Differential Poly(I:C) Responses of Human Vγ9Vδ2 T Cells Stimulated with Pyrophosphates *Versus* Aminobisphosphonates

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Abstract: Bacterial pyrophosphates or aminobisphosphonates induce cytokine secretion and expansion of freshly isolated (resting) human Vγ9Vδ2 γδ T cells. The initial expansion of γδ T cells strictly depends on co-stimulatory signals of accessory cells and IL-2. However, resting γδ T cells produce cytokines after stimulation with pyrophosphates combined with TLR3 ligand poly(I:C) in the absence of accessory cells. We observed that aminobisphosphonate together with poly(I:C) stimulation did not induce cytokine secretion in resting γδ T cells. Moreover, proliferation of γδ T cells within PBMC was enhanced after stimulation with pyrophosphates and poly(I:C), but not after stimulation with aminobisphosphonates plus poly(I:C), even though accessory cells were present. Aminobisphosphonate together with poly(I:C) induced a delayed up-regulation of co-stimulatory molecules and cell survival of monocytes within PBMC. In contrast to resting γδ T cells, activated Vγ9Vδ2 T cells produced IFN-γ after stimulation with pyrophosphate antigens as well as with aminobisphosphonate in the absence of accessory cells and poly(I:C). However, aminobisphosphonate stimulation resulted in much lower IFN-γ production than pyrophosphate stimulation, which fits well with the weaker aminobisphosphonate induced activation of extracellular regulated kinase (ERK) involved in IFN-γ secretion. Poly(I:C) failed to enhance cytokine production and proliferation of activated γδ T cell lines after aminobisphosphonate stimulation unless accessory cells were added. Taken together, the results suggest a differential regulation of Vγ9Vδ2 T cell activation by pyrophosphates and aminobisphosphonates, especially after co-stimulation with poly(I:C). This might be relevant when using such pyrophosphates or aminobisphosphonates together with TLR3 agonists as adjuvants in γδ T cell-based immunotherapy.

INTRODUCTION

Peripheral blood Vγ9Vδ2 T cells exert their potent effector function against various bacteria, viruses and tumours by the release of pro-inflammatory cytokines and cytotoxic mediators [1, 2]. Several pilot studies described γδ T cells as a promising strategy for immunotherapy due to their potent MHC-nonrestricted cytotoxicity [3-6]. Activation and expansion of Vγ9Vδ2 T cells is stimulated by the recognition of small phosphorylated intermediates (such as (E)-4-hydroxy-3-methyl-but-2-enyl-diphosphate, HMBPP) of the desoxyxylulose-5-phosphate (DOXP) isoprenoid biosynthesis pathway of non-mammalian cells (so called phosphoantigens, PAg) [7]. In contrast, the mevalonate isoprenoid pathway-derived Isopentenylpyrophosphate (IPP) produced by mammalian cells does not activate Vγ9Vδ2 T cells at concentrations that are produced in physiological non-transformed cells [8]. However, the inhibition of farnesyl diphosphate (FPP) synthase, an IPP-processing enzyme of the eukaryotic isoprenoid pathway, results in an accumulation of the substrates of FPP synthase, IPP and its stereoisomer dimethylallyl diphosphate (DMAPP), then leading to Vγ9Vδ2 T cell expansion [9, 10]. Inhibitors of the FPP synthase include aminobisphosphonates (e.g. pamidronate, zolendronate or alendronate) and alkylamines [11, 12]. Aminobisphosphonates (nitrogen-containing bisphosphonates, N-BPs) prevent the biosynthesis of isoprenoid lipids required for the prenylation of small GTPases, which result e.g. in an abolishment of pathological bone-resorption through osteoclasts in osteoporosis patients [13]. N-BPs are licensed drugs for the treatment of osteoporosis, and tumour-associated bone diseases and carry perspectives for γδ T cell-based immunotherapy [14-16]. Roelofs and colleagues have shown that the accumulation of IPP/DMAPP in peripheral blood monocytes or in tumour cells with a dysregulated mevalonate pathway is responsible for the Vγ9Vδ2 T cell activation [10]. The stimulation of PBMC with PAgs or N-BPs results in a release of cytokines including IFN-γ, TNF-α and IL-6 and in the presence of IL-2 in a selective expansion of γδ T cells [17, 18]. In addition to the activation of Vγ9Vδ2 T cells by PAgs and N-BPs, other cell surface molecules such as activating natural killer receptor NKG2D (CD314) and Toll-like receptor (TLR) 3 modulate the effector function of γδ T cells [18, 19]. NKG2D binds to stress-inducible MHC class I-chain-related antigens (MIC) A/B as well as UL16-binding proteins (ULBP) 1-4 inducibly

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expressed on peripheral blood monocytes or constitutively on tumour cells, and deliver stimulatory signals [19-22]. On the other side, TLR have been identified as primary sensors of bacterial and viral components leading to the production of pro-inflammatory cytokines and chemokines, up-regulation of co-stimulatory molecules, enhanced proliferation and regulation of cell survival as well as apoptosis [23-25]. We and others previously reported that both the NKG2D/NKG2DL- and the TLR3/TLR3L-pathway positively affect the activation of freshly isolated γδ T cells and modulate the cytotoxic activity of activated Vγ9Vδ2 T cells [18, 19, 26].

