Antiviral Vaccines License T Cell Responses by Suppressing Granzyme B Levels in Human Plasmacytoid Dendritic Cells

Dorit Fabricius, Benedikt Nußbaum, Daniel Busch, Verena Panitz, Birgit Mandel, Angelika Vollmer, Mike-Andrew Westhoff, Christof Kaltenmeier, Oleg Lunov, Kyrylo Tron, G. Ulrich Nienhaus, Bernd Jahrsdörfer and Klaus-Michael Debatin

*J Immunol* 2013; 191:1144-1153; Prepublished online 19 June 2013;
doi: 10.4049/jimmunol.1203479
http://www.jimmunol.org/content/191/3/1144

---

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2013/06/19/jimmunol.1203479.DC1

**References**

This article cites 54 articles, 26 of which you can access for free at:
http://www.jimmunol.org/content/191/3/1144.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Antiviral Vaccines License T Cell Responses by Suppressing Granzyme B Levels in Human Plasmacytoid Dendritic Cells

Dorit Fabricius,* Benedikt Nußbaum,**† Daniel Busch,*,† Verena Panitz,*, Birgit Mandel,*, Angelika Vollmer,*, Mike-Andrew Westhoff,*, Christof Kaltenmeier,† Oleg Lunov,‡ Kyrylo Tron,§ U. Nienhaus,‡ Bernd Jahrsdörfer,‡ and Klaus-Michael Debatin*‡

Human plasmacytoid dendritic cells (pDC) are important modulators of adaptive T cell responses during viral infections. Recently, we found that human pDC produce the serine protease granzyme B (GrB), thereby regulating T cell proliferation in a GrB-dependent manner. In this study, we demonstrate that intrinsic GrB production by pDC is significantly inhibited in vitro and in vivo by clinically used vaccines against viral infections such as tick-borne encephalitis. We show that pDC GrB levels inversely correlate with the proliferative response of coincubated T cells and that GrB suppression by a specific Ab or a GrB substrate inhibitor results in enhanced T cell proliferation, suggesting a predominant role of GrB in pDC-dependent T cell licensing. Functionally, we demonstrate that GrBhigh but not GrBlow pDC transfer GrB to T cells and may degrade the γ-chain of the TCR in a GrB-dependent fashion, thereby providing a possible explanation for the observed T cell suppression by GrB-expressing pDC.

Modulation of pDC-derived GrB activity represents a previously unknown mechanism by which both antiviral and vaccine-induced T cell responses may be regulated in vivo. Our results provide novel insights into pDC biology during vaccinations and may contribute to an improvement of prophylactic and therapeutic vaccines. The Journal of Immunology, 2013, 191: 1144–1153.

Plasmacytoid dendritic cells (pDC) are a unique subset of DC linking innate and adaptive immunity, thereby modulating immune responses in the course of viral, autoimmune, and neoplastic diseases (1–4). Apart from production of extraordinarily high IFN-α levels and their capacity to rapidly initiate Ag-specific antiviral CD8+ T cell responses by direct proteasome-independent cross-presentation of exogenous viral Ags (5), pDC are able to secrete large amounts of the serine protease granzyme B (GrB), particularly after treatment with IL-3 and IL-10, which can result in a suppression of T cell proliferation (6).

Granzymes including GrB represent a major component of the granules of CTL and NK cells, classically known for apoptosis induction in target cells recognized by CTL (7, 8). Lately, certain pathological conditions were associated with the presence of extracellular GrB. The occurrence of extracellular GrB may be explained either by escape of GrB from the immunological synapses of cytotoxic lymphocytes (9) or by production of GrB by nonclassical sources. This possibility is supported by recent findings that immune cell subsets other than CTL or NK cells are able to produce and secrete active GrB under certain circumstances.

Subsets capable of GrB production include B cells (10, 11), hematopoietic progenitor cells (12), basophils (13), mast cells (14), IFN-α–activated monocyte-derived DC (15), and pDC (6, 16). Of note, pDC can produce GrB in amounts that considerably exceed GrB levels produced by classical cytotoxic lymphocytes (6). Apart from its cytotoxic functions, GrB can exert alternative effects such as degradation of viral proteins important for assembly and replication (17), cytokine-enhancing effects (18), receptor cleavage (19), matrix degradation and remodeling (20), and immunosuppression (9, 21, 22), including the recently published pDC–GrB-mediated inhibition of T cell proliferation (6).

Antimicrobial vaccines are powerful tools to decrease the severity of an infection whose effects on B cells and Ab production are fairly well understood (23, 24). Certain viral vaccines appear to depend on both humoral and cellular immune responses to be protective (e.g., yellow fever vaccine (25) or varicella-zoster vaccine (26)). It has become clear that cellular immunity contributes to a more solid and long-lasting protection involving B cell memory and long-lived Ab responses. The precise mechanisms by which vaccines confer sustainable immunity are not fully understood but hints point to an activation of certain DC subsets such as pDC (27). So far, it remains unclear how pDC as mediators between innate and adaptive immunity contribute to B and T cell activation following vaccination, but it can be supposed that certain vaccines that contain viral nucleic acid motifs may exert their effects in part via TLR-mediated activation of pDC. On the basis of our recent...
findings, one contributing factor for an efficient T cell response may be the modulation of pDC-derived GrB by viral nucleic acid-containing vaccines, which may allow enhanced T cell expansion after vaccination.

