Toll-like Receptors 3 and 7 Agonists Enhance Tumor Cell Lysis by Human γδ T Cells

Hamed Shojaei,[1] Hans-Heinrich Oberg,[1] Matthias Juricke,[1] Lothar Marischen,[1] Monika Kunz,[1] Christoph Mundhenke,[2] Frank Gieseler,[3] Dieter Kabelitz,[1] and Daniela Wesch[1]

[1]Institute of Immunology, [2]Department of Gynecology, and [3]First Department of Medicine, University Hospital Schleswig-Holstein, Campus Kiel, Germany

Abstract

Toll-like receptor (TLR) agonists are considered adjuvants in clinical trials of cancer immunotherapy. Here, we investigated the modulation of γδ T cell–mediated tumor cell lysis by TLR ligands. γδ T-cell cytotoxicity and granzyme A/B production were enhanced after pretreatment of tumor cells with TLR3 [poly(I:C)] or TLR7 ligand (imiquimod). We examined TLR3- and TLR7-expressing pancreatic adenocarcinomas, squamous cell carcinomas of head and neck and lung carcinomas. Poly(I:C) treatment of pancreatic adenocarcinomas followed by coculture with γδ T cells resulted in an upregulation of CD54 on the tumor cells. The interaction of CD54 and the corresponding ligand CD11a/CD18 expressed on γδ T cells is responsible for triggering effector function in γδ T cells. Moreover, treatment with imiquimod downregulated MHC class I molecules on tumor cells possibly resulting in a reduced binding affinity for inhibitory receptor NKG2A expressed on γδ T cells. These results indicate that TLR3 or TLR7 ligand stimulation of tumor cells enhances the cytotoxic activity of expanded γδ T cells of cancer patients *in vitro*. [Cancer Res 2009;69(22):8710–7]

Introduction

Circulating, as well as tumor-infiltrating, γδ T cells exert their effector function by the production of high amounts of cytokines [e.g., IFN-γ, tumor necrosis factor-α (TNF-α)] and cytotoxic mediators, including perforin, granzymes, and TNF-related apoptosis-inducing ligand (1, 2). Vγ9Vδ2 γδ T cells recognize aminobisphosphonates (APC dependent) and phosphorylated intermediates of the bacterial nonmevalonate isoprenoid pathway (APC independent), so-called phosphoantigens (3). A synthetic phosphoantigen analogue is bromohydrin pyrophosphate (BrHPP; ref. 4). Moreover, high concentrations of isopententyl pyrophosphate, possibly produced due to a dysregulated mevalonate pathway in malignant cells as well as ectopically expressed mitochondrial F1-ATPase/apolipoprotein I complex on tumor cells, can selectively induce γδ T-cell expansion (5, 6). In addition to the TCR-dependent recognition, activating killer receptor NKG2D (CD314) is involved in the cytotoxic effector activity of γδ T cells (7, 8). NKG2D binds to stress-inducible MHC class I chain–related antigens (MIC) A/B, as well as UL16-binding proteins (ULBP) 1–4 (9, 10). Other natural killer receptors (NKR) including the inhibitory receptor NKG2A (CD159a), which binds to classic MHC class I molecules, can also modulate the reactivity pattern of γδ T cells (11–13). Furthermore, the CD11a/CD18 (LFA-1)-CD54 [intercellular adhesion molecule-1 (ICAM-1)] interaction and Toll-like receptors (TLR) also modulate γδ T-cell effector function (14–16).

Pattern recognition receptors, such as TLR, have been identified as primary sensors of bacterial and viral components. The cytoplasmic Toll/interleukin-1 receptor (IL-1R) domain of the TLRs is required for intracellular signaling, leading to the production of proinflammatory cytokines and chemokines, type I IFNs, and upregulation of costimulatory molecules (17). TLR agonists are suggested as immunoresponse enhancers with the potential to overcome the tolerance to tumor antigens, the decreased expression of costimulatory molecules on tumor cells, the immunosuppressive function of regulatory T cells, and the production of antiproliferative cytokines produced in the tumor microenvironment (18). TLR agonists mediate their effect by enhancing CTL activity or by triggering apoptosis in tumor cells. On the other hand, TLR agonists can provide survival, proliferation, chemoresistance (e.g., upregulation of antiapoptotic proteins, such as cFLIP and XIAP), and invasiveness of tumor cells (19–21). Thus, the effects of TLR agonists on tumor cells need to be carefully examined before their application in immunotherapy.

In this report, we focused on TLR3, which recognizes dsRNA or surrogate ligand poly(I:C), and TLR7, which recognizes ssRNA or surrogate ligand imiquimod (22, 23). We observed that the *in vitro* stimulation of tumor cells with TLR3 and TLR7 agonists enhances cytotoxicity of γδ T cells.

Materials and Methods

Establishment of T-cell lines and clones. Buffy coats from healthy adult blood donors were obtained from the Department of Transfusion Medicine in Kiel/Lübeck, Germany. Heparinized blood was obtained from cancer patients suffering from pancreatic or ovarian cancer. Informed consent was obtained from all donors, and research was approved by relevant institutional review boards. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (Biochrom, Berlin, Germany). γδ T-cell lines were established as previously described (8, 24). γδ T-cell clones were established by limiting dilution and further expansion as described (25). In addition, $CD8^+$ αβ T cells were established from PBMC of cancer patients 1 and 2, both suffering from ovarian cell carcinoma. PBMCs were stimulated with phytohemagglutinine to expand T cells. $CD8^+$ T cells were purified by magnetic depletion of non-$CD8^+$ T cells and expanded by feeder cells and phytohemagglutinine as described (8, 24).

Tumor cell lines. The following epithelial tumor cell lines were used: pancreatic adenocarcinoma Pt45P1, Panc89, PancTu1, Colo357 (kindly provided by Prof. H. Kalthoff, Section of Molecular Oncology, Kiel), squa-

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

D. Kabelitz and D. Wesch are equally contributing senior authors.

Current address for F. Gieseler: First Department of Medicine, University Hospital Schleswig-Holstein, Campus Lübeck, Germany.