In this report, we examined whether TLR3, the receptor for dsRNA, which can be mimicked by the surrogate ligand poly(I:C), induces a co-stimulatory signal in N-BP stimulated γδ T cells comparable to the observed results with PAg-stimulated cells. We describe a differential outcome of PAg- versus N-BP-stimulation in the presence of poly(I:C), which should be considered as relevant when using such drugs as selective activators in γδ T cell based immunotherapy.

MATERIALS AND METHODS

Isolation of Cells and Establishment of γδ T Cell Lines. PBMC were isolated from buffy coats from healthy adult blood donors by Ficoll-Hypaque (Biochrom, Berlin, Germany) density gradient. Buffy coats were obtained from the Department of Transfusion Medicine in Kiel/Lübeck, Germany. Informed consent was obtained from all donors and research was approved by the relevant institutional review boards. γδ T cells were negatively separated from freshly isolated PBMC using T cell receptor (TCR) γ/δ T Cell Isolation Kits (Miltenyi Biotec, Bergisch-Gladbach, Germany). For negative selection, the non-γδ T cells within PBMC were indirectly stained with a cocktail of biotinylated monoclonal anti-human antibodies against antigens not expressed by γδ T cells followed by anti-biotin microbeads. To improve γδ T cell purity after magnetic separation and to eliminate residual non-γδ T cells by sorting the cells with a FACS Aria (BD Biosciences), enriched γδ T cells were stained with a cocktail of the following mAbs: pan anti-TCR αβ BMA031 (Beckman Coulter, Krefeld, Germany), anti-CD19, anti-CD56, anti-CD14 (all from BD Biosciences, Heidelberg, Germany), anti-CD303 (BDCA-2) and CD141 (BDCA-3) (both from Miltenyi Biotec). The purity was > 99% $\gamma\delta^+$ T cells. The majority of the freshly isolated, negatively sorted γδ T cells expressed a Vγ9Vδ2 TCR. To establish Vγ9Vδ2 γδ T cell lines, PBMC were stimulated with 200 nM bromohydrin pyrophosphate (BrHPP, kindly provided by Innate Pharma, Marseille, France) in the presence of 100 U/ml IL-2. The selectively expanded γδ T cells within the PBMC were re-stimulated with irradiated (40 gray), autologous PBMC, BrHPP and IL-2 after 10 days. After the second re-stimulation, γδ T cells were separated from remaining non-γδ T cells by magnetically depleting residual $TCR\alpha\beta^+$ T cells. To expand Vγ9Vδ2 T cell lines, cells were re-stimulated in a 14-day interval with BrHPP in the presence of irradiated PBMC (40 gray) and EBV-transformed B cell lines (60 gray) as feeder cells. IL-2 was added two days after re-stimulation. Dead feeder cells were removed 4-5 days after re-stimulation by Ficoll-Hypaque density gradient. Autologous $CD14^+$ monocytes were isolated from PBMC of the same healthy donors by using CD14 microbeads (Miltenyi Biotec) for positive selection. The separated $CD14^+$ monocytes were frozen in liquid nitrogen until autologous γδ T cell lines were established.

Cell Culture and Determination of IFN-γ. PBMC or isolated γδ T cells were stimulated in the presence or absence of $CD14^+$ monocytes with 200 nM PAg BrHPP or 5 μM N-BP alendronate (Merck, Darmstadt, Germany) in the presence or absence of 25 μg/ml poly(I:C) or with poly(I:C) alone in RPMI 1640 (supplemented with 2 mM L-glutamine, antibiotics, 25 mM Hepes, and 10 % heat-inactivated pooled human AB serum obtained from male donors). Supernatants of duplicate cultures were collected after 24 hours and stored at -20°C to determine IFN-γ by a sandwich ELISA kit (Quantikine, R&D Systems, Wiesbaden, Germany) following the procedures outlined by the manufacturer. To analyze proliferation of γδ T cells, 50 U/ml IL-2 were added at the beginning of the culture to the assays.

Measurement of T Cell Expansion. Proliferation was analyzed by uptake of [^{3}H]-thymidine incorporation after 6 to 9 days of culture. Microculture wells with 10^5 stimulated PBMC were pulsed during the last 16 hours of a 6 to 9-day culture with 1 μCi [^{3}H]-TdR per well (sp. act.: 5 Ci/mmol; GE Healthcare, München, Germany). Cell cultures were harvested onto filtermats and radioactivity was determined by a Wallac 1450 Microbeta β-counter (Perkin Elmer, Rodgau, Germany). The absolute cell number of expanded γδ T cells was measured by a previously developed flow cytometric method termed standard cell dilution assay (SCDA) [27, 28]. Briefly, cultured cells were incubated with PE-labelled anti-TCR Vδ2 mAb (BD Biosciences). After being washed, cells were resuspended in 100 μl sample buffer containing a constant number (10^5/ml) standard cells and 0.2 μg/ml propidium iodide (PI; Sigma, Taufkirchen, Germany) to exclude dead cells. Standard cells were prepared by staining purified T cells with FITC-labelled anti-HLA class I mAb W6/32 and fixed in 1% paraformaldehyde. From the ratio of viable $V\delta2^+$ γδ T cells (PE^+/PI^-) to standard cells ($FITC^+PI^+$), the absolute number of $V\delta2^+$ γδ T cells can be determined by multiplication with the known input number of standard cells (10^4 per sample = 100 μl). Results of [^{3}H]-TdR incorporation assay and SCDA are given as mean values of three replicates. SD of mean values was always less than 10 %.