In this study, we demonstrate that intrinsic GrB production by pDC is significantly inhibited in vitro and in vivo by clinically used vaccines against viral infections. We show that pDC GrB levels are inversely correlated with the proliferative response of cocultured T cells, with tick-borne encephalitis (TBEV)-stimulated pDC exhibiting the highest T cell–licensing capacity. Importantly, we also show that GrB-expressing pDC, but not GrB-suppressed pDC, deliver GrB to T cells and may degrade the ζ-chain of the TCR in a GrB-dependent manner, thereby providing a possible explanation for the regulatory effect of GrB-expressing pDC on T cell proliferation. Our study provides novel insights into how pDC fine-tune and license antiviral T cell responses and may contribute to an improvement of prophylactic and therapeutic vaccination approaches.

Materials and Methods

Human subjects and cell culture

The present study was approved by the Ethics Committee at Ulm University. Peripheral blood from healthy volunteers was acquired after obtaining informed consent. For some experiments, peripheral blood from healthy individuals was collected up to 7 d before and 4 d after vaccination against TBEV (FSME Immun, Baxter Vaccines, Deerfield, IL). Mononuclear cells from PBMC were isolated, and RBC were removed. pDC were isolated either by positive selection using the BDCA4+ cell isolation kit II or by negative selection with the Plasmacytoid Dendritic Cell Isolation Kit, according to the manufacturer’s instructions (Miltenyi Biotec, Bergisch Gladbach, Germany), resulting in >95% cells with a lin−1 BDCA-2+ CD123+ MHC II+ phenotype. For in vitro cultures, cells were suspended in adaptive immunotherapy medium (AIMV; Life Technologies, Grand Island, NY) and, if not indicated otherwise, supplemented with 10 ng/ml of the human pDC growth factor IL-3 (R&D Systems, Minneapolis, MN). Cells were incubated for 16–24 h as indicated on 96-well flat-bottom plates (5 × 10^5 cells/ml, 200 µl/well, 37°C, and 5% CO2).

Reagents for functional assays

The CpG oligodeoxynucleotide ODN 2006 (2.5 µg/ml CpG B) with the specific sequence 5’-tcg tcg ttt tgt cgt ttt gtc gtt-3’ of potently TLR9 agonist was purchased from Invivogen (San Diego, CA). The vaccines used in our study are listed in Table I. Aluminum hydroxide (Al(OH)3) served as control for adjuvant-containing TBEV and HBV vaccines (Sigma-Aldrich, St. Louis, MO). Anti-CD3/CD28 Ab–coated beads (0.25 µg/l/well) were purchased from Dynal (Invitrogen, Karlsruhe, Germany). For GrB inhibition in functional assays, the GrB inhibitor III (1 μM Z-IE(OMe)T-DFMK; Calbiochem, Darmstadt, Germany) and a neutralizing anti-GrB Ab (1 µg/ml; R&D Systems) were used. As a TLR7 antagonist, we used the oligonucleotide IRS 661 (28) with the sequence 5’-tgc tc ttt gtc gta a a g c c t t g g t g g a g c c a a g g c a-3’ and as TLR9 antagonist, we used the oligonucleotide IRS 869 (28) with the sequence 5’-t tc c t t g g a a g g g c t g t g r s-3’ with * indicating phosphorothioate modifications (http://www.biomers.net, Ulm, Germany).

Flow cytometry

Cells were harvested at the indicated time points and stained as described previously (29). FITC-, PE-, PE-Cy5, PE-Cy7, PerCP-Cy5.5, Pacific blue, or allophycocyanin–labeled Abs to lin-1, CD3, CD4, CD123, CD80, CD86, CD54, CD40, Annexin V, MHC class I (MHC I), and MHC II were purchased from BioLegend, PE- or APC–labeled Abs to BDCA-2 (CD303) were purchased from Miltenyi Biotec, PE-labeled anti-GrB Ab, and an appropriate isotype control were purchased from Sanquin (Amsterdam, The Netherlands). 7-Aminomethionin (7-AAD) was purchased from Merck (Darmstadt, Germany). For flow cytometric intracellular GrB detection, pDC were cultured at 5 × 10^5/ml for 16 h, followed by a 4-h incubation with brefeldin A (Epitope Technologies, Madison, WI) at 1 µg/ml. Intracellular staining was performed as described previously (6). Flow cytometric analysis was performed on a FACScan, a FACSort, or an LSRII (BD Immunocytometry Systems, San Jose, CA), and data were analyzed using FlowJo software (version 8.7.1; Tree Star, Stanford, CA).