Requests for reprints: Daniela Wesch, Institute of Immunology, UK S-H, Campus Kiel, Arnold-Heller Strasse 3, Haus 17, D-24105 Kiel, Germany. Phone: 49-431-5973379; Fax: 49-431-5973335; E-mail: wesch@immunologie.uni-kiel.de.

doi:10.1158/0008-5472.CAN-09-1602

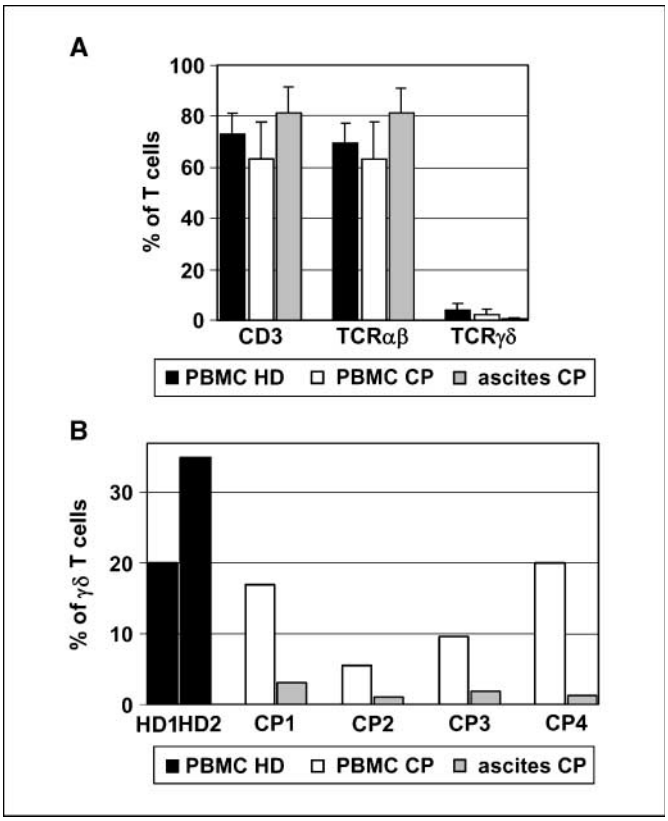

Figure 1. Relative proportion and responsiveness of γδ T cells from peripheral blood and ascites. *A,* the relative proportion of $CD3^+$, $TCR\alpha\beta^+$, and $TCR\gamma\delta^+$ T cells from PBMC of healthy donors (*HD*) and from PBMC or ascites of cancer patients (CP1–CP4) was analyzed by flow cytometry. *Columns,* mean of six (HD) and four (CP) experiments; *bars,* SD. *B,* the same cells as presented in *A* were stimulated with 200 nmol/L BrHPP in the presence of 50 units/mL IL-2, and the relative proportion of γδ T cells was determined by staining with anti-TCRγδ mAb after 9 d of culture. Data of only two representative donors (HD1, HD2) are shown.

mous cell carcinoma of head and neck (SCCHN) BHY and PCI-1 (kindly provided by Prof. B. Wollenberg, Department of Otorhinolaryngology, Lübeck), malignant melanoma MeWo [American Type Culture Collection (ATCC)], primary cell line (lung-ca 459) established from the malignant pleural effusion of a patient with a non–small cell lung carcinoma, and two tumor cell lines established from ascites of patients with ovarian cell carcinoma. All tumor cell lines were cultured in RPMI 1640 with 10% FCS supplemented with antibiotics, 2 mmol/L L-glutamine, and 25 mmol/L HEPES. Adherent tumor cells were trypsinized by treatment with 0.5% trypsin/0.2% EDTA for splitting and functional assays and by Accutase (PAA, Cölbe, Germany) for staining.

Flow cytometry. The following monoclonal antibody (mAb) were used as FITC or PE conjugates for cell surface staining: anti-CD3, anti-TCRγδ, anti-TCRαβ, corresponding isotype controls (all from BD Biosciences), anti-TCRVδ2 (Beckman Coulter), anti-TCRVγ9 (clone 7A5; ref. 26), and anti-TCRVδ1 (Thermo Fisher). For intracellular staining, 3 to 5×10^5 cells were washed, fixed, permeabilized with the Cytofix/Cytoperm kit (BD Biosciences), and stained with PE-conjugated anti-TLR3 mAb clone TLR3.7 (e-Biosciences), unconjugated anti-TLR7 mAb clone 4F4 (Alexis) or Alexa Fluor 647-labeled granzyme B mAb (BD Biosciences), or the appropriate isotype controls for 30 min. For detection of unconjugated TLR7, PE-labeled goat $F(ab')_2$ anti-mouse IgG (Invitrogen) was used as a second step reagent.

To characterize NKRs and their corresponding ligands as well as adhesion molecules, we used the following mAb for tumor cells: FITC-labeled HLA class I (W6/32; immunoglobulin purified from supernatants of hybridoma obtained from ATCC); PE-labeled anti-CD54 (from BD Biosciences). All samples were measured on a FACS-Calibur (BD Biosciences) using the CellQuestPro software.

Cell culture and determination of cytokines and granzymes. To investigate the effect of TLR ligands, a final concentration of 50 μg/mL poly(I:C) (Calbiochem) and 5 μg/mL imiquimod (InvivoGen; both previously titrated) were added to the coculture of γδ T cells and tumor cells. Alternatively, tumor cells were pretreated for 20 h with poly(I:C) or imiquimod. The cells were then washed, and cell number and viability were determined. Supernatants of duplicate cultures were collected after 20 h and stored at −20°C to determine cytokines (e.g., IL-6, TNF-α, type I IFNs) produced by tumor cells. IL-6 and TNF-α were measured by Immulite, an automatic chemiluminescence immunoassay analyzer (DPC Biermann GmbH), and type I IFN (IFNα/β) by human sandwich IFN-α and IFN-β ELISA kit (41105 and 41410; PBL Biomedical Laboratories), all according to the manufacturers' instructions. Human granzyme A and B produced by γδ T cells in the coculture with tumor cells were measured by a sensitive sandwich ELISA (BMS2026 and BMS2027), following the procedures outlined by the manufacturer (Bender MedSystems).

Cytotoxicity assay. Untreated or TLR ligand pretreated tumor cells were labeled with 50 μCi sodium ^{51}Cr and used as targets in a standard of 4-h ^{51}Cr release assay with titrated numbers of γδ T-effector cells. Supernatants were measured in a MicroBeta Trilux β-counter (Perkin- Elmer). Specific lysis was calculated as $((cpm_{test} - cpm_{spontaneous}) / (cpm_{max} - cpm_{spontaneous})) \times 100$, where spontaneous release was determined in medium only and maximal release was determined in Triton-lysed target cells. Spontaneous release did not exceed 15% of the maximal release. Where indicated, γδ effector T cells were preincubated for 30 to 60 min before the