Flow Cytometry. To characterize the CD69 expression on the surface of γδ T cells, negatively isolated γδ T cells were stained before and after stimulation with antigens with anti-CD69 mAb or the appropriate isotype control (both from BD Biosciences).

To determine the expression of co-stimulatory molecules or NKG2D ligands on $CD14^+$ monocytes, the following mAb were used: PE-conjugated anti-CD80, PE-labelled anti-CD86 (all from BD Biosciences), unconjugated ULBP-2 or ULBP-3, (R&D Systems) followed by PE-conjugated goat $F(ab')_2$ anti-mouse IgG (Invitrogen, Karlsruhe, Germany) as a second step reagent or the corresponding isotype controls combined with staining with FITC-labelled anti-CD14 mAb. A gate was set on the $CD14^+$ monocytes within the PBMC to analyze the expression of the co-stimulatory molecules or NKG2D ligands on $CD14^+$ monocytes.

For cell death analysis of monocytes, PBMC were stained with FITC-conjugated anti-CD14 mAb after 24 and 48 hours and after 6-9 days of culture, washed and resuspended in buffer containing 2.5 µg/ml PI. A gate was set on the $CD14^+$ monocytes within the PBMC to determine $CD14^+PI^+$ cells. All samples were measured on a FACSCalibur (BD Biosciences) using the CellQuestPro software.

Phospho-ERK1/2 Western Blot. γδ T cell lines were stimulated in the absence or presence of autologous $CD14^+$ monocytes with 5 µg/ml anti-CD3 mAb OKT3 cross-linked with 1.7 µg/ml rabbit-anti-mouse (ram), 500 nM BrHPP or 50 µM alendronate for the indicated timepoints. Cells were lysed in Nonidet P40 lysis buffer (Fluka Chemie, Buchs, Switzerland). The lysis buffer contains 1% (v/v) of detergent in 20 mM Tris-HCL (pH 8.1) and 50 mM NaCl with phosphatase and protease inhibitors. After complete denaturation, samples were separated on 10% SDS-PAGE, and proteins were transferred to nitrocellulose membranes (Hybond-C-Extra, GE Healthcare). Blots were blocked with BSA (Sigma-Aldrich), stained with phosho-ERK1/2 mAb (Cell Signaling, Frankfurt, Germany) followed by HRP-conjugated donkey anti-rabbit antibody (GE Healthcare) and analyzed with chemiluminescence reagent (ECL, GE Healthcare).

Statistical Analysis. The paired, two-tailed Student´s t-Test was performed where applicable.

RESULTS

Poly(I:C) Increases CD69 Expression and IFN-γ Production of PAg-Activated- but not of N-BP-Activated γδ T Cells. We recently observed that CD69 expression on highly purified, positively selected γδ T cells was slightly increased when cells were cultured for 24 hours in medium alone [18]. This effect seemed to be mediated by the mitogenic effect of the pan TCRγδ mAb clone 11F2, which was used for the positive selection of γδ T cells. As shown in Fig. (**1**), CD69 expression was not enhanced on negatively isolated γδ T cells from healthy donors when cells were cultured for 24 hours in medium alone. In line with the results with positively isolated γδ T cells, CD69 expression (Fig. **1A**) and IFN-γ production (Fig. **1B**) were up-regulated after TCR activation with the PAg BrHPP (200 nM) and further up-regulated in the presence of 50 µg/ml poly(I:C), whereas poly(I:C) alone did not induce CD69 expression and IFN-γ production. This confirmed our recent data that poly(I:C) has a co-stimulatory effect on TCR-activated freshly isolated γδ T cells [18]. Moreover, the experiments clearly show that the co-stimulatory effect was independent of the isolation procedure of γδ T cells. We also investigated whether other γδ T cell-activating reagents such as the N-BP alendronate also induced IFN-γ production in γδ T cells in the presence of poly(I:C) and absence of accessory cells as PAg stimulation did. We observed that the stimulation of negatively isolated γδ T cells for 24 hours with alendronate did not enhance CD69 expression or IFN-γ expression on purified γδ T cells and was not further modulated by the addition of poly(I:C) (Fig. **1A**, **B**).

