IL-21 May Promote Granzyme B-Dependent NK/Plasmacytoid Dendritic Cell Functional Interaction in Cutaneous Lupus Erythematosus

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Autoimmune skin lesions are characterized by a complex cytokine milieu and by the accumulation of plasmacytoid dendritic cells (pDCs). Granzyme B (GrB) transcript is abundant in activated pDCs, though its mechanisms of regulation and biological role are largely unknown. Here we report that IL-21 was the only T helper 1/T helper 17 cytokine able to induce the expression and secretion of GrB by pDCs and that this action was counteracted by the autocrine production of type I IFNs. In lupus erythematosus skin lesions, the percentage of GrB^+ pDCs directly correlated with the IL-21/MxA ratio, indicating that the interplay between these two cytokines finely tunes the levels of pDC-dependent GrB also in vivo. In lupus erythematosus, pDCs colocalized with professional cytotoxic cells at sites of epithelial damage, suggesting a role in keratinocyte killing. Accordingly, we demonstrate that supernatants of IL-21-activated pDCs promoted autologous keratinocyte killing by natural killer cells and this action was dependent on GrB. These results propose a GrB-dependent functional interaction between pDCs and natural killer cells and highlight a negative feedback regulation by type I IFNs in vitro and in vivo that may function to limit excessive tissue damage.

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INTRODUCTION

Autoimmune diseases are pathological conditions generally dominated by T helper 1 (Th1)/T helper 17 (Th17) immune responses and characterized by the so-called "interferon signature." In fact, because of their immune-modulatory effects, type I IFNs have been associated with several pathogenic pathways in various autoimmune conditions (Ronnblom and Eloranta, 2013; Sozzani et al., 2010a). The main cell type responsible for the production of type I IFNs is plasmacytoid dendritic cells (pDCs). pDCs represent a minor subset of leukocytes that normally localizes in the circulation and in primary and secondary lymphoid organs. Accumulation of pDCs in both lymphoid and nonlymphoid tissues is however observed in several pathological conditions, including autoimmune diseases (e.g., systemic lupus erythematosus, psoriasis, lichen planus, and rheumatoid arthritis) (Colonna et al., 2004; Facchetti et al., 2003a; Sozzani et al., 2010b; Swiecki and Colonna, 2015), where exposure to self RNA and DNA immunocomplexes results in a strong type I IFN response (Conrad et al., 2009; Furuzawa-Carballeda et al., 2007; Gilliet et al., 2008; Sozzani et al., 2010b; Ueno et al., 2007). For this reason, pDCs have been implicated in the pathogenesis of diseases characterized by a type I IFN signature and are considered as a promising target for new intervention strategies.

In addition to type I IFNs, one of the most expressed transcripts in pDCs is granzyme B (GrB) (Bratke et al., 2010; Rissoan et al., 2002). Granzymes are a family of structurally related proteases constitutively synthesized and stored in association with other proteins, such as perforin, in the granules of natural killer (NK) cells, NKT cells, cytotoxic T lymphocytes, and γδ T cells (Afonina et al., 2010; Anthony et al., 2010; Heusel et al., 1994). GrB is a potent inducer of programmed cell death, and experiments performed with genetically deficient mice have highlighted the crucial role of this protein in host protection against viral and bacterial infections (Heusel et al., 1994). GrB is also expressed by activated basophils (Tschopp et al., 2006), mast cells (Strik et al., 2007), keratinocytes (Hernandez-Pigeon et al., 2006), and B cells (Hagn et al., 2009). Indeed, GrB may also exert functions different from apoptosis, including a role in the pathogenesis of autoimmune diseases and in the regulation of cell migration (Boivin et al., 2009; Buzza and Bird, 2006; Darrah and Rosen, 2010). GrB expression by pDCs can be detected in both circulating cells and pDCs located within secondary lymphoid organs (Facchetti et al., 2003b; Rissoan et al., 2002). Recent work performed in vitro with blood-purified pDCs has shown that GrB

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Abbreviations: GrB, granzyme B; LE, lupus erythematosus; NK, natural killer; pDC, plasmacytoid DC; Th1, T helper 1; Th17, T helper 17

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expression is upregulated by IL-3, IL-10, and IL-21, whereas it is inhibited by cell activation (Bratke et al., 2010; Jahrsdorfer et al., 2010; Karrich et al., 2013). Because pDCs do not express perforin (Bratke et al., 2010), a cytotoxic role for pDC-derived GrB remains uncertain. Instead, pDC-derived GrB was proposed to have regulatory functions, being able to inhibit T-cell proliferation (Jahrsdorfer et al., 2010; Karrich et al., 2013).

The purpose of this study was to characterize the regulation of GrB production in pDCs in vitro and in vivo in the context of lupus erythematosus (LE) skin biopsies characterized by Th1/Th17 and type I IFN skewed immune responses.