GrB ELISA

pDC from healthy individuals were magnetically purified to over 95% purity as outlined above, and incubated in AIMV medium in the presence of human IL-3 (10 ng/ml) and vaccines at different concentrations as indicated. After 16 h, supernatants were collected, and GrB concentration was determined using a human GrB ELISA kit, according to the manufacturer’s instructions (Mabtech, Nacka Strand, Sweden). The lower limit of GrB detection was 13 pg/ml.

IFN-α ELISA

pDC were isolated as previously described and cultured in IL-3–containing (10 ng/ml) AIMV medium (5 × 10^5 pDC/well) with various vaccines at the concentrations indicated. After 16 h, supernatants were harvested, and IFN-α concentration was determined using an ELISA kit for multisubtype human IFN-α (PBL Biomedical Laboratories, Piscataway, NY). The lower detection limit for IFN-α was 76 pg/ml.

CFSE staining and proliferation assay

For coculture experiments, pDC were preincubated for 24 or 48 h. T cells from healthy individuals were isolated using the CD4+ T cell isolation kit II (Miltenyi Biotec). T cells were labeled with CFSE (Life Technologies, Madison, WI) (27) and cocultured with pDC. Particles were harvested after 4 d, and CFSE staining was performed as described previously (6). Flow cytometric analysis was performed on a FACScan, a FACSort, or an LSRII (BD Immunocytometry Systems, San Jose, CA), and data were analyzed using FlowJo software (version 8.7.1; Tree Star, Stanford, CA).

Figure 1. TBEV vaccine induces substantial IFN-α secretion by pDC in a concentration-dependent manner. Purified pDC were incubated in medium containing IL-3 (10 ng/ml) with various antiviral vaccines overnight. IFN-α secretion was determined by ELISA. (A) Vaccines were concentrated or diluted as follows: rubella, measles, yellow fever, polio, and varicella virus vaccine: 1:20; HBV and TBEV vaccine concentration: 100 ng/ml. Bar graphs show average values of IFN-α secretion from at least three independent experiments, error bars indicate SEM, and p values indicate significant differences compared with no vaccine. (B) pDC were incubated overnight in IL-3–containing medium with TBEV vaccine as indicated. Bar graphs show average values of IFN-α secretion from at least three independent experiments, error bars indicate SEM, and p values indicate significant differences compared with no vaccine.
Carlsbad, CA) for 10 min at a concentration of 2.5 μM. CD4+ T cells (2 × 10^5) were coincubated with pretreated allogeneic pDC at a pDC:T cells ratio of 1:250 for 5 d and then analyzed by FACS.

Fluorescence and spinning-disk confocal microscopy
Purified pDC were cultured for 48 h in AIMV medium containing IL-3 (10 ng/ml) with or without TBEV vaccine (100 ng/ml). CD4+ T cells were isolated as previously described and cultured in RPMI 1640 medium for 24 h. Enzymatically active GrB was visualized by incubation of 2 × 10^7 pDC for 1 h with 25 μl GranToxiLux fluorogenic GrB substrate in 75 μl AIMV medium at 37°C and 5% CO2 (OncoImmunin, Gaithersburg, MD). pDC were cocultivated with T cells at a ratio of 1:5 for 2 h in the presence of the substrate. Cells were stained with PE Mouse Anti-Human CD3 (BD Biosciences) for 15 min at room temperature and washed with medium. Cells were placed on collagen-coated chamber slides, and fluorescence microscopy was performed using an Olympus AX70 microscope with Cell F imaging software.

For confocal microscopy, purified pDC were cultured for 60 h in AIMV medium containing IL-3 (10 ng/ml) with or without TBEV vaccine (100 ng/ml). CD4+ T cells were isolated as previously described and cultured in RPMI 1640 medium for 24 h. Cell membranes were stained with Cell Mask deep red membrane dye (Invitrogen, Paisley, U.K.) at 0.5 μg/ml for 5 min at 37°C. Then, T cells were seeded on ibiTreat chamber slides (Integrated BioDiagnostics, Munich, Germany) for an immobilization period of 1 h. Enzymatically active GrB was visualized as described above. pDC were subsequently added to immobilized T cells, and the coculture was followed up for several hours. Fluorescence images were acquired as described previously (6).

FIGURE 2. Antiviral vaccines mildly modulate the expression of surface molecules on pDC. pDC were incubated in IL-3–containing medium with vaccines in concentrations as follows: measles and yellow fever virus: 1:10; polio and varicella virus: 1:20; and TBEV and HBV, 100 ng/ml. Surface molecule expression was analyzed via flow cytometry. (A) Representative histograms are shown. (B) Bar graphs represent average values relative to no vaccine from at least four independent experiments. Error bars indicate SEM; p values indicate significant differences compared with no vaccine.
TCR \( \zeta \)-chain Western blot