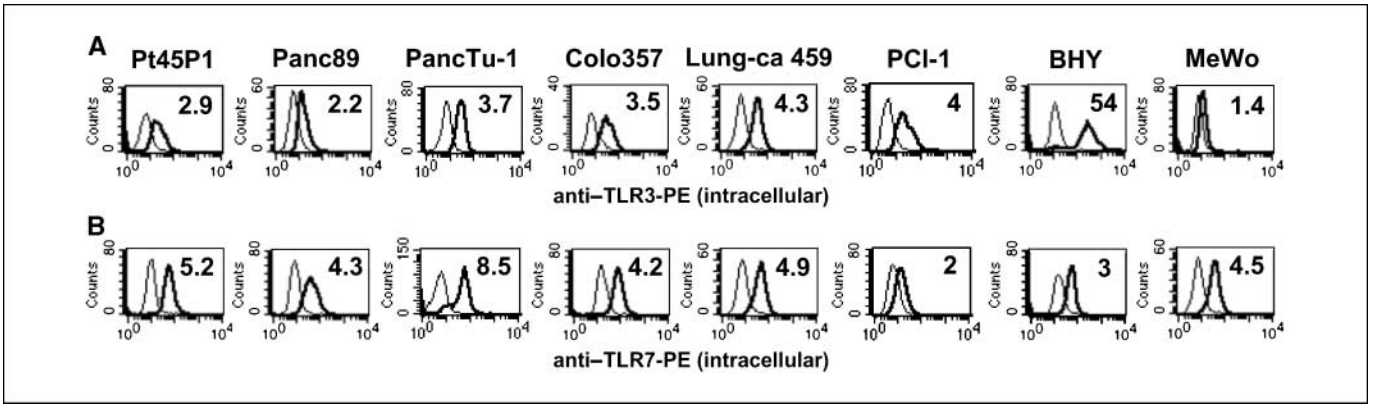

Figure 2. Expression of TLRs in tumor cells. TLR3 (*A*) and TLR7 (*B*) expression was analyzed intracellularly in the indicated tumor cells. The cells were stained by anti-TLR3 mAb or anti-TLR7 mAb (*bold lines*) and by control immunoglobulin (*thin lines*). Histograms of one representative of four. For comparison, median fluorescence intensity was calculated as an *x*-fold increase in relation to the staining with immunoglobulin control as shown by the indicated numbers.

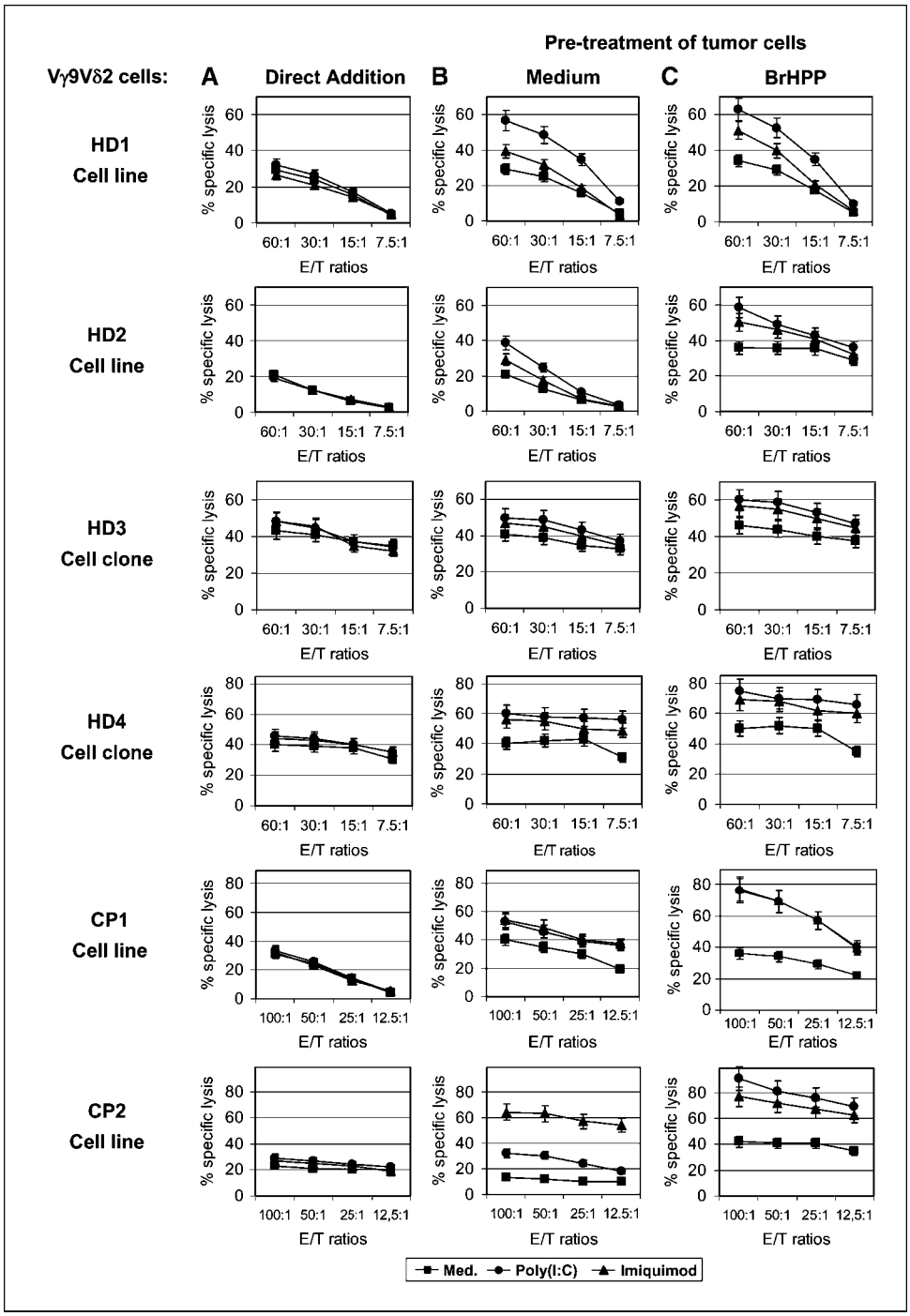

Figure 3. Enhancement of γδ T-cell cytotoxicity by pretreatment of tumor cells. Vγ9Vδ2 T-cell lines/clones from healthy donors (HD1–HD4) and cancer patients (CP1–CP2) were used as effector cells against Pt45P1 at the indicated effector/target (*E/T*) ratios. Poly(I:C) or imiquimod were directly added to the coculture of tumor cells and effector γδ T cells (*A*). Alternatively, tumor cells (*B, C*) were pretreated for 20 h with the indicated TLR ligands, washed, and cocultured with γδ T cells. In some experiments γδ T-cell lines/clones were preincubated with 200 nmol/L BrHPP at 37°C before addition of ^{51}Cr-labeled target cells (*C*). Mean values of triplicate cultures are calculated (SD < 10%). *Points,* mean of two to four independent experiments each with the same donor in one graph; *bars,* SD. Similar reactivity patterns were obtained in independent experiments with two other Vγ9Vδ2 effector T-cell lines of healthy donors (HD5–HD6) or cancer patients (CP3–CP4).

assay with one of the following reagents: anti-NKG2D mAb M585 (Amgen), control mouse IgG (Sigma), or 200 nmol/L BrHPP before the addition of ^{51}Cr-labeled tumor target cells.