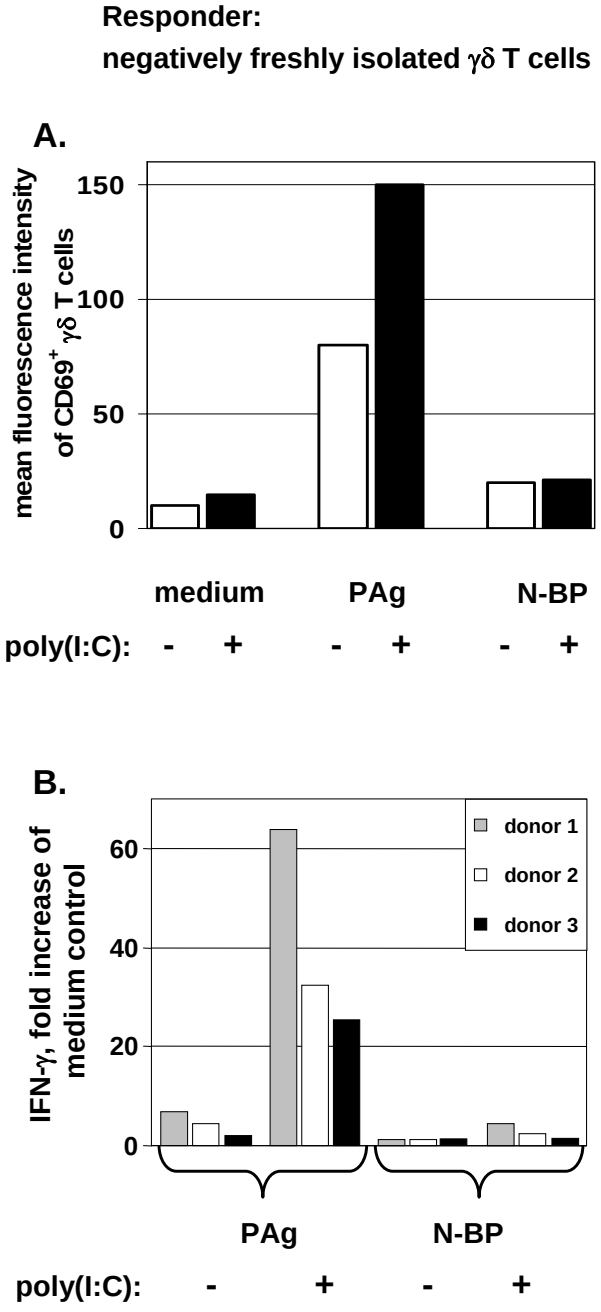

Fig. (1). CD69 expression and IFN-γ production of γδ T cells in response to PAg- *versus* N-BP stimulation with or without poly(I:C). 2.5 x 10^5 negatively isolated γδ T cells were cultured in medium, with PAg (BrHPP) or N-BP (alendronate) +/- poly(I:C) as indicated. (**A**) Cells were stained with PE-labeled CD69 mAb and CD69 expression on γδ T cells was analyzed by flow cytometry after 24 hours. The result of one representative donor out of four is shown. (**B**) IFN-γ was measured in the supernatants after 24 hours by ELISA. The results of 3 representative blood donors are shown.

Differential Responses of γδ T Cell Lines to PAg or N-BP in the Presence of poly(I:C). We also compared the response of freshly isolated γδ T cells and activated γδ T cell lines to PAg and N-BP in the presence of poly(I:C). In contrast to freshly isolated γδ T cells, N-BP-activated γδ T cell lines produced slightly increased levels of IFN-γ in the absence of accessory cells (e.g. monocytes/macrophages) as compared to the medium control (Fig. **2A**). While an elevated level of IFN-γ produced by PAg-stimulated γδ T cell lines was further enhanced in the presence of poly(I:C), IFN-γ production of N-BP-stimulated γδ T cell lines was not increased with poly(I:C) in the absence of monocytes/ macrophages (Fig. **2A**). However, co-culture of these γδ T cell lines with monocytes/macrophages resulted in a slightly enhanced IFN-γ production compared to parallel culture experiments without accessory cells. (Fig. **2A**, **B**). Moreover, we observed a minor up-regulation of IFN-γ production in the presence of poly(I:C) even without TCR stimulus, which seems to be caused by the pre-activated state of the γδ T cell lines (Fig. **2B**). In line with the weak responsiveness of γδ T cell lines to N-BP in the absence of accessory cells, we observed that unless accessory cells were added, alendronate only mildly activated the extracellular regulated kinase (ERK)1/2 (Fig. **3A**, **B**) and p38 (data not shown), two mitogen-activated protein kinases involved in the TCR-stimulated IFN-γ secretion. However, under these conditions monocytes alone seem to induce MAP kinase ERK1/2 after N-BP stimulation, making it difficult to distinguish the ERK1/2 activation in the co-culture of γδ T cell lines and $CD14^+$ monocytes. As a positive control for phosphorylation of ERK1/2 in γδ T cell lines, we used stimulation with anti-CD3 mAb OKT3 cross-linked with rabbit-anti-mouse or BrHPP, respectively. As expected, anti-CD3 as well as BrHPP induced an activation of MAP-kinases in γδ T cell lines (Fig. **3**). In contrast, we found that alendronate did not induce activation-induced cell death in γδ T cell lines (data not shown). Of note, we previously published that proliferation of BrHPP-stimulated γδ T cell lines was increased in the presence of poly(I:C) in the absence of accessory cells [18], whereas N-BP-stimulated γδ T cell lines failed to proliferate under these conditions unless accessory cells were present (data not shown).