pDC were isolated from buffy coats of healthy individuals using a negative selection kit (Miltenyi Biotec) and preincubated for 24 h at 10^6 cells/ml in AIMV medium containing IL-3 (10 ng/ml) with or without IL-10 (25 ng/ml). CD4^+ T cells were isolated with a negative selection kit (Miltenyi Biotec) and treated with 5 \mu g/ml cycloheximide for 30 min at 37°C. pDC were washed and coincubated with T cells at a T cell:pDC ratio of 5:1 in the presence or absence of a neutralizing anti-GrB Ab (5 \mu g/ml AF2906; R&D Systems). T cells and pDC incubated in separate wells served as controls. After 20 h, cells were lysed, and proteins were run on an 18% SDS-PAGE. Immunoblotting was performed with a specific anti-CD247 Ab (clone 6B10.2; BioLegend, San Diego, CA) 1:5,000 and a secondary Ab (1:10,000; Dako-Cytomation, Glostrup, Denmark). TCR \( \zeta \)-chain was detected at 19 kDa.

Statistics

Data are expressed as means ± SEM. To determine statistical differences between the means of two data columns, the paired two-tailed Student \( t \) test was used. The \( p \) values were corrected using the Bonferroni method where applicable. Results were considered significant with \( p < 0.05 \) and highly significant with \( p < 0.01 \). To determine a correlation between two data columns, the one-sided Pearson’s correlation coefficient was calculated.

FIGURE 3. Both GrB production and secretion are decreased by nucleic acid-containing antiviral vaccines in a concentration-dependent fashion. Purified pDC were incubated overnight in IL-3–containing medium with vaccines in dilutions/concentrations as indicated. (A) Bar graphs show median fluorescence intensities of intracellular GrB relative to IL-3 alone from at least three independent experiments. Error bars indicate SEM; \( p \) values indicate significant differences compared with no vaccine. (B) GrB secreted into the supernatants was measured by ELISA. Bar graphs represent average values of medium and maximum vaccine concentrations from at least four independent experiments. (C) Bar graphs represent average values of indicated concentrations normalized to IL-3 alone from at least four independent experiments. Error bars indicate SEM; \( p \) values indicate significant differences compared with no vaccine.

Results

Various clinically used antiviral vaccines do not induce substantial IFN-α expression in pDC

Recently, it was reported that clinically used prophylactic vaccines modulate pDC-orchestrated immune responses (30, 31). A detailed understanding of the mechanisms involved may be important for further improving such vaccines as both antiviral prophylaxis agents and as adjuvants in cancer immunotherapy. The first step to address regulation of pDC by clinically used antiviral vaccines was to investigate their effect on IFN-α, which is considered the key cytokine pDC produce after viral challenge. To our surprise, only the TBEV vaccine induced substantial IFN-α levels when added to cultured pDC (Fig. 1). All other vaccines tested had either no relevant (rubella, measles, yellow fever, polio, and HBV) or only mild effects (varicella) on IFN-α secretion (Fig. 1A). Importantly, the effect of TBEV vaccine on IFN-α expression occurred dose-dependently manner (Fig. 1B) and did not depend on the adjuvant Al(OH)₃ (Supplemental Fig. 1).
Antiviral vaccines mildly modulate surface molecules expression in pDC

When considering pDC as APC, their immunogenic surface phenotype appears critical in terms of their T cell stimulatory capacity. We therefore screened a series of markers that are presumably involved in immunological cross-talk between pDC and T cells. Such markers involve cell adhesion molecules such as CD54, Ag-volved in immunological cross-talk between pDC and T cells. Such markers include CD40, CD83, and CD86. Again, we found that most of the antiviral vaccines considered in our study were not able to induce significant upregulation in the expression of above-mentioned markers. As shown above for IFN-α, only varicella and TBEV vaccine had a significant impact on certain markers such as CD40, CD54, CD86, and MHC I (Fig. 2A, 2B).

Expression of GrB by pDC is downmodulated by nucleic acid–containing antiviral vaccines in a TLR-dependent manner

The fact that most antiviral vaccines tested did not substantially affect IFN-α expression nor the pDC surface phenotype implies that additional parameters may be involved in the efficacy of pDC to license T cell–dependent immune responses. Recently, we found that pDC produce the serine–protease GrB at levels exceeding even those expressed by CTL, suggesting a significant role of GrB for human pDC biology (6). In the current study, we tested a variety of antiviral vaccines with regard to their impact on pDC-produced GrB. We found that most vaccines containing nucleic acids were able to suppress pDC-derived GrB in a concentration-dependent fashion (Fig. 3). Although we observed marked differences in the capacity of such vaccines to inhibit pDC-derived GrB, we found TBEV vaccine to be the strongest GrB suppressor among all vaccines tested (Supplemental Fig. 2). In contrast to nucleic acid–based vaccines (Fig. 3, Table I), the protein-based vaccine against HBV showed a tendency to not suppress but rather enhance GrB expression by pDC (Supplemental Fig. 3). To test TLR dependency of the observed effects, pDC were treated with TBEV in the presence and absence of TLR antagonists. The TLR7 antagonist IRS 661 was ameliorated, and the TLR9 antagonist IRS 869 completely ablated TBEV-mediated GrB suppression (Supplemental Fig. 3B).