Statistical analysis. The paired, two-tailed Student's *t* test was performed.

Results and Discussion

Differential response to BrHPP stimulation of γδ T cells from the peripheral blood versus ascites from cancer patients. There is substantial interest to explore γδ T cells for immunotherapy and also a partial success (2, 27–31). We examined whether γδ T cells in the peripheral blood or in the ascites of cancer patients respond differentially to *in vitro* stimulation and which population is better for large-scale expansion of γδ T cells. We observed that the relative proportion of $CD3^+$ γδ T cells within the peripheral blood of healthy donors and cancer patients is higher than in the ascites of the same cancer patients (Fig. 1*A*). The majority of the γδ T cells were Vγ9Vδ2 γδ T cells (data not shown). In contrast, the relative proportion of $CD3^+TCR\alpha\beta^+$ T cells was slightly higher in the ascites of cancer patients compared with the peripheral blood (Fig. 1*A*). Additionally, we recognized that γδ T cells from the ascites of patients with pancreatic, ovarial, or

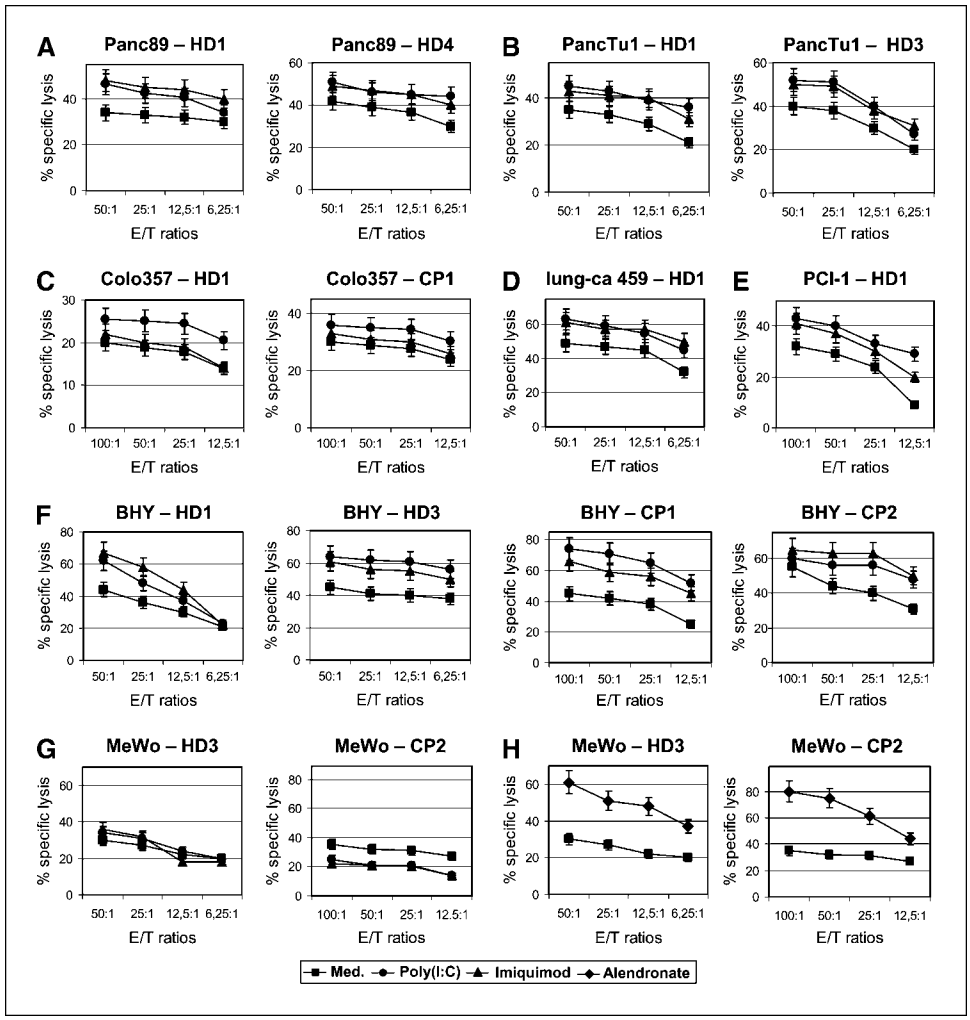

Figure 4. Increased lysis of different tumor cells after pretreatment of tumor cells with TLR ligands. Vγ9Vδ2 T-cell lines (HD1, HD2)/clones (HD3, HD4) of healthy donors and cancer patients (CP1, CP2) were preincubated with BrHPP before the addition of medium (*filled squares*), poly(I:C) (*filled circles*), or imiquimod (*filled triangles*) pretreated tumor cells (*A–H*). As a positive control for lysis, MeWo were pretreated for 20 h with 5 μmol/L alendronate (*filled diamonds*; *G, H*). The mean values of triplicate assays were analyzed (SD < 10%). *Points,* mean of two to three independent experiments each with the same donor; *bars,* SD.

breast cancer were unable to proliferate in response to BrHPP even in the presence of IL-2 (Fig. 1*B*) and even after depletion of $CD4^+$ $CD25^+$ regulatory T cells (data not shown), whereas γδ T cells from peripheral blood of the same cancer patients proliferated (Fig. 1*B*). We and others could correlate the nonresponsiveness of the γδ T cells in the ascites with high amounts of soluble MICA in the ascites inhibiting the binding of membrane bound MICA and thus an internalization of NKG2D, which is important for activation of the NKG2D/NKG2D ligand pathway (32). Furthermore, immunosuppressive cytokines in the microenvironment of the tumor, including transforming growth factor-β and IL-10, chemotherapy as well as apoptose-sensitivity of γδ T cells could be responsible for the nonresponsiveness of γδ T cells from the tumor side.[4]

Therefore, we established γδ T-cell lines and clones from the peripheral blood of healthy donors and cancer patients to investigate whether the addition of TLR agonists enhances the cytotoxicity of γδ T cells toward tumor cells.