RESULTS

IL-21 is the only Th1/Th17 cytokine able to induce GrB secretion by pDCs

To evaluate GrB production, blood-purified pDCs were incubated in the presence of IL-3, a pDC survival factor (Grouard et al., 1997), and one of the following Th1 and Th17 cytokines, IFNγ, IL-17, IL-20, IL-21, IL-22, or IL-23 for 24 hours. Figure 1a and b show that among the cytokines tested, only IL-21 was able to upregulate GrB secretion in a dose-dependent fashion. This observation was paralleled by the increase of GrB mRNA (Figure 1c). As previously described, IL-21 induced GrB steady-state mRNA also in the absence of IL-3 (Figure 1c; Karrich et al., 2013). GrB protein could be detected intracellularly in freshly purified pDCs (8.3 ± 1.5 ng/10^6 cells; $n = 4$), and this concentration was further increased after 5- and 24-hour culture in the presence of IL-3 or IL-3/IL-21 (Figure 1d). At 24-hour stimulation, GrB was also detected in the cell supernatants with levels that were 2.1 ± 0.7-fold higher ($n = 8$) in the presence of IL-21 than in the presence of IL-3 alone (Figure 1e).

Type I IFNs negatively regulate GrB expression in pDCs

Previous work has shown that pDC maturation is associated with the reduction of GrB production, but the mechanism underlying this effect remains unknown (Karrich et al., 2013). Results reported in Figure 2a confirm and extend this observation showing that pDCs, activated with CpG oligodeoxynucleotide 2216 (TLR9 ligand) or imiquimod and R848 (TLR7 ligands), produce significantly less GrB than resting cells either in the presence or absence of IL-21. Because TLR7 and TLR9 engagement is known to induce type I IFNs as well as proinflammatory cytokines (Colonna et al., 2004; Facchetti et al., 2003a; Gilliet et al., 2008), the possible autocrine negative regulation of GrB production by these mediators was investigated. Figure 2b shows that B18R, a type I IFN-neutralizing protein encoded by vaccinia virus, significantly restored the secretion of GrB in the presence of CpG. By contrast, this effect was not observed when a TNF-α blocker (Etanercept) or an IL-6Rα blocking antibody was used. These results strongly suggest that autocrine type I IFNs play a major role in the inhibition of GrB production by activated pDCs. In support of this finding, recombinant IFNα strongly inhibited IL-21-induced GrB release and GrB production was restored in the presence of an anti-IFNAR blocking mAb (Figure 2c). Kinetics experiments were performed to further highlight the opposite regulation of type I IFNs and GrB in activated pDCs. Figure 2d shows that IL-21 was able to induce GrB but not IFNα, whereas CpG induced IFNα but not GrB. When combined together, the two agonists partially counter-regulated the action of each other with a reduced production of IFNα and a slight increase in the production of GrB. If pDCs were pretreated with IL-21 for 5 hours before CpG stimulation, the effect of IL-21 predominated over CpG stimulation, with the induction of GrB but not of IFNα (Figure 2d).

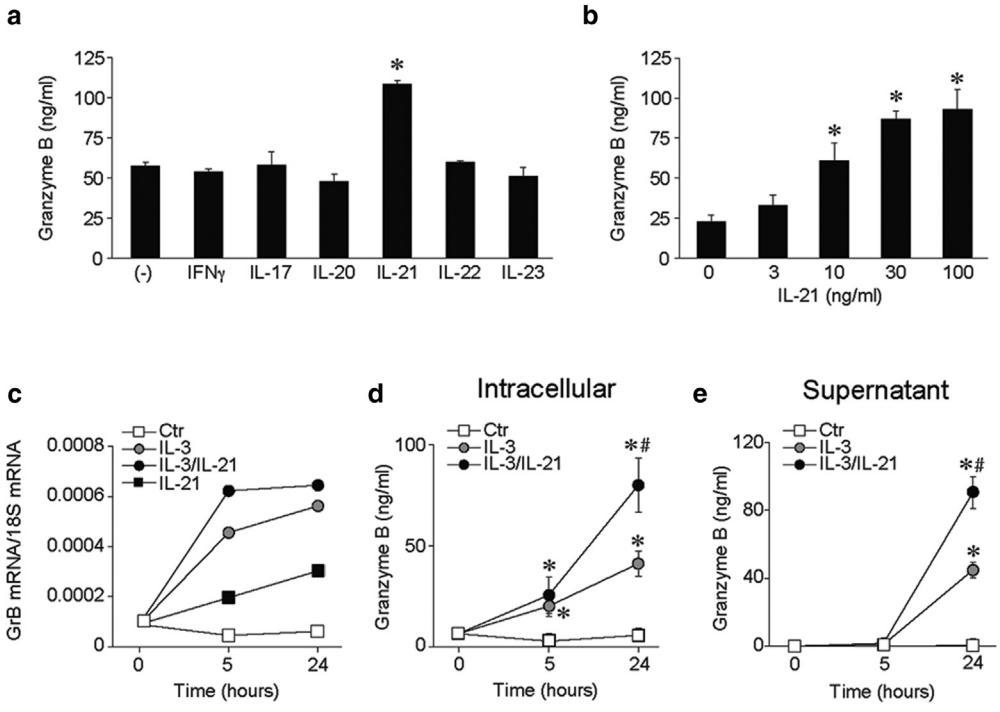

Figure 1. IL-21 induces GrB secretion in pDCs. (**a, b**) pDCs were incubated in the presence of IL-3 (−) and one of the indicated cytokines for 24 hours. GrB production was evaluated by ELISA. Data are expressed as mean ± SD ($n = 3$); $^*P < 0.01$ versus (−) by one-way ANOVA with Dunnet's post hoc test. (**c**–**e**) pDCs were stimulated in the absence (Ctr) or in the presence of IL-3 or IL-21 alone, or with IL-3/IL-21 for the indicated time points. (**c**) mRNA levels were evaluated by real-time PCR. The results are representative of two independent donors. Intracellular (**d**) and secreted (**e**) GrB were evaluated by ELISA. Results are expressed as mean ± SEM ($n = 8$); $^*P < 0.05$ versus Ctr, $^{\#}P < 0.05$ versus IL-3 by paired Student's *t*-test. ANOVA, analysis of variance; GrB, granzyme B; pDC, plasmacytoid dendritic cell; SD, standard deviation; SEM, standard error of the mean.