PDC from TBEV-vaccinated donors produce significantly less GrB than pDC from unvaccinated donors

The results presented above suggest that GrB expression by pDC may be an important parameter in pDC function after antiviral vaccination. Because the TBEV vaccine had the strongest effect on pDC expression of GrB, we compared the GrB potential of pDC freshly isolated from healthy subjects before and after vaccination against TBEV. To this purpose, pDC were purified from healthy individuals 7 d before and 4 d after TBEV vaccination and were stimulated in vitro for 48 h with IL-3 in the presence or absence of TBEV vaccine. We found that pDC harvested from healthy donors after TBEV vaccination exhibited a significantly reduced capacity to express GrB as compared with pDC harvested before vaccination (Fig. 4).

IL-3-activated pDC deliver GrB to T cells and may induce degradation of the TCR ζ-chain in a GrB-dependent manner

In our recent study, we demonstrated that pDC transfer their intrinsically produced GrB to T cells, where it suppresses T cell proliferation in a cell contact– and GrB-dependent manner. We therefore tested the impact of the TBEV vaccine on GrB transfer to T cells. pDC were preincubated with IL-3 in the presence or absence of TBEV vaccine. Then, pDC were coincubated with CD4+ T cells, and GrB transfer was visualized by fluorescence and spinning-disk confocal microscopy using a GrB-specific fluorescent substrate. Quantification of GrB transfer from pDC to T cells was carried out using flow cytometry with the same cells and staining as for fluorescence microscopy. Although GrB delivery to T cells was detected with pDC stimulated with IL-3 in the absence of TBEV vaccine, no transfer was observed after pDC activation in the presence of TBEV vaccine (Fig. 5A, Supplemental Fig. 4A).

Table I. Vaccines used in this study

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Virus</th>
<th>Genome</th>
<th>Type of Vaccine</th>
<th>Adjuvants and Other Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSME Immun</td>
<td>TBEV</td>
<td>(+) ssRNA</td>
<td>Inactivated, adsorbed</td>
<td>Al(OH)₃, human albumin, saccharose</td>
</tr>
<tr>
<td>IPV Merieux</td>
<td>Poliovirus</td>
<td>(+) ssRNA linear</td>
<td>Inactivated</td>
<td>Polysorbate 80, 2-phenoxethanol, ethanol</td>
</tr>
<tr>
<td>Stamaril</td>
<td>Yellow fever virus</td>
<td>(+) ssRNA</td>
<td>Live attenuated</td>
<td>Lactose, sorbitol, L-histidine, L-alanine</td>
</tr>
<tr>
<td>Masern-Impfstoff</td>
<td>Measles virus</td>
<td>(-) ssRNA</td>
<td>Live attenuated</td>
<td>Human albumin, amino acids, lactose, dextran 70, sorbitol, urate</td>
</tr>
<tr>
<td>Röteln-Impfstoff</td>
<td>Rubella virus</td>
<td>(+) ssRNA</td>
<td>Live attenuated</td>
<td>Dextran 70, glutamine acid, urate, human albumin, glucose</td>
</tr>
<tr>
<td>Engerix B</td>
<td>HBV</td>
<td>dsDNA</td>
<td>Recombinant HBsAg, adsorbed</td>
<td>Al(OH)₃</td>
</tr>
<tr>
<td>Varilrix</td>
<td>Varicella zoster virus</td>
<td>dsDNA</td>
<td>Live attenuated</td>
<td>Amino acids, human albumin, lactose</td>
</tr>
</tbody>
</table>

FIGURE 4. pDC from TBEV-vaccinated donors produce significantly less GrB than pDC from unvaccinated donors. pDC were purified from the peripheral blood of healthy donors before and 4 d after vaccination against TBEV and cultured in the presence of IL-3 and TBEV vaccine (100 ng/ml) for 48 h as indicated. GrB expression was determined using flow cytometry before and 4 d after vaccination in freshly isolated pDC (day 0) and after incubation (day 2). Bar graphs represent normalized average mean fluorescence intensities (MFI) from at least seven independent experiments, dots represent individual experimental data points, error bars indicate SEM. The p values indicate significant differences of GrB production before and after vaccination.
Hence, the percentage of GrB+ T cells was significantly lower in the presence of IL-3 + TBEV-pretreated pDC than with pDC pretreated with IL-3 alone (Fig. 5B, 5C).

Previously, the ζ-chain of the TCR was shown to be a substrate for GrB (19). Because the TCR ζ-chain is involved in T cell proliferation, we hypothesized that the antiproliferative effect of GrB-expressing pDC on T cells may be due to GrB-dependent degradation of the TCR ζ-chain. To test this hypothesis, we purified pDC from healthy volunteers and stimulated them with the GrB-inducing cytokines IL-3 ± IL-10. Subsequently, pretreated pDC were washed and coincubated with T cells. We found that the amount of detectable TCR ζ-chain protein was reduced when T cells were coincubated with IL-3–treated GrB+ pDC but not when T cells were incubated separately from pDC (Fig. 6A, 6B).