Poly(I:C) and imiquimod increase γδ T-cell lysis of different tumor cells. We analyzed tumor cells of different origins (pancreatic adenocarcinomas, SCCHN, ovarian carcinomas, melanoma) for TLR3 and TLR7 expression by flow cytometry. As can be seen in Fig. 2*A*, all tested carcinomas (Pt45P1, Panc89, PancTu1, Colo357, lung-ca 459, PCI-1, BHY) expressed TLR3 intracellularly, except of the melanoma MeWo. The SCCHN tumor cell BHY expressed TLR3 intracellularly with the highest intensity as already described (33). As expected, TLR3 was not expressed on the cell surface (data not shown). All cell lines expressed TLR7 intracellularly with comparable intensity (Fig. 1*B*). The staining of the two ovarian carcinomas was not possible due to the spontaneous cell death of these tumor cells occurring during staining procedure.

Cytotoxicity assays with tumor target cells cocultured with γδ T-effector cells showed that the direct addition of TLR3 ligand poly(I:C) or TLR7 ligand imiquimod did not enhance the cytotoxic activity of γδ T cells from healthy donors (HD1–HD4) or cancer patients (CP1–CP2). These results were repeated in two to four independent experiments with the indicated γδ T-cell lines or clones cocultured with pancreatic adenocarcinoma Pt45P1 as shown in Fig. 3*A*. The data suggest that the 4-hour period of ^{51}Cr release assay is too short to trigger effects of TLR ligands on tumor cells or γδ T cells (Fig. 3*A*). Therefore, Pt45P1 was pretreated for 20 hours with either poly(I:C) or imiquimod followed by two washing steps, resulting in increased sensitivity to γδ T-cell killing (Fig. 3*B*). Moreover, an additional stimulation of γδ T cells with the phosphoantigen

[4] D. Wesch, et al., unpublished observation.

BrHPP further increased cytotoxicity toward pretreated Pt45P1 (Fig. 3*C*). In comparison to γδ T cells, $CD8^+$ TCRαβ T cells from the same donors did not lyse the tested tumor cells, independently of preincubation with medium or TLR ligands (data not shown).

Furthermore, similar patterns were observed with the indicated γδ T-cell lines or clones when other pancreatic adenocarcinomas (Panc89, PancTu1, Colo357), SCCHN (PCI-1, BHY), or lung carcinoma (lung-ca459) instead of Pt45P1 were used (Fig. 4*A–F*). Pretreatment of Panc89, PancTu1, Colo357, lung-ca459, PCI-1, and BHY with poly(I:C) or imiquimod increased the sensitivity to BrHPP-activated γδ T-cell killing (Fig. 4*A–F*). Interestingly, we observed a high increase in γδ T-cell cytotoxicity, when the tumor cell line with the highest TLR3 expression (BHY) was preincubated with poly(I:C) (Fig. 4*F*). However, pretreatment of Colo357 with imiquimod did not enhance γδ T-cell cytotoxicity (Fig. 4*C*). In contrast, melanoma MeWo proved to be resistant to the treatment with poly (I:C) or imiquimod in repetitive experiments (Fig. 4*G*). The lack of TLR3 in MeWo could explain the resistance to the increased lysis after poly(I:C) pretreatment. However, imiquimod pretreatment did not enhance the susceptibility of TLR7-expressing MeWo, suggesting that other or additional mechanisms were involved in the enhanced tumor cell lysis mediated by TLR ligand pretreatment (see below). To show that MeWo is principally susceptible to γδ T-cell killing, we pretreated MeWo with the aminobisphosphonates alendronate (Fig. 4*H*). Aminobisphosphonates induce an accumulation of isopententyl pyrophosphate and an upregulation of NKGD2 ligands, such as ULBP2 and ULBP3 (ref. 34; data not shown), which results in an increased γδ T-cell effector function.

In accordance with the enhancement of tumor cell lysis, we measured an increased production of granzymes A and B, when poly(I:C)- or imiquimod-treated tumor cells were cocultured with γδ T-cell lines, and a significantly increased accumulation of intracellular granzyme B in γδ T cells (Supplementary Fig. S1).

Differential effects of poly(I:C) versus imiquimod on tumor cells. Based on these findings, we were interested in the potential mechanism of enhanced γδ T-cell cytotoxicity after TLR ligand pretreatment of tumor cells. Therefore, we examined whether poly(I:C) and imiquimod influence (*a*) tumor cell survival or apoptosis, (*b*) production of proinflammatory cytokines, and (*c*) modulation of costimulatory molecules.

(*a*) Others have recently reported that TLR3 or TLR7 agonists trigger apoptosis resulting in a direct cytostatic and cytotoxic effect in several breast, skin, or bladder cancer cell lines in malignant melanoma and in basal cell carcinoma patients (35–38). Poly(I:C)-induced cell death involves the Toll/IL-1R domain–containing adaptor–dependent production of IFN-β as well as the activation of NF-κB p65 and extrinsic caspases, which all seem to be required for apoptosis (35, 39). Similarly, imiquimod is described to induce caspase-dependent cell death via the adaptor molecule MyD88 and activation of transcription factor NF-κB. However, we excluded a direct poly(I:C)- or imiquimod-induced cell death in tested tumor cell lines, except for Colo357, of flow cytometric Annexin/propidium iodide staining (data not shown). The pretreatment of Colo357 for 20 hours with TLR3 or TLR7 ligands induced enhanced cell death (70%). However, for the ^{51}Cr assay, viable Colo357 cells were counted and no further cell death was observed during the 4-hour period of the ^{51}Cr assay. In accordance with the failure of TLR ligands to trigger apoptosis in the majority of the tested tumor cell lines, we were unable to detect type I IFN in the supernatant of tumor cells by ELISA after TLR ligand stimulation (data not shown).

(*b*) On the other hand, we measured high levels of IL-6 produced by Pt45P1 and BHY after poly(I:C) but not after imiquimod treatment (Fig. 5*A*) and slightly enhanced concentrations of TNF-α after poly(I:C) treatment of Pt45P1 and BHY or imiquimod treatment of Pt45P1 (Fig. 5*B*). An enhanced proliferation or survival of some tumor cells mediated by an increased autocrine IL-6 production after stimulation with TLR7/8 agonist, a polarization of immune cells toward tumor supporting cells by cytokines including IL-6, TNF-α, and MIF, and an induction of regulators of cell survival such as XIAP and phospho-AKT by TLR ligands have been reported by others (40–42). Interestingly, we observed a weak reduction of enhanced spontaneous cell death in SCCHN after pretreatment with poly(I:C) in the absence of γδ T cells (data not shown). However, poly(I:C) treatment of SCCHN cocultured with γδ T cells resulted in enhanced killing instead of survival (as shown in Fig. 4*E, F*). One explanation for the enhanced killing by γδ T cells could be the low level of IL-6 possibly secreted during the 4 hours of ^{51}Cr release assay. Tumor cells treated for 20 hours with poly(I:C) produced high amounts of IL-6 (Fig. 5*A, B*), which was washed out when the tumor cells were prepared for the ^{51}Cr release assay. To examine the effects of IL-6, we added recombinant IL-6 to the coculture of γδ T cells and tumor cells during the 4 hours of ^{51}Cr release assay. The addition of recombinant IL-6 did not enhance the γδ T cell–mediated killing of the tumor cells, whereas lysis