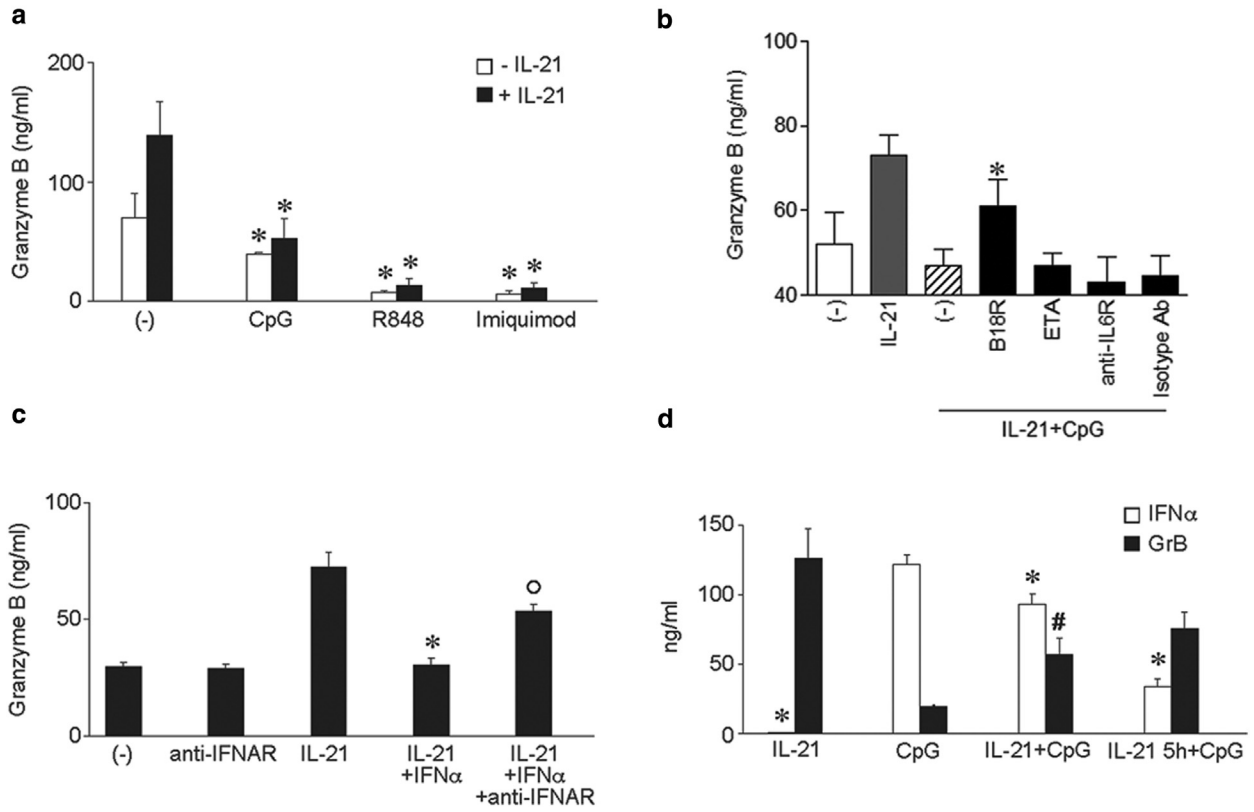

Figure 2. Negative regulation of GrB expression by type I IFNs in pDCs. (**a**–**d**) GrB and IFNα were evaluated by ELISA (mean ± SD; n = 3). (**a**) pDCs were stimulated as indicated for 24 hours without (white bars) or with (black bars) IL-21; $^{*}P < 0.05$ by one-way ANOVA with Dunnet's post hoc test. (**b**) pDCs were pretreated for 1 hour with specific cytokine blockers and then stimulated with IL21+CpG for 24 hours; $^{*}P < 0.05$ versus IL-21+CpG by paired Student's *t*-test. (**c**) pDCs were incubated for 24 hours with IL-21 alone or IL-21+IFNα in the presence or absence of anti-IFNAR mAb; $^{*}P < 0.05$ versus IL-21 or $^{\circ}P < 0.05$ versus IL-21 + IFNα by paired Student's *t*-test. (**d**) pDCs were stimulated with IL-21 or CpG or IL-21+CpG for 24 hours; $^{*}P < 0.05$ versus CpG or $^{\#}P < 0.05$ versus IL-21 by paired Student's *t*-test. ANOVA, analysis of variance; GrB, granzyme B; pDC, plasmacytoid dendritic cells; SD, standard deviation.