Inhibition of pDC-derived GrB results in enhanced potential of pDC to license T cell proliferation

Having shown that pDC-derived GrB can induce a decrease of TCR ζ-chain expression in cocultured T cells, we finally tested whether these effects impact on T cell proliferation. To this purpose, pDC were pretreated for 24 h in the presence of IL-3, IL-10, and TBEV.
GrB did not affect T cell viability. T cell viability (Supplemental Fig. 4B), suggesting pDC-derived both CD4+ and CD8+ T cell proliferation in a GrB-dependent than cytotoxic cells and that pDC are able to efficiently suppress IL-3 and IL-10 produce substantially more GrB on a per-cell basis and coincubation in the absence or presence of a neutralizing GrB Ab (right bar graph panel). Error bars indicated SEM; p values indicate significant differences of TCR β/β-actin band intensity ratios.

Discussion

Although the importance of pDC in antiviral immunity is widely accepted, their exact role during viral infections and vaccinations is not fully understood, and significant controversy exists regarding their immunogenic versus their tolerogenic potential (31, 32). The general opinion is that pDC express and secrete large amounts of IFN-α after viral stimulation by which Ag-specific T cells and NK cells are activated (33). Recently, pDC also were shown to efficiently cross-present exogenous Ags, thereby directly contributing to the activation of CD8+ CTL (5, 34). In contrast, pDC appear to hold an important position in the fine-tuning and modulation of T cell responses including those T cell responses that are initiated by other types of APC (3, 32, 35). As we and others have shown, pDC are able to express and secrete a series of molecules with immunomodulatory effects on T cells including IDO (36), ICOS ligand (37), and GrB (6). GrB takes an exceptional position in this context because it was shown to be necessary for the function of regulatory T cells (22, 38) and to be expressed by other regulatory cell subsets such as human regulatory B cells (39). Furthermore, we could demonstrate in our recent study that pDC activated with IL-3 and IL-10 produce substantially more GrB on a per-cell basis than cytotoxic cells and that pDC are able to efficiently suppress both CD4+ and CD8+ T cell proliferation in a GrB-dependent manner (6).

Lately, the interest in cellular mechanisms of antiviral and antitumor vaccines has dramatically increased. Even though prophylactic antiviral vaccines have been clinically used for decades, their precise impact on DC function is currently not well defined. Because T cell expansion, induced by various DC types, may contribute to a more profound cellular antiviral immune response after vaccination, the impact on DC appears to be of eminent importance for the effectiveness of an antiviral vaccine. Recent studies started to explore the effect of antiviral vaccines on the Ag-presenting and tumoricidal capacity of DC including pDC (30, 31, 40). In our current study, we investigated how clinically used antiviral vaccines may impact on the GrB-mediated immunoregulatory potential of pDC. On the basis of our recent findings on pDC-mediated regulation of T cell expansion (6), we hypothesized that an effective antiviral vaccination may suppress pDC-derived GrB, resulting in an enhanced induction of T cell expansion. Our study showed that antiviral vaccines, most pronounced the TBEV vaccine, could suppress pDC GrB expression and that GrB but not IFN-α expression by pDC exhibited a strong and significant negative correlation with the proliferative potential of CD4+ T cells. These findings were confirmed by blocking experiments, demonstrating the functional involvement of GrB in the licensing of T cell proliferation. Importantly, we also demonstrated that these effects may play a role in vivo, because pDC isolated from recently vaccinated individuals expressed significantly less GrB than pDC from healthy control subjects.

Of note, the regulatory potential of pDC-expressed GrB may represent just one side of the same coin. Recently, pDC were shown to acquire an NK-like phenotype after stimulation with various vaccines, resulting in cytotoxic potential toward typical NK cell targets (31). Therefore, pDC may combine both regulatory effects on T cells and cytotoxic potential toward virus-infected or tumor cells. Very interesting in this context are recent reports indicating that GrB is not only involved in Ag uptake and cross-presentation (41, 42) but also in the initiation of immune responses against autoantigens (42–44). Therefore, expression of GrB in pDC may allow this protease to cleave Ags in the early phase of an immune response so that they are optimally prepared for Ag loading and presentation on MHC I molecules. During this early phase of Ag
processing and loading, peripheral T cells may be suppressed in their function by secreted GrB to avoid unspecific activation of potentially autoreactive T cells (45). Then, in a later phase, danger signals such as TLR ligands, or CD40L expressed by highly activated T cells returning from the draining lymph nodes, may turn off GrB expression by pDC (6). Instead, expression of Ag-presenting and costimulatory molecules may be induced, resulting in optimal Ag cross-presentation by pDC (5, 34). Interestingly, our findings regarding pDC-expressed GrB exhibit remarkable parallels to other APC including myeloid DC, IFN-α–matured monocyte-derived DC and B cells. All these APC types have been shown to be capable of expressing GrB and of cross-presenting Ags to T cells. Obviously, the immune system has evolved APC that unify the capabilities of killing target cells, of providing certain proteases such as GrB that may contribute to the appropriate digestion and cross-presentation of Ags, and of suppressing T cell responses during the early phase of the immune response. Importantly, cells with some of these properties have meanwhile been observed in both mice (46–48) and humans (15, 31, 49).