PAg- ***versus*** **N-BP-Induced γδ T Cell Proliferation with poly(I:C) Might be Due to Differential Effects on Cell Death and Co-Stimulatory Molecules.** On the basis of necessity of accessory cells for N-BP induced γδ T cell cytokine production and proliferation, we used PBMC in the following experiments. As expected, PBMC proliferated after stimulation with BrHPP or alendronate in the presence of exogenous IL-2 and in the absence of poly(I:C). Surprisingly, we observed that the proliferation was enhanced after BrHPP stimulation by adding poly(I:C), whereas proliferation was reduced when alendronate stimulation was combined with poly(I:C) (Fig. **4A**). BrHPP selectively activates γδ T cells, but alendronate and poly(I:C) could also stimulate accessory cells. Since the ^{3}[H]-TdR incorporation does not allow direct measurements of proliferating subpopulations, the expansion of γδ T cells within PBMC was analyzed using the SDCA method. By determining the absolute cell numbers, we could demonstrate that $V\gamma9^+$ γδ T cells expand after BrHPP or alendronate stimulation (Fig. **4B**). Moreover, we determined that the presence of poly(I:C) increased the BrHPP- but decreased the N-BP-stimulated proliferation of $V\gamma9^+$ γδ T cells (Fig. **4B**).The increased γδ proliferation could be explained by a direct effect of BrHPP and poly(I:C) on TLR3-expressing

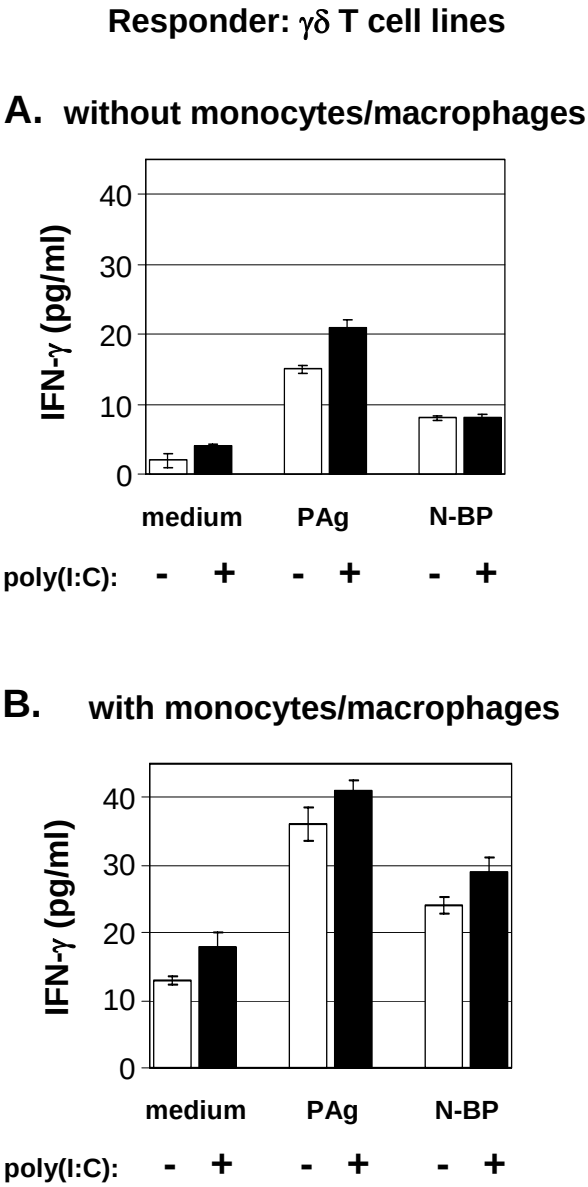

Fig. (2). Poly(I:C) did not enhance IFN-γ production of N-BP-stimulated γδ T cell lines in the absence of APC. 2.5 x 10^5 short-term γδ T cell lines were activated with the indicated stimuli in the absence (**A**) or presence (**B**) of 5 x 10^4 autologous $CD14^+$ monocytes for 24 hours. Supernatants were taken and the concentration of IFN-γ was determined by ELISA. Mean +/- SD of duplicate determinations of one representative experiment out of three is shown.

γδ T cells and an additional indirect effect of TLR3-expressing $CD11c^+$ $CD1c^+$ myeloid dendritic cells producing e.g. type-I-interferons within PBMC. This goes in line with the previous notion that, in contrast to PAg, N-BP indirectly stimulate γδ T cell proliferation by activating monocytes/ macrophages [10] to produce significantly more PAg IPP/DMAPP. Of note, we observed an increased cell death after 7 days of culturing $CD14^+$ monocytes/ macrophages with N-BP alendronate (Fig. **5A**), which suggests that accumulated IPP/DMAPP is released only after cell death of monocytes/macrophages and then binds and activates