The positive IL-21/type I IFNs ratio correlates with GrB production by pDC in vivo

To explore the possibility that a similar regulation might take place in vivo, LE was selected as a pathological condition characterized by pDC infiltration and by the expression of both IL-21 and type I IFNs. In physiological conditions, IL-21 is mainly expressed in lymph nodes and tonsils but absent in skin (Tangye, 2015; Uhlen et al., 2010). By contrast, IL-21 is strongly expressed in lesional skin of patients with LE (Caruso et al., 2009). These data were confirmed by real-time PCR experiments (Figure 3a). The cellular source of IL-21 in LE biopsies was investigated by immunohistochemistry. Compared with normal skin, where the anti-IL-21 antibody showed only scant reactivity (not shown), LE biopsies were characterized by strong IL-21 staining in keratinocytes (Figure 3b and inset) together with some dermal expression by infiltrating lymphoid-like cells (Figure 3c) that localize in pDC-rich areas (Vermi et al., 2009). Double immunohistochemistry showed that the large majority of dermal IL-21-expressing cells were $CD3^{+}$, $ZAP70^{+}$, $CD4^{+}$, $PAX5^{-}$, and $CD8^{-}$ with a small fraction of cells also expressing CD30; these results indicate a predominant activated T-cell identity (Figure 3d–h). More rarely, IL-21 expressing cells were positive for the NK/NKT cell marker CD56 (Figure 3i). To assess the expression and the overall levels of type I IFNs in these tissues, we evaluated the expression of MxA, a type I IFN-inducible gene often used as a readout for type I IFN production. Figure 3j shows that MxA mRNA was very low in healthy skin and virtually absent in tonsils and lymph nodes. Conversely, MxA was strongly expressed in LE skin lesions. MxA staining and specificity was also evaluated by immunohistochemistry in sections of healthy skin and LE biopsies (Figure 3k).

Next, the expression of GrB by pDCs was investigated by double immunohistochemistry. Accumulation of GrB immunoreactive (GrB^{+}) pDCs was observed in LE biopsies (Figure 4a) as well as in reactive lymph nodes (Figure 4b) and tonsils (not shown) (Facchetti et al., 2003a). However, the differential counting of GrB^{+} cells within the whole population of infiltrating pDCs revealed that the percentage of GrB^{+} cells was lower in LE skin lesions as compared with other tissues (Figure 4c). In addition, in LE biopsies, a marked heterogeneity among donors was observed, ranging from 41.2% to 89.7% (mean 65.5 ± 15.4%) of GrB^{+} pDCs.

These results suggested that the cytokine milieu could indeed modulate the production of GrB by pDCs, as demonstrated by our in vitro experiments. To corroborate this hypothesis, the ratio between IL-21 and MxA expression was determined in different samples of lymph nodes, tonsils, and LE skin, and it was found to correlate with the percentage of GrB^{+} pDCs observed in each sample. Figure 4d shows that a

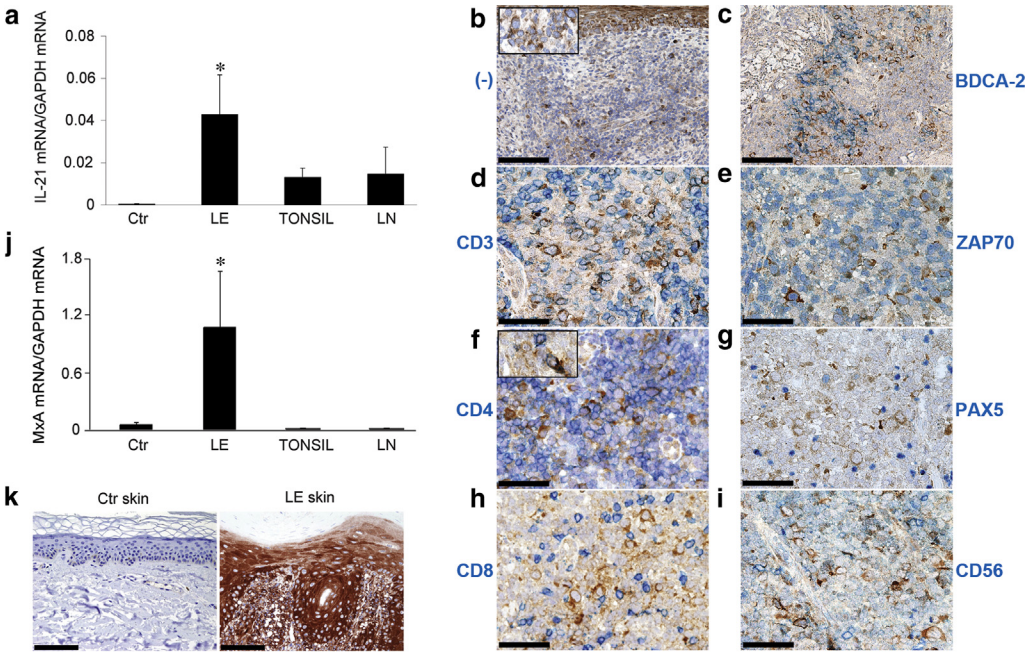

Figure 3. Tissue expression of IL-21 and MxA in LE skin biopsies. Real-time PCR for (**a**) IL-21 or (**j**) MxA mRNA transcripts in skin biopsies from healthy donors (Ctr; n = 5) and patients with LE (n = 5) or in reactive tonsils (n = 10) and reactive lymph nodes (n = 3). IL-21 and MxA expression was normalized based on GAPDH content. Data are expressed as mean ± SEM; $*P < 0.05$ by one-way ANOVA with Dunnet's post hoc test. (**b**–**i**) Sections from one LE skin biopsy are stained for IL-21 (brown) and indicated markers (blue). Magnification ×200 (**b, c**; bar =100 μm), ×400 (**d**–**i**; bar = 50 μm), and ×600 (inset in **f**, CD30; bar = 33 μm). (**k**) Immunohistochemistry for MxA on formalin-fixed tissue sections from healthy skin and LE skin biopsy. Magnification ×200, bar = 100 μm. ANOVA, analysis of variance; BDCA, blood DC antigen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LE, lupus erythematosus; SEM, standard error of mean.

