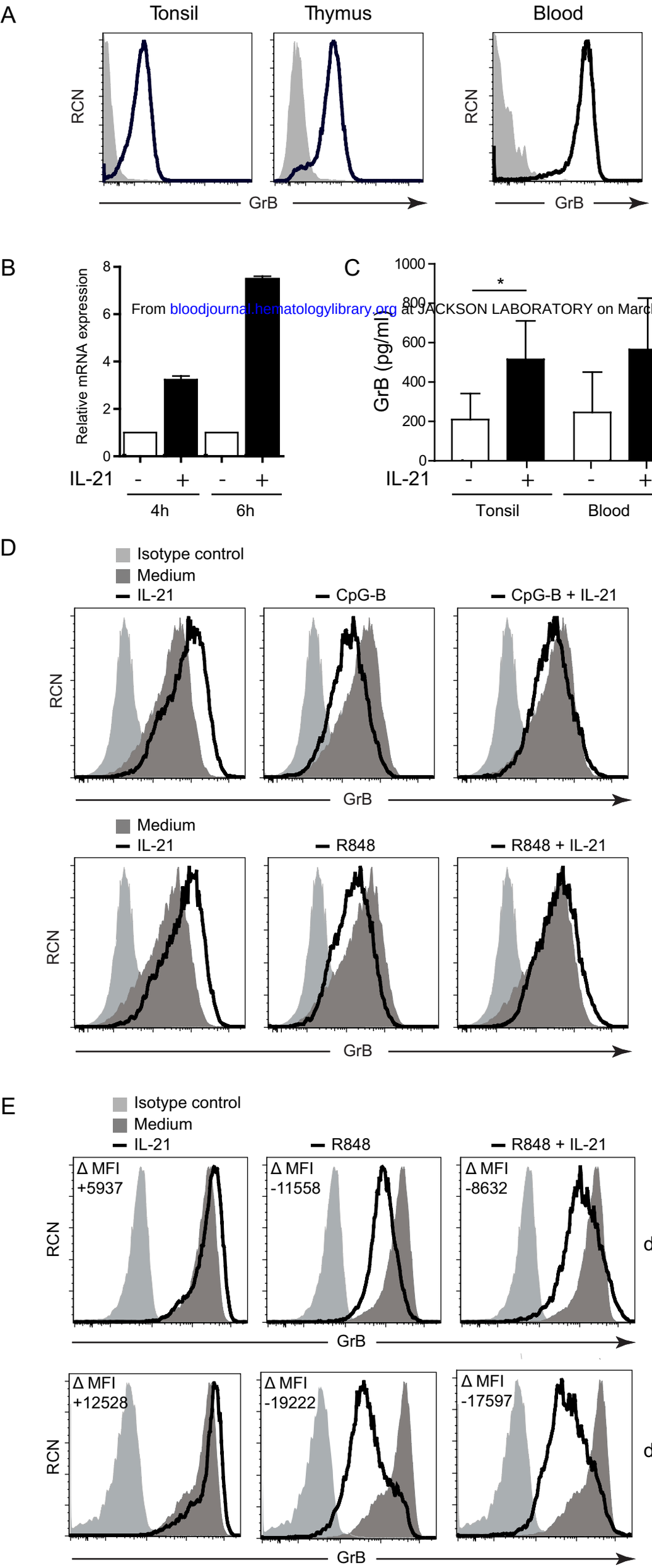






Karrich et al Figure 3





Karrich et al. Figure 5