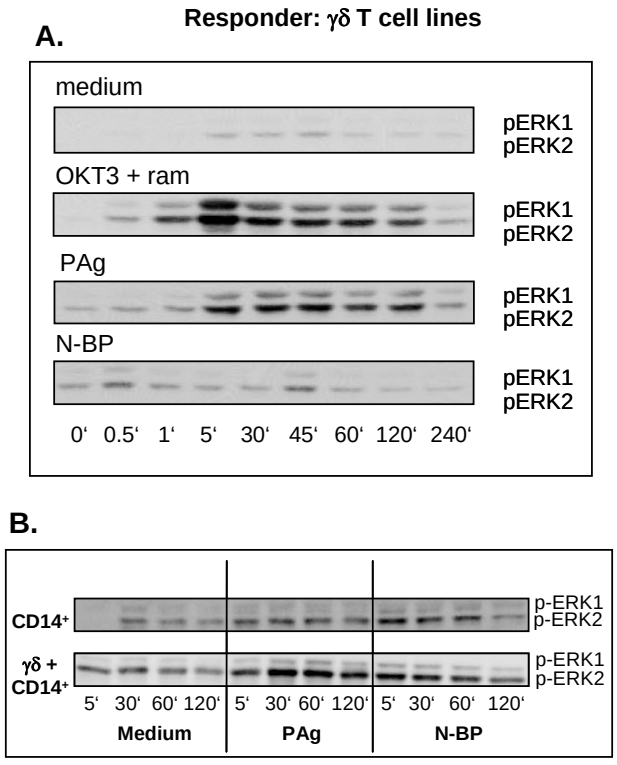

Fig. (3). Different kinetics of ERK1/2 phosphorylation in PAg- and N-BP-stimulated γδ T cell. (**A**) 10^6 γδ T-cells or (**B**) 10^6 $CD14^+$ monocytes without or with γδ T cells were cultured with medium, 500 nM PAg (BrHPP) or 50 μM N-BP (alendronate) for the indicated time range. (**A**) As a positive control, γδ T cell was activated with 5 μg/ml OKT3 crosslinked with 1.7 μg/ml rabbit-anti-mouse (ram). MAPK activity was analyzed in total cell lysates and Western blotting using polyclonal rabbit anti-phospho-ERK1/2 antibodies. Data of one representative experiment out of six are shown.

γδ T cells. Moreover, we observed that poly(I:C) prevents cell death of monocytes/macrophages under these conditions (Fig. **5A**) and thus possibly also the release of γδ T cell-stimulating IPP/DMAPP. Notably, TLR3 expression was nearly absent in $CD14^+$ monocytes/macrophages [18], but was up-regulated after stimulation [29]. In comparison to poly(I:C) stimulation alone, alendronate plus poly(I:C) induced a delayed up-regulation of co-stimulatory molecules such as CD80, CD86, ULBP-2 and ULBP-3 on $CD14^+$ monocytes/ macrophages. This could also account for the reduced γδ T cell proliferation after stimulation of PBMC with alendronate and poly(I:C) (Fig. **5B**).

DISCUSSION

In the present study, we demonstrate a differential regulation of $V\gamma9V\delta2^+$ γδ T cell-activation by PAg and N-BP in the presence of poly(I:C). We previously reported a direct co-stimulatory effect of poly(I:C) on the CD69 expression and IFN-γ production of PAg-stimulated positively isolated γδ T cells [2, 18]. These data were confirmed with negatively isolated γδ T cells. In contrast to this co-stimulatory effect of PAg, we demonstrate here that