An important question is how the GrB-suppressive effect in pDC by various vaccines including TBEV vaccine is mediated. Recent studies demonstrated that both TLR7 and TLR9 ligation suppress pDC GrB expression (6, 49). This matches our current finding that CpG B–activated pDC were at least as effective as pDC activated with TBEV vaccine to license alloreactive T cell responses. Of note, we excluded that the adjuvant Al(OH)3, which is contained in the TBEV vaccine formulation, was responsible for the observed effects. A possible mechanism may therefore be that viral ssRNA in the TBEV vaccine mediates the observed effects via ligation of TLR7 and/or TLR9. Three independent observations support this hypothesis. First, only vaccines containing nucleic acid components were able to suppress pDC GrB expression. In
VACCINE-INDUCED GrB SUPPRESSION IN pDC

Disclosures
The authors have no financial conflicts of interest.

References
21. Co, M. D., M. Terajima, J. Cruz, F. A. Ennis, and A. L. Rothman. 2002. Human cytosolic T lymphocyte responses to live attenuated 17D yellow fever vaccine: contrast, HBV vaccine, which contains HbsAg protein, but no nucleic acid component, had no significant impact on pDC GrB production. Second, when CpG B and TBEV vaccine were combined, there was no additive but rather an antagonistic effect on GrB suppression in pDC (data not shown), suggesting a competitive inhibition between TBEV vaccine and CpG B. Third, a TLR9 antagonist ameliorated and a TLR7 antagonist abolished the GrB-suppressing effect of the TBEV vaccine in pDC.

To date, literature on the recognition of both inactivated and live attenuated virus vaccines by pattern recognition receptors is limited. Very recent reports demonstrate that stimulation of DC in general and pDC in particular by prophylactic vaccines including the TBEV vaccine used in our study results in activation patterns resembling TLR-mediated activation (30, 31, 40). Moreover, effects on DC mediated by both inactivated and live attenuated viruses could be suppressed by using a TLR9 inhibitor or the endosomal maturation inhibitor chloroquine (30), confirming dependency on TLRs. As far as live attenuated virus vaccines such as varicella vaccine are concerned, we suggest that pDC may get activated after vaccination without actually being infected by the virus. This assumption is in line with observations by others showing that an attenuated form of HIV-1, namely HIV-2, may not directly infect pDC but still cause activation by HIV virions in vivo (50). Furthermore, after i.m. injection of live attenuated influenza vaccine, TLR-dependent activation of pDC in vivo was detected (51), suggesting that sufficiently high amounts of live attenuated viral vaccine compounds are internalized by pDC in vivo. We therefore hypothesize that suppression of GrB release from pDC by viral nucleic acids may allow increased T cell expansion at the site of infection or after vaccination. The overall result may be the development of a strong antiviral immune response, supported by both TLR-activated pDC and possibly other APC types. Importantly, this assumption is also supported by the clinical observation that high GrB levels may be associated with a failure of antiviral immunity. Examples are infections with CMV after renal transplantation, with dengue fever virus or with HIV, all of which have been associated with high serum levels of GrB (9).

In conclusion, we have shown that a series of clinically used antiviral vaccines potently suppress expression of GrB by pDC. Because GrB-expressing pDC are thought to suppress potentially autoreactive T cells at the site of acute viral infections (6), antiviral vaccines may bypass this security circuit, thereby endowing pDC with T cell licensing properties. Importantly, our findings do not only provide novel insights into the role pDC play for the generation of cellular immune responses but also may have implications for the improvement of antiviral and antitumor vaccination approaches. Antitumor immune responses are generally considered to be weaker than antiviral immune responses because of the absence of danger signals such as TLR ligands (52). Because pDC are found to infiltrate a series of tumors, they are thought to play an important role for cancer immunity (2). Although the expression of GrB in tumor-infiltrating pDC has not been investigated so far, the main cytokines responsible for the induction of GrB in pDC, namely IL-3 and IL-10 (6), can be detected in the environment of neoplastic diseases (53, 54). This suggests that GrB-secreting pDC in such an environment may be involved in suppressing the expansion of tumor-specific T cells, thereby contributing to a potential tumor evasion. In summary, suppression of pDC-expressed GrB, for example, by clinically approved antiviral vaccines such as the TBEV vaccine, may represent a novel strategy to induce more robust and comprehensive cellular immune responses to vaccinations against both tumors and viruses.