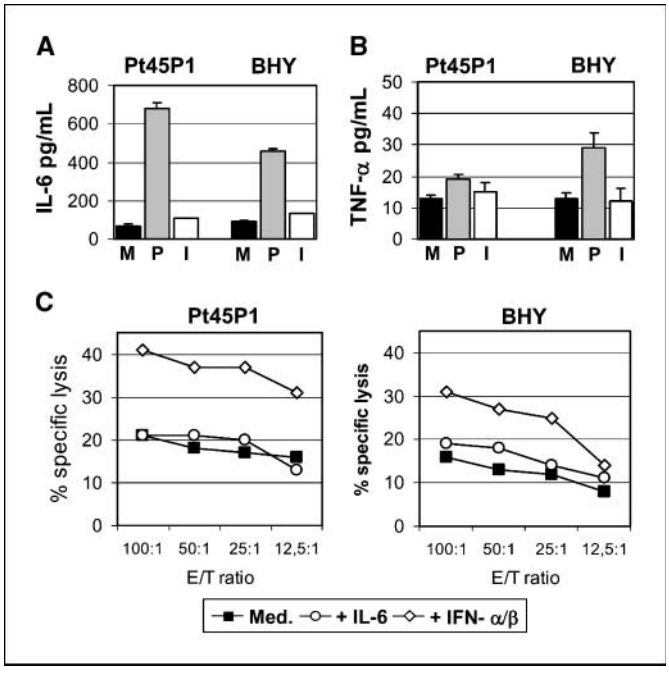

Figure 5. IL-6 has no effect on γδ T-cell cytotoxicity. *A*, Pt45P1 and BHY were cultured in medium (*M*) or stimulated with either poly(I:C) (*P*) or imiquimod (*I*) for 20 h. IL-6 and TNF-α were determined by Immunlite. Results of three experiments with duplicate determinations (*A, B*). *C*, tumor cells Pt45P1 and BHY were tested for γδ T cell–mediated lysis at the indicated effector/target (*E/T*) ratio with γδ T-cell lines generated from healthy donor 1 in the absence (*filled squares*) or presence of 10 ng/mL recombinant IL-6 (*open circles*) or IFN-α/β (*open diamonds*). Mean values of triplicate cultures of one of two similar experiments (SD < 10%). The usage of other γδ T cells generated from other donors (HD2, HD3; CP1, CP2) delivered similar results.

could be increased in the presence of recombinant type I IFNs (IFNα/β, mainly produced by dendritic cells; Fig. 5*C*). Moreover, the nonresponsiveness of γδ T cells to IL-6 is well in line with the failure of the tested γδ T-cell lines to express IL-6R (data not shown).

(*c*) Furthermore, we examined whether costimulatory molecules were upregulated after TLR ligand stimulation. However, none of the tumor cells expressed CD80/CD86 and CD40 ligand, except Panc89, which slightly expressed CD40 ligand (data not shown). Moreover, we did not observe an upregulation of costimulatory molecules after pretreatment of tumor cells with poly(I:C) or imiquimod (data not shown).

We and others reported that the NKG2D/NKG2D ligand pathway also has a costimulatory effect on γδ T cells, thereby regulating activation and cytolytic effector function (7, 8). Therefore, we examined the effect of TLR ligands on NKRs in the coculture of γδ T cells and tumor cells. As expected, most of the Vγ9Vδ2 γδ T-cell lines of healthy donors as well as of cancer patients expressed the activating NKG2D receptor on the cell surface, whereas the inhibitory NKG2A receptor was differentially expressed (Supplementary Fig. S2*A*). Other activating receptors, such as NKG2C, NKp30, NKp46, KIR2DS1, and KIR2DS2, were not expressed (Supplementary Fig. S2*A*). The inhibitory receptors KIR2DL1 and KIR2DL2 were also not detectable, and the inhibitory KIR3DL1 was barely expressed (Supplementary Fig. S2*A*). Therefore, we concentrated on a possible modulation of NKG2D and NKG2A or their ligands. All tested tumor cell lines expressed the NKGD2 ligands MICA/B and ULBP 2-3 (except for MeWo) and the NKG2A ligands MHC-class I molecules (data not shown).

We observed that the NKG2D expression on γδ T cells decreased already at 4 hours after TCR stimulation (BrHPP) in the presence of tumor cells but did not further decrease by coculturing γδ T cells with poly(I:C)- or imiquimod-treated tumor cells (Supplementary Fig. S2*B*). NKG2A expression was also not modulated in the presence of poly(I:C)- or imiquimod-treated tumor cells (data not shown). Additionally, NKG2D ligand expression on tumor cells was unaffected by pretreatment of tumor cells with TLR ligands (data not shown), suggesting that the NKG2D/NKG2D ligand pathway was not involved in the enhancement of γδ T-cell cytotoxicity after pretreatment of tumor cells with poly(I:C) or imiquimod. This was confirmed by the usage of anti-NKG2D mAb M585. γδ T-cell cytotoxicity was decreased after the addition of