strong direct correlation existed between the IL-21/MxA mRNA ratio and the percentage of GrB$^+$ pDCs (Spearman $r = 0.77$; $P = 0.007$), supporting the notion that also in vivo type I IFNs may function as a negative regulator of IL-21-dependent GrB production by pDCs.

pDCs colocalize with professional cytotoxic cells at sites of epithelial damage

GrB is a protease responsible for the cytotoxic activity of NK and CD8$^+$ T cells. In LE, as previously observed for lichen planus lesions (Parolini et al., 2007), blood DC antigen-2 (BDCA-2)$^+$ pDCs were found to localize in strict proximity to CD56$^+$ NK cells (Figure 5a) and CD8$^+$ T cells (Figure 5b) in areas of keratinocyte cell death, as demonstrated by triple immunostaining for BDCA-2, GrB, and Casapase 3 (Figure 5c). These histopathological observations raised the question whether pDCs may cooperate with professional cytoxic cells in inducing keratinocyte killing.

pDC-derived GrB increases NK cell cytotoxic activity

Preliminary experiments demonstrated that recombinant IL-21 did not directly induce NK cell degranulation as assessed by CD107 membrane expression (not shown) and that pDC supernatants (either control or IL-3/IL-21-activated)

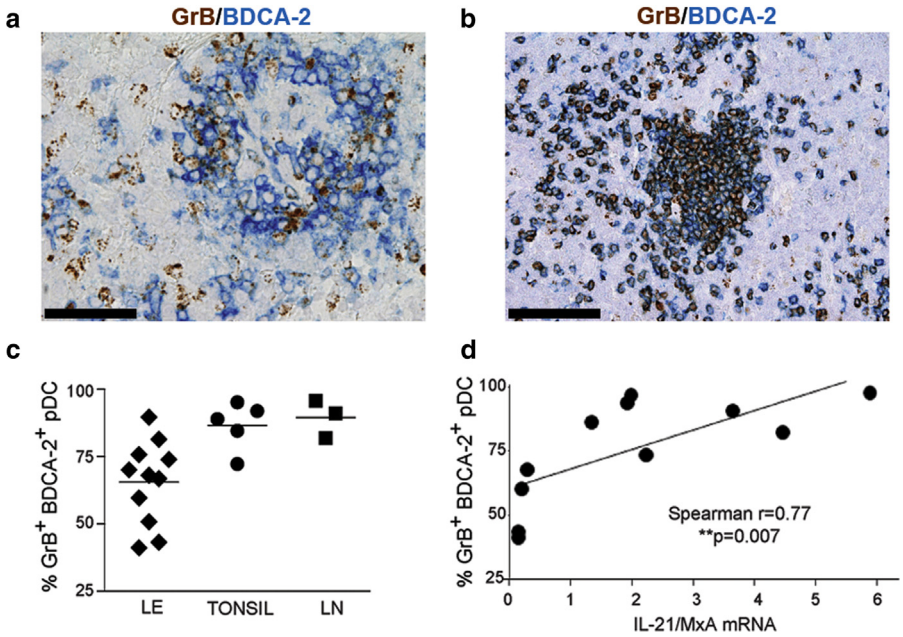

Figure 4. Regulation of GrB expression by the IL-21/MxA expression ratio in tissue pDCs. (**a**) Immunohistochemistry for GrB (brown) and BDCA-2 (blue) on a representative LE skin biopsy and (**b**) reactive lymph node; magnification ×200 (**b**; bar = 100 μm) and ×400 (**a**; bar = 50 μm). Results are representative of at least five different patients. (**c**) Percentage of GrB$^+$ pDCs among BDCA-2$^+$ pDCs in LE skin biopsies (n = 11), reactive tonsils (n = 5) and reactive lymph nodes (n = 3). (**d**) Correlation between GrB$^+$ pDCs and IL-21/MxA mRNA levels in tissue biopsies from LE (4 cases), reactive tonsils (5 cases), and reactive lymph nodes (2 cases) ($y = 7.7531x + 60.57$, $r^2 = 0.51$; Spearman $r = 0.77$, $^{**}P = 0.007$). BDCA, blood DC antigen; GrB, granzyme B; LE, lupus erythematosus; pDC, plasmacytoid dendritic cell.