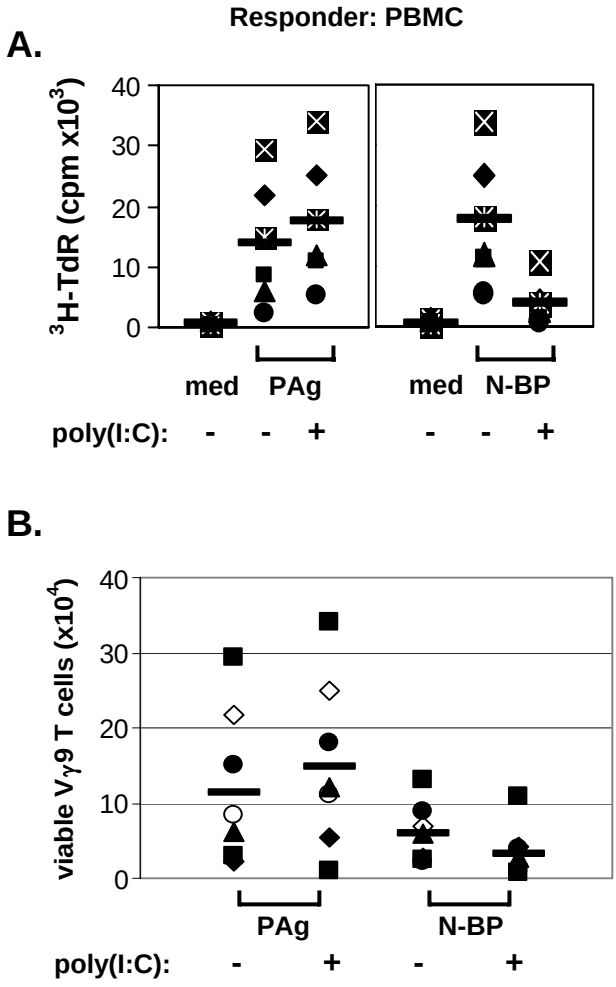

Fig. (4). Different effects of poly(I:C) on the proliferation of PAg- or N-BP-stimulated PBMC. 10^5 PBMC were cultured with medium (med) or activated with 200 nM PAg (BrHPP) or 5 μM N-BP (alendronate) in the presence or absence of 25 μg/ml poly(I:C) as indicated plus exogenous 50 U/ml IL-2. Symbols represent the measurement for individual donors and each dot represent the mean values of triplicate cultures. Mean values for all donors are presented as horizontal bars. (**A**) Proliferation was measured by ^{3}[H]-TdR-incorporation after 6 days. (**B**) The absolute cell number of γδ T cells was determined by flow cytometry after 7 days. The absolute number of $V\delta2^+$ γδ T cells at the onset of culture was donor-dependent between 200 and 5000 cells/ per well (not shown).

poly(I:C) does not co-stimulate N-BP-activated, freshly isolated γδ T cells. The reason for the discrepancy seems to

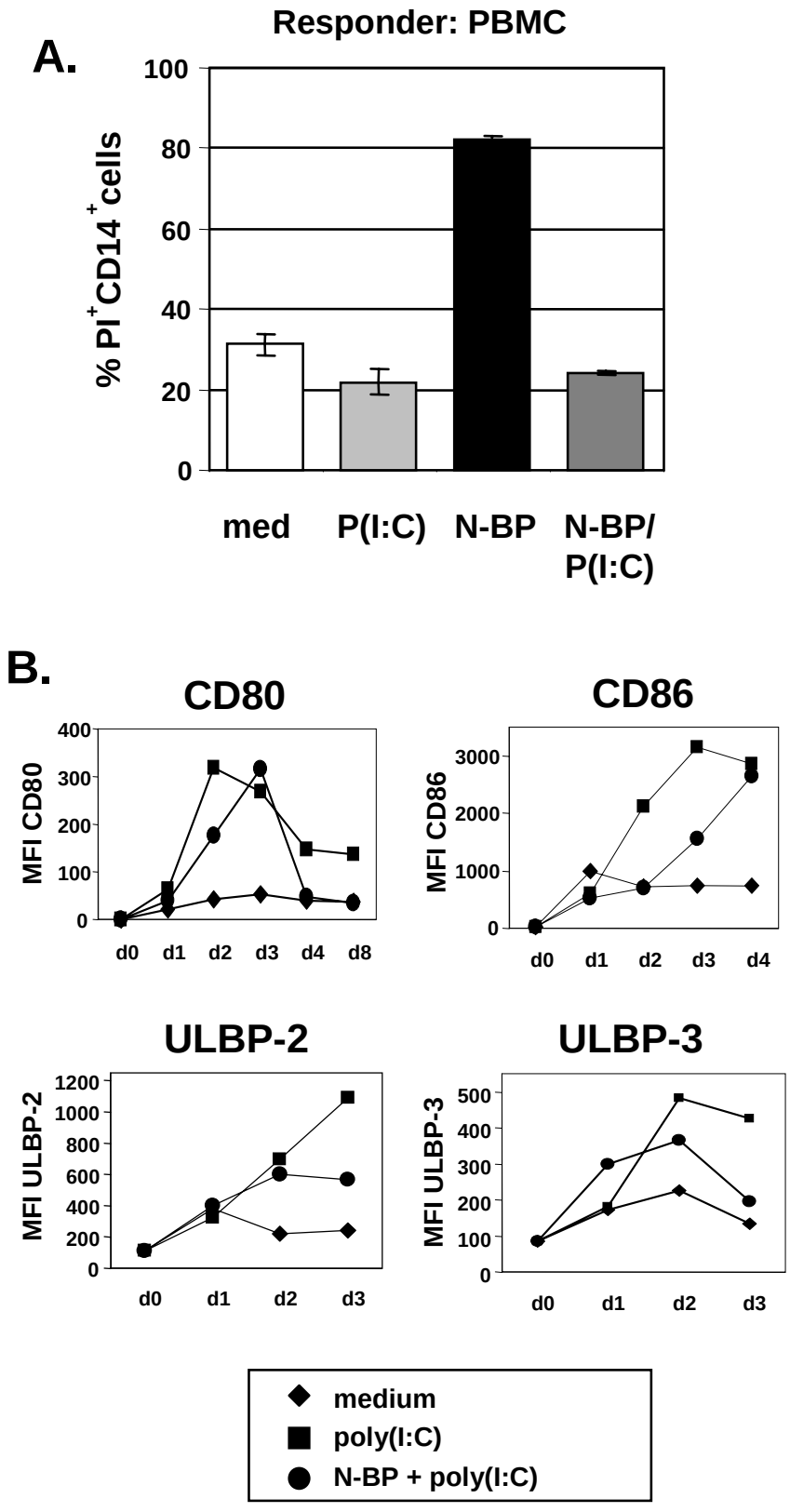

Fig. (5). Poly(I:C) prevents the N-BP induced cell death in CD14$^+$ monocytes and delays the expression of co-stimulatory molecules. 10^5 PBMC were stimulated with the indicated stimuli in the presence of exogenous IL-2. (**A**) The proportion of PI$^+$ CD14$^+$ monocytes was measured after 7 days of culture by flow cytometry. Mean +/- SD of three independent experiments with duplicate determinations are shown. (**B**) CD14$^+$ monocytes within PBMC were stained with anti-CD14 mAb in combination with anti-CD80, anti-CD86, anti-ULBP-2 or anti-ULBP-3 mAbs before (day 0) and at the indicated days after stimulation. A gate was set on the total population of CD14$^+$ monocytes within the PBMC to determine the expression of the indicated co-stimulatory molecules on CD14$^+$ monocytes. The expression of co-stimulatory molecules is presented as mean fluorescence intensity. Results of one representative donor out of three are shown.