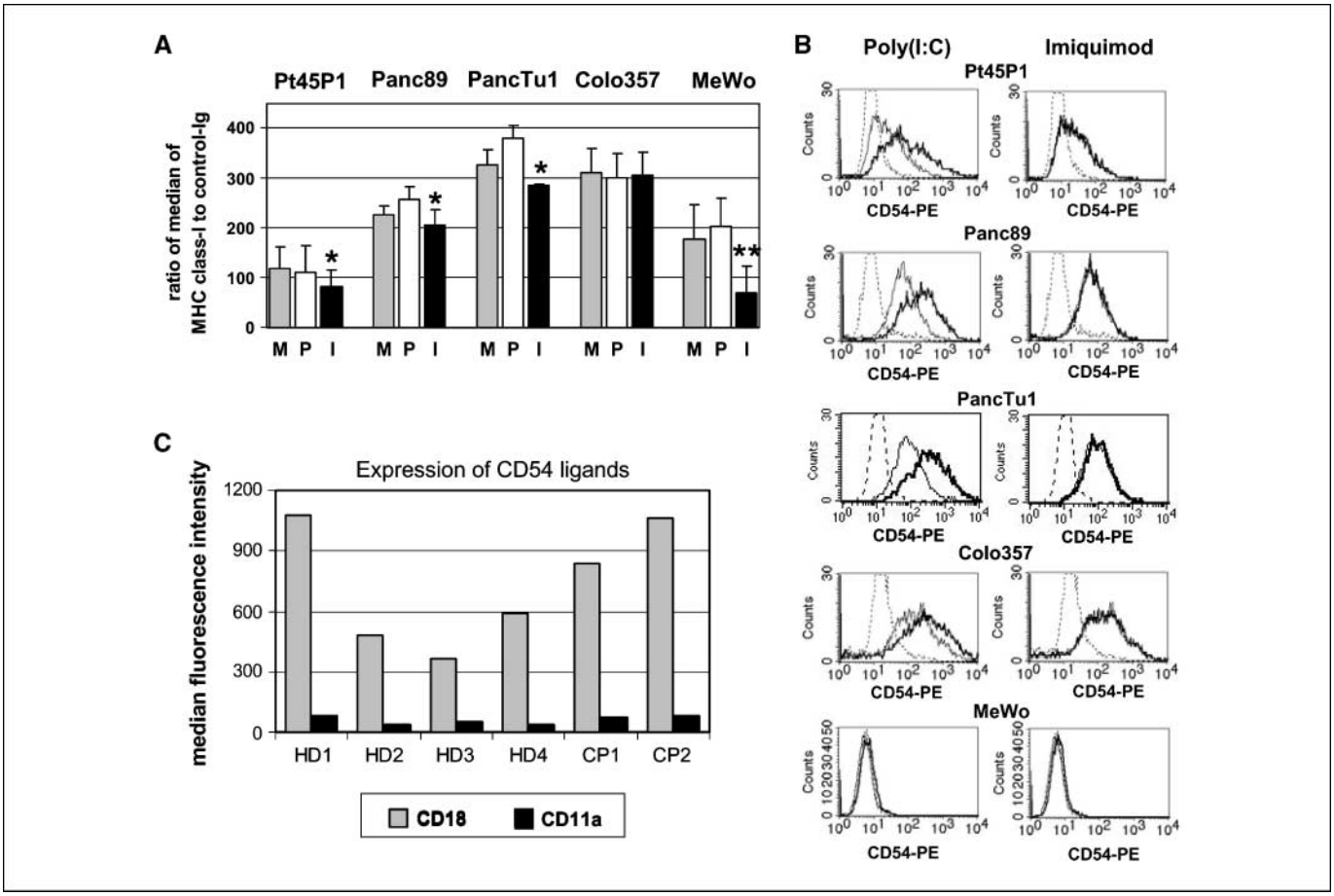

Figure 6. Downregulation of MHC class I on tumor cells after pretreatment with imiquimod and upregulation of CD54 after pretreatment with poly(I:C). Pancreatic adenocarcinomas or MeWo were pretreated for 20 h with medium (*M*), poly(I:C) (*P*), or imiquimod (*I*). *A,* tumor cells were stained by anti–MHC class I mAb W6/32 or appropriate isotype control. The ratio of median fluorescence intensity of MHC class I expression to isotype control was calculated from four independent experiments with healthy donor 1 and cancer patient 1. Levels of significance are represented as *, $P < 0.05$; **, $P < 0.01$. *B,* CD54 (ICAM-1) expression was measured on the cell surface of the indicated tumor cell lines after preincubation with medium (*thin lines*), poly(I:C) (*bold line*), or imiquimod (*bold line*). The dotted lines represent the isotype control. Similar reactivity patterns were obtained in three independent experiments. *C,* median fluorescence intensity of CD11a and CD18 for γδ T-cell lines/clones of the indicated donors. One representative staining of two is presented.

anti-NKG2D mAb M585 to the coculture of γδ T cells and tumor cells. However, the pretreatment of Pt45P1 with poly(I:C) or imiquimod in the presence of anti-NKG2D mAb M585 could not completely inhibit the γδ T cell–mediated lysis. Pretreatment of Pt45P1 with TLR ligands reduced γδ T-cell cytotoxicity only to the level of γδ T-cell lysis in the presence of untreated tumor cells without anti-NKG2D mAb M585 (Supplementary Fig. S2*C*).

Interestingly, pretreatment of tumor cells with imiquimod significantly downregulated MHC class I molecules on tumor cells, which are sensitive to enhanced γδ T-cell killing (Fig. 6*A*). The data suggest a reduced binding affinity to inhibitory receptor NKG2A and thus an enhancement of tumor cell lysis. In contrast, pretreatment of Colo357 with imiquimod did not downregulate MHC class I molecules on these cells, which correlated with the failure to enhance γδ T cell–mediated lysis after pretreatment with imiquimod. In contrast, MHC class I molecules were downregulated by imiquimod on MeWo. However, the failure of imiquimod to enhance lysis of MeWo by γδ T cells could be explained by the lack of NKG2D ligands on MeWo, which are usually required for tumor cell lysis (8).

In contrast, poly(I:C) pretreatment of tumor cells did not downregulate MHC class I molecules (Fig. 6*A*). Therefore, we also investigated a possible modulation of adhesion molecules, which also play an important role in the interaction between cytotoxic γδ T cells and tumor cells. Antibody blockade with anti-CD54 or anti-CD18 has been reported to block γδ T cell–mediated killing of tumor cells (14). In accordance with these data (14), we observed that a failure of CD54 expression, together with a lack of NKG2D ligand expression on MeWo, could explain the reduced capacity of γδ T cells to lyse MeWo cells. Furthermore, pretreatment of tumor cells with poly(I:C) but not with imiquimod for 20 hours enhanced the CD54 expression on these cells (Fig. 6*B*), which nicely correlated with an increased lysis of poly(I:C)-treated tumor cells (Figs. 3*B, C* and 4*A–F*). A kinetic analysis over 24 hours with tumor cells cocultured with γδ T cells in the absence or presence of TLR ligands clearly showed that MHC class I molecules were not downregulated and CD54 was not upregulated after 4 hours with imiquimod- or poly(I:C)-treated tumor cells, respectively (data not shown). These results could explain that the direct addition of TLR3 and TLR7 ligands for 4 hours in the ^{51}Cr release assay did not enhance γδ T-cell cytotoxicity (Fig. 3*A*). Moreover, we observed that poly(I:C) treatment enhanced TNF-α production (Fig. 5*B*), which is reported to enhance CD54 expression (14). Furthermore, the enhancement of γδ T-cell cytotoxicity toward poly(I:C)-treated tumor cells (Figs. 3 and 4) correlated with the intensity of CD11a/CD18 expression on γδ T cells (Fig. 6*C*). Our data suggest that different mechanisms are involved in the modulation of tumor cell lysis by TLR3 versus TLR7 ligand.