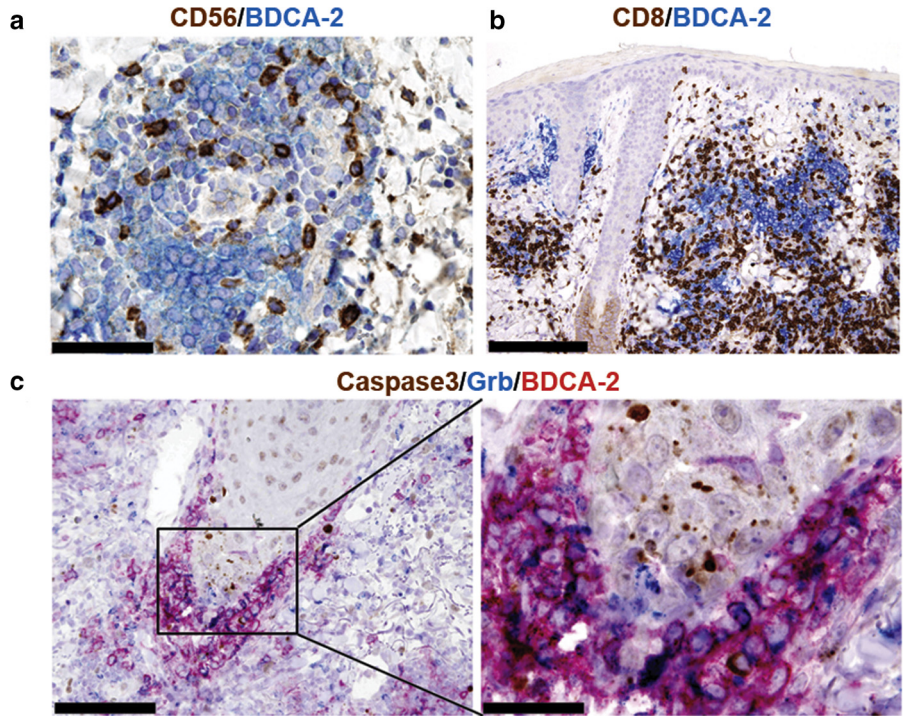

Figure 5. pDCs colocalize with NK and T cells in areas of keratinocyte cell death. Immunohistochemistry for (**a**) CD56 (brown)/BDCA-2 (blue) and (**b**) CD8 (brown)/BDCA-2 (blue) on a representative LE skin biopsy. Magnification ×100 (b; bar = 200 μm) and ×400 (a; bar = 50 μm). (**c**) LE skin stained for BDCA-2 (red), GrB (blue), and activated caspase 3 (brown) (magnification ×200 (left panel), bar = 100 μm; ×600 (right panel), bar = 33 μm) illustrating colocalization of GrB-producing pDCs and areas of epithelial damage. BDCA, blood DC antigen; GrB, granzyme B; LE, lupus erythematosus; NK, natural killer; pDC, plasmacytoid dendritic cell.

in the absence of NK cells induced a basal degree of keratinocyte killing that was independent of the culture condition used (Figure 6b). By contrast, the addition of supernatants of IL-3/IL-21-activated pDCs, but not of control pDCs, to co-cultures of primary keratinocytes with autologous NK cells (either resting or IL-2-activated) was able to increase NK cell-mediated keratinocyte killing (Figure 6a and b). To confirm that the cytotoxic effect of pDC supernatants was dependent on GrB, supernatants of IL-3/IL-21-activated pDCs were GrB immunodepleted (Figure 6c, upper panel). Figure 6c (lower panel) shows that immunodepletion of GrB significantly abrogated the increase in NK cell killing. Finally, IL-21 was found to upregulate the mRNA of PI-9 in pDCs (Figure 6d). PI-9 is a serpin that efficiently inhibits GrB; this mechanism is likely to protect pDCs by GrB-mediated apoptosis as previously demonstrated for cytotoxic T cells (Bird et al., 1998).