be related to a differential recognition of PAg and N-BP [17, 30-33]. As shown by others, monocytes are required for activation of freshly isolated γδ T cells by N-BP [10]. Thus, N-BPs induced an accumulation of PAg such as IPP/DMAPP in monocytes, which triggered γδ T cell lines activation in a cell contact-dependent manner. The mechanism of the release of IPP/DMAPP by monocytes seems is still unclear. As expected, the co-culture of freshly isolated γδ T cells lines with monocytes induced a N-BP-stimulated IFN-γ production of γδ T cells, which was further enhanced by the addition of poly(I:C) (data not shown). This fits well with our recent observation that γδ T cells have to be activated *via* the TCR (e.g. with IPP/DMAPP) to become able to react to TLR3 ligands [18]. However, in contrast to freshly isolated γδ T cells, we found that γδ T cell lines slightly produced some IFN-γ 24 hours after stimulation with N-BP in the absence of monocytes. Hewitt and colleagues also observed a rapid production of TNF-α and IL-6 after N-BP stimulation of activated γδ T cell clones, but they used HeLa cells as (antigen-presenting) cells in their assays [17]. An explanation for the IFN-γ production of N-BP-stimulated γδ T cell lines could be the recently reported APC function of activated γδ T cells [34, 35]. Roelofs *et al*. showed that N-BPs are internalized *via* fluid-phase endocytosis by highly endocytic monocytes [10]. In contrast to monocytes, freshly isolated T cells were not or barely able to internalize fluorescently labelled N-BP [10]. Thus, we propose that resting γδ T cell are unable to internalize N-BP, whereas activated γδ T cells lines might endocytose N-BP, but probably less efficient than monocytes. In agreement with this, we observed that the addition of monocytes to N-BP-stimulated γδ T cell lines further up-regulated the IFN-γ production of the γδ T cell lines. Moreover, the MAP kinases ERK1/2 and p38 were also more active in the co-culture of γδ T cell lines and monocytes compared to γδ T cell lines alone. However, this activation mainly seems to result from the N-BP stimulation of monocytes. Unfortunately, we were not able to distinguish MAP kinases activation between γδ T cell lines and monocytes in this assay. Furthermore, the failure of poly(I:C) to enhance IFN-γ production of N-BP-stimulated γδ T cell lines might be due to the weak TCR stimulus of N-BP in the absence of monocytes. Thus, the addition of monocytes in the presence of N-BP induced a stronger IFN-γ production in γδ T cell lines, possibly mediated by a stronger TCR stimulus, which might be necessary for a poly(I:C) response. Besides, IFN-γ treatment of TCR-stimulated γδ T cell lines with poly(I:C) resulted in an enhanced production of TNF-α, granzyme A/B and increased cytotoxicity (Shojaei *et al*., submitted). In contrast, we observed that proliferation of poly(I:C) activated γδ T cells was only increased after the stimulation with PAg, but not with N-BP. As shown by others, N-BPs such as alendronate induce cell death in macrophages after several days of culture [36]. We observed that the N-BP induced cell death was abolished by the addition of poly(I:C). Moreover, N-BP and poly(I:C) stimulation together induced a delayed expression of co-stimulatory molecules in monocytes compared to poly(I:C) stimulation alone.

TLR3 agonists are suggested as adjuvants in clinical trials of cancer immunotherapy [37, 38]. However, a dual role of some TLR ligands, e.g. TLR3 ligand has been described [24, 39]. TLR 3 ligand poly(I:C) induced either apoptosis or cell survival of myeloid tumour cells [40, 41]. Further, TLR3 ligands increased the production of pro-inflammatory cytokines/chemokines or induced hypersensitivity reactions, shock and renal failure [38, 42]. Our data indicate an enhancement of γδ T cell effector function after PAg stimulation in the presence of poly(I:C). An enhancement of γδT cell effector function could be useful for γδ T cell-based immunotherapy. In contrast, N-BP often used for expansion of γδ T cells *in vivo* or *in vitro* with the aim to eliminate tumour cells, should not be used together with TLR3 ligand poly(I:C) as adjuvant due to negative effects on cytokine production and proliferation.

In summary, our results demonstrate a differential outcome of PAg- and N-BP-stimulation on γδ T cells in the presence of poly(I:C).

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ABBREVIATIONS

poly(I:C) = Polyinosinic-polycytidylic acid

TLR = Toll-like receptor

N-BP = Nitrogen-containing bisphosphonate

PAg = Phosphoantigens

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