Downloaded from http://www.jimmunol.org/ by guest on July 26, 2017
identification of HLA-B15-restricted CTL epitopes on nonstructural proteins NS1, NS2b, NS3, and the structural protein E. Virology 293: 151–163.
Supplemental Figure 1. The adjuvant aluminum hydroxide does not significantly impact on pDC expression of GrB and IFN-alpha. Purified pDC were cultured in IL-3-containing medium and stimulated with increasing concentrations of TBEV vaccine or Al(OH)₃ for 24 hrs. Al(OH)₃ concentrations were adjusted to the vaccine dose (100 ng/mL of TBEV vaccine correspond to 14.6 µg/mL Al(OH)₃). Then cells and supernatants were harvested for further analysis. (A) After culture pDC were stained for intracellular GrB and analyzed by flow cytometry. Shown is one representative set of dot plots. (B) Bar graphs represent average median fluorescence intensities (MFI) of pDC-expressed GrB normalized to medium from at least four different experiments. (C) IFN-alpha levels in the supernatants were determined by ELISA. Bar graphs represent mean IFN-alpha concentrations from four different experiments. Error bars indicate SEM, * p-values indicate significant differences, ** p-values indicate highly significant differences.

Supplemental Figure 2. Clinically used anti-viral vaccines inhibit both GrB production and secretion by pDC. Purified pDC were incubated overnight with IL-3 and antiviral vaccines as indicated. Rubella, measles and yellow fever virus vaccines were diluted 1:5; polio and varicella virus vaccines were diluted 1:10; TBEV vaccine concentration was 100 ng/mL. After culture, pDC and supernatants were harvested. GrB production was determined by intracellular GrB staining and FACS analysis. (A) Representative dot blots are shown. (B) Bar graphs represent average MFI of intracellular GrB in vaccine-stimulated pDC relative to unstimulated pDC from at least 3 individual experiments. (C) Secreted GrB was determined from supernatants by ELISA. Bar graphs represent GrB concentrations in vaccine-stimulated pDC cultures relative to GrB concentrations in unstimulated pDC cultures. In both panels, error bars indicate SEM and *p-values indicate significant differences.

Supplemental Figure 3. The protein-based anti-HBV vaccine does not significantly suppress GrB secretion by pDC and TLR-7 and TLR-9 antagonists abrogate TBEV-mediated GrB suppression. (A) Purified pDC were incubated overnight with IL-3 and HBV vaccine in the indicated concentrations. GrB secretion was measured in supernatants by ELISA. Bar graphs represent average values normalized to IL-3 alone from at least 4 independent experiments. Error bars indicate SEM. (B) Purified pDC were pre-treated for 48 hrs in the presence of IL-3 (10 ng/mL) and TBEV vaccine (100 ng/mL), and either the TLR-7
antagonist IRS 869 or the TLR-9 antagonist IRS 661 as indicated. GrB expression by pDC was evaluated by intracellular GrB staining and subsequent flow cytometry analysis. Bar graphs represent MFI of intracellular GrB. Error bars indicate SEM and *p-value indicates a significant difference.

**Supplemental Figure 4. PDC incubated with TBEV vaccine do not transfer GrB to T cells and T cell viability is not affected by co-culture with differentially activated pDC.**

**(A)** Purified pDC were pre-incubated in IL-3-containing medium with or without TBEV vaccine (100 ng/mL) for 60 hrs. In parallel, purified CD4⁺ T cells were incubated in medium overnight. After pDC pre-incubation, pDC were pulsed for 1 hr with GranToxiLux fluorescent substrate to stain enzymatically active GrB. T cells were immobilized on chamber slides and stained with CellMask Deep Red. Subsequently pDC (green arrows) were added to T cells and their interaction was visualized using spinning-disk confocal microscopy. Over an observation time of 400 min, GrB transfer from pDC to T cells (red arrows) was analyzed. Images from two representative experiments are shown. **(B)** Purified pDC were pre-treated for 48 hrs in the presence of IL-3 (10 ng/mL) and different vaccines as indicated. Then, pDC were washed and co-incubated for 6 days with CFSE-stained allogeneic CD4⁺ T cells at a pDC:T cell ratio of 1:250. Then, cells were harvested, stained with fluorescently labeled anti-CD3 monoclonal antibody and 7-AAD, and analysed by FACS. Bar graphs show average percentages of CD3⁺7-AAD⁻ viable CD4⁺ T cells from 3 individual experiments, error bars indicate SEM.
**Suppl. Fig. 1**

**A**
- Side Scatter plots for TBEV and Al(OH)_3 with Granzyme B production indicated.

**B**
- Bar graph showing Relative MFI of granzyme B with significance levels indicated:
  - **p < 0.003**
  - **p < 0.006**
  - *p < 0.02*

**C**
- Bar graph showing Relative IFN-alpha with media conditions:
  - Medium
  - 1ng/mL
  - 10ng/mL
  - 100ng/mL

Legend:
- **TBEV**
- **Al(OH)_3**
Suppl. Fig. 2
Suppl. Fig. 3

A

Relative GrB secretion (%)

HBV vaccine concentration (ng/mL)

Med 10 100

HBV

n.s.

B

MFI GrB

* p < 0.02

n.s.

MFI GrB

Medium

TBEV

no IRS IRS 869 IRS 661

Suppl. Fig. 3
Suppl. Fig. 4