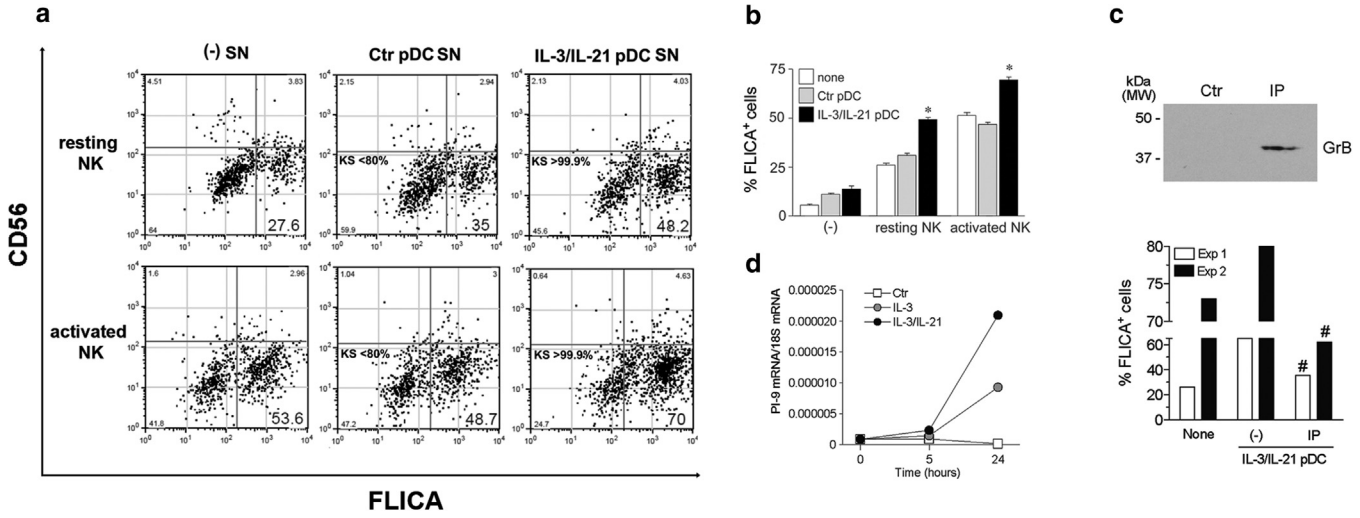

Figure 6. pDC-derived GrB increases NK cell cytotoxicity. (**a, b**) Keratinocytes were cultured for 6 hours without (−) or with Ctr or IL-3/IL-21 pDC supernatants (SN) in the presence or absence of resting or activated autologous $CD56^+$ NK cells. Numbers in LR quadrants show the percentage of $FLICA^+CD56^-$ keratinocytes. Kolmogorov-Smirnov (KS) probability as compared with "(−) SN." (**b**) Mean ± SD (n = 3); $^*P < 0.05$ versus Ctr pDCs by paired Student's *t*-test. (**c**) Upper panel: western blot showing GrB immunoprecipitation in IL-3/IL-21 pDC supernatants; Lower panel: Keratinocytes were cultured in the presence of activated NK cells (None) and with IL-3/IL-21-treated supernatants either native (−), or GrB-depleted (IP); #: KS>99.9% compared with native IL-3/IL-21-treated supernatants (−). (**d**) PI-9 mRNA levels in pDCs stimulated as indicated. Representative of two donors. FLICA, fluorochrome-labeled inhibitor of caspases assay; GrB, granzyme B; NK, natural killer; pDC, plasmacytoid dendritic cell; SD, standard deviation.

DISCUSSION

This study investigates the regulation and function of GrB in human pDCs and reports two main findings. First, it identifies a central role for autocrine type I IFNs in the negative regulation of GrB production by IL-21-activated pDCs in vitro and in vivo. Second, it shows that pDC-derived GrB is biologically active and may play a pathogenic role in autoimmune diseases, such as LE, by increasing keratinocyte killing mediated by professional cytotoxic cells.

IL-21 is a cytokine produced by $CD4^+$ T cells, in particular T follicular helper cells and Th17 cells (Sarra et al., 2013; Tangye, 2015), and it was shown to induce GrB expression in B cells and in pDCs (Hagn et al., 2009; Karrich et al., 2013). This study extends these observations showing that among the Th1/Th17 cytokine tested, IL-21 was the only cytokine able to induce GrB expression in pDCs. IL-21 was previously found upregulated in LE, an autoimmune disease characterized by Th1/Th17 polarization (Caruso et al., 2009; Sarra et al., 2013). In this study we identify $CD4^+$ T lymphocytes as the major IL-21-expressing cells in skin LE lesions. As expected (Vermi et al., 2009), the same LE skin lesions were heavily infiltrated by GrB^+ pDCs although donor-to-donor heterogeneity was present in terms of percentage of GrB^+ pDCs, suggesting that the local cytokine milieu may finely tune the induction of GrB expression (Facchetti et al., 2003a). Analysis of the different types of cutaneous LE apparently did not reveal any direct correlation with GrB expression, although the number of samples investigated does not allow any definitive conclusions. In vitro experiments showed that pDC activation by TLR ligands counteracted the effect of IL-21, inducing a strong inhibition of GrB production. This effect was largely dependent on the autocrine production of type I IFNs with no involvement of two other proinflammatory mediators, namely IL-6 and TNF-α, known to be produced under the same experimental conditions. However, we cannot exclude a minor contribution of other cytokines released by TLR-activated pDCs. The statistically significant correlation between the percentage of GrB^+ pDCs and the IL-21/MxA ratio in pathological tissues supports the concept that also in vivo high local expression of type I IFNs inhibits the production of GrB by pDCs. This regulation may also be responsible for the heterogeneous expression of GrB observed by infiltrating pDCs in LE skin biopsies. Wenzel et al. (2005a) reported a positive correlation between GrB and MxA expression in LE biopsies. GrB is known to be produced by many different cells, including professional cytotoxic cells (e.g., CTLs and NK cells). Therefore, these results are not in contradiction with our study that concentrated on GrB solely produced by pDCs.

Type I IFNs constitute a group of more than 20 cytokines that possess complex immunoregulatory functions (Capobianchi et al., 2015; Sozzani et al., 2010a). For example, type I IFNs were shown to regulate either in a positive or negative manner the production of both pro- and anti-inflammatory cytokines, although the molecular mechanisms underlying this modulatory capacity are not completely understood (Howes et al., 2016). In the present setting, at least two alternative hypotheses can be considered. Both IFNAR and IL-21R activate STAT1 and STAT3 (Ivashkiv and Donlin, 2014; Leonard and Wan, 2016), two transcription factors known to play opposing effects on IL-21-activated genes (Wan et al., 2015). Thus, by competing for the activation of these transcription factors, type I IFNs may interfere with the IL-21R signaling and the regulation of downstream genes. Alternatively, type I IFNs might regulate IL-21-induced GrB production at the receptor level by reducing the expression of IL-21R in pDCs, as described in human NK and T cells (Strengell et al., 2004).

LE skin lesions are characterized by extensive tissue damage due to the killing of keratinocytes by autoreactive cytotoxic T cells and NK cells (Vermi et al., 2009). pDCs were suggested to contribute to epithelial cell death either indirectly, via the recruitment of $CXCR3^+$ cytotoxic cells (Wenzel et al., 2005b, 2007), or directly, by the production of effector molecules such as TRAIL (Gilliet et al., 2004). Here, we show that the supernatants of IL-21-activated pDCs cooperate with NK cells in the killing of autologous keratinocytes in a GrB-dependent manner. Although the exact molecular mechanism responsible for this effect awaits further investigation, it is tempting to speculate that pDC-derived GrB may support NK cell-derived GrB/perforin cytotoxicity and possibly contribute also to $CD8^+$ cell-mediated epithelial cell damage previously observed in autoimmune skin lesions (Vermi et al., 2009). The colocalization of pDCs with NK cells further supports the emerging concept of innate cooperation between pDCs and NK cells (Moretta et al., 2008).

In addition to apoptosis, extracellular matrix modification is a hallmark of many chronic inflammatory and autoimmune disorders that is closely linked to neoantigen generation. As a potent serine protease, extracellular GrB plays a predominant role in this process (Boivin et al., 2009). Thus, pDC-derived GrB might also exert critical perforin-independent enzymatic functions in autoimmune disease pathogenesis. In this context, the negative regulation of GrB production by type I IFNs may be interpreted as an extreme feedback strategy to limit tissue damage and autoantigen spreading.

In conclusion, this study extends our understanding on the regulation and function of GrB production by pDCs and highlights additional roles for infiltrating pDCs in skin LE lesions.

MATERIALS AND METHODS

Purification and stimulation of peripheral blood pDCs

Peripheral blood mononuclear cells were obtained from buffy coats (through the courtesy of the Centro Trasfusionale, Spedali Civili, Brescia) by Ficoll gradient. Peripheral blood pDCs were magnetically sorted with Diamond Plasmacytoid Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). pDCs (1×10^6 cells/ml) were cultured in RPMI 1640 (Gibco, Invitrogen, Carlsbad, CA) with 10% fetal calf serum (Lonza Group, Switzerland) and 20 ng/ml IL-3 (ProSpec, Rehovot, Israel).

Cytokine ELISA

GrB was detected by ELISA (Diaclone, Besançon, France); IFNα was detected using a specific Module Set (Bender MedSystems, Vienna, Austria). To detect intracellular GrB, cells were washed twice with PBS and lysed in ice-cold buffer (5 mM EDTA, 1% Triton X100 and a

protease inhibitor cocktail in phosphate buffered saline; all reagents were from Sigma, St. Louis, MO).

Immunohistochemistry

Tissues were obtained from the archive of the Department of Pathology, Spedali Civili di Brescia. Samples included LE skin biopsies (11 cases), healthy skin (4 cases), reactive tonsils (5 cases), and lymph nodes (3 cases). This retrospective study was conducted in compliance with the Declaration of Helsinki and with policies approved by the Delibera del garante n. 52 del 24/7/2008 and DL 193/2003. Specifically, for a retrospective and observational study on archival material obtained for diagnostic purpose, patient consent is not needed. Four-micron formalin-fixed paraffin embedded tissue sections were stained with primary antibodies as listed in Supplementary Materials online. On appropriate antigen retrieval, reactivity was revealed using Real EnVision Mouse/Rabbit-HRP (Dako, Santa Clara, CA) or Novolink polymer (Novocastra Laboratories, Newcastle upon Tyne, UK) followed by 3,3′-diaminobenzidine. For double/triple immunohistochemistry, the second immune reaction was visualized using Mach 4 MR-AP (Biocare Medical, Concord, CA), followed by Ferangi Blue (Biocare Medical) as chromogen and New Fucsin (Dako) for triple stain. Sections were photographed using the DP-70 Olympus digital camera mounted on the Olympus BX60 microscope, and the digital pictures (0.036 mm^2) were used for cell count. Because of the heterogeneity of pDC density and distribution in different biopsies, the number of images evaluated in each case varied from 4 to 11.

Induction of keratinocyte apoptosis

To obtain NK cells, the $CD3^-CD56^+$ fraction of peripheral blood mononuclear cells was purified by immunomagnetic negative selection using the NK cell Isolation kit (Miltenyi Biotec). The resulting NK cells (<2% $CD3^+$ and >90% $CD56^+$) were suspended in RPMI supplemented with 10% fetal calf serum and activated or not with rhIL-2 (80 U/ml) (Novartis, Origgio, Italy). Autologous keratinocyte cultures were prepared from skin blister roofs obtained from healthy donors as previously described (Carbone et al., 2010) and used as target cells for NK cell-mediated cytotoxicity. Keratinocyte apoptosis was determined using the caspase 3 and 7 fluorochrome-labeled inhibitor of caspases assay kit (FLICA; ImmunoChemistry Technologies, Bloomington, MN) and evaluated by FACS (Carbone et al., 2010).

GrB immunodepletion

IL-3/IL-21-stimulated pDC supernatant was incubated overnight at 4 °C with an anti-GrB antibody (sc-71173, Santa Cruz Biotechnology, Santa Cruz, CA) or with its isotype control. Immunocomplexes were precipitated at 4 °C by a 4-hour incubation with an anti-mouse-agarose antibody (Sigma), released following a "soft" elution protocol (Antrobus and Borner, 2011) and analyzed by western blotting under nonreducing conditions using the same anti-GrB antibody. GrB-immunodepleted supernatants underwent a second round of anti-mouse-agarose antibody to eliminate residual immunoglobulins and then used in the assay.

Statistical analysis

Results are expressed as mean ± standard error of the mean or mean ± standard deviation. Statistical significance was determined using paired Student's *t*-test or one-way analysis of variance with Dunnet's post hoc test; statistical significance in Figure 4d was determined by Spearman rank correlation (GraphPad Prism version 4.00). Kolmogorov-Smirnov probability assessing statistically significant differences between cell populations was calculated by FlowJo (version 10.1).

CONFLICTS OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2017.03.016.

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