In summary, pretreatment of tumor cells with TLR ligands resulted in an increased production of granzymes A and B, as well as in an enhanced killing capacity of γδ T-cell lines/clones. The treatment of tumor cells with poly(I:C) upregulated CD54, and imiquimod downregulated MHC class I molecules. To date, imiquimod is used as first line topical therapy for, e.g., genital condyloma, actinic keratosis, and basal cell carcinomas (43). Our data raise a note of caution with regard to a possible *in vivo* application of poly (I:C) or imiquimod (except for topical application) due to (*a*) additional side effects described by others (44) including poly(I:C)-induced shock, renal failure, and hypersensitivity reactions; (*b*) enhanced survival in the majority of the tested tumor cells (except of Colo357); (*c*) no enhancing effect on costimulatory molecules; and (*d*) insufficient examination of effects on other TLR3- or TLR7-expressing cells of the innate and adaptive immune system. In context of (*d*), we observed in preliminary experiments that the cytotoxicity of γδ T cells from healthy donors or cancer patients could also be enhanced by the pretreatment of the expanded γδ T cells with poly(I:C) or imiquimod in combination with TCR stimulation.[5] The preliminary data suggest that pretreatment of expanded γδ T cells with TLR ligands followed by several washing steps (to eliminate TLR agonists) could be useful in the optimization of protocols for adoptive transfer. Pretreatment of γδ T cells seems to be more expedient than *in vivo* application of TLR ligands with unknown effects on diverse cell populations. Moreover, in ongoing experiments, we test whether poly(I:C(12)U) (Ampligen), a GMP grade synthetic analogue of poly(I:C), which is suggested as an adjuvant for immunotherapy of cancer, also enhances the cytolytic effector function of γδ T cells (44).

[5] D. Wesch, et al., unpublished data.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 5/5/09; revised 8/31/09; accepted 9/15/09; published OnlineFirst 11/3/09.

Grant support: Werner und Klara Kreitz Stiftung.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Hoa Ly, Christine Wilhelms, Frank Rösel, and Sandra Ussat for their technical assistance; Sabine Adam-Klages for fruitful discussions; Dr. Helene Sicard (Innate Pharma, Marseille, France) for the gift of BrHPP; and Dr. David Cosman (Amgen, Seattle, WA) for providing anti-NKG2D mAb M585.

This work forms part of the MD theses of H. Shojaei and M. Juricke.

References

1. Mattarollo SR, Kenna T, Nieda M, Nicol AJ. Chemotherapy and zoledronate sensitize solid tumour cells to Vγ9Vδ2 T cell cytotoxicity. Cancer Immunol Immunother 2007;56:1285–97.
2. Dieli F, Vermijlen D, Fulfaro F, et al. Targeting human {γδ} T cells with zoledronate and interleukin-2 for immunotherapy of hormone-refractory prostate cancer. Cancer Res 2007;67:7450–7.
3. Kunzmann V, Bauer E, Feurle J, Weissinger F, Tony HP, Wilhelm M. Stimulation of γδ T cells by aminobisphosphonates and induction of antiplasma cell activity in multiple myeloma. Blood 2000;96:384–92.
4. Espinosa E, Belmant C, Pont F, et al. Chemical synthesis and biological activity of bromohydrin pyrophosphate, a potent stimulator of human γ δ T cells. J Biol Chem 2001;276:18337–44.
5. Gober HJ, Kistowska M, Angman L, Jeno P, Mori L, De Libero G. Human T cell receptor γδ cells recognize endogenous mevalonate metabolites in tumor cells. J Exp Med 2003;197:163–8.
6. Bonneville M, Scotet E. Human Vγ9Vδ2 T cells: promising new leads for immunotherapy of infections and tumors. Curr Opin Immunol 2006;18:539–46.
7. Rincon-Orozco B, Kunzmann V, Wrobel P, Kabelitz D, Steinle A, Herrmann T. Activation of Vγ9Vδ2 T cells by NKG2D. J Immunol 2005;175:2144–51.
8. Wrobel P, Shojaei H, Schittek B, et al. Lysis of a broad range of epithelial tumour cells by human γδ T cells: involvement of NKG2D ligands and T-cell receptor- versus NKG2D-dependent recognition. Scand J Immunol 2007;66:320–8.
9. Bauer S, Groh V, Wu J, et al. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science 1999;285:727–9.
10. Cosman D, Mullberg J, Sutherland CL, et al. ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. Immunity 2001;14:123–33.
11. O'Connor GM, Hart OM, Gardiner CM. Putting the natural killer cell in its place. Immunology 2006;117:1–10.

12. Halary F, Peyrat MA, Champagne E, et al. Control of self-reactive cytotoxic T lymphocytes expressing γδT cell receptors by natural killer inhibitory receptors. Eur J Immunol 1997;27:2812–21.
13. Wesch D, Kabelitz D. Differential expression of natural killer receptors on Vδ1 γδ T cells in HIV-1-infected individuals. J Acquir Immune Defic Syndr 2003;33:420–5.
14. Guo BL, Liu Z, Aldrich WA, Lopez RD. Innate anti-breast cancer immunity of apoptosis-resistant human γδ-T cells. Breast Cancer Res Treat 2005;93:169–75.
15. Beetz S, Wesch D, Marischen L, Welte S, Oberg HH, Kabelitz D. Innate immune functions of human γδ T cells. Immunobiology 2008;213:173–82.
16. Wesch D, Beetz S, Oberg HH, Marget M, Krengel K, Kabelitz D. Direct costimulatory effect of TLR3 ligand poly(I:C) on human γδT lymphocytes. J Immunol 2006;176:1348–54.
17. Kawai T, Akira S. TLR signaling. Semin Immunol 2007;19:24–32.
18. Paulos CM, Kaiser A, Wrzesinski C, et al. Toll-like receptors in tumor immunotherapy. Clin Cancer Res 2007; 13:5280–9.
19. Smits EL, Ponsaerts P, Berneman ZN, Van Tendeloo VF. The use of TLR7 and TLR8 ligands for the enhancement of cancer immunotherapy. Oncologist 2008;13: 859–75.
20. Conroy H, Marshall NA, Mills KH. TLR ligand suppression or enhancement of Treg cells? A double-edged sword in immunity to tumours. Oncogene 2008;27:168–80.
21. Yu L, Chen S. Toll-like receptors expressed in tumor cells: targets for therapy. Cancer Immunol Immunother 2008;57:1271–8.
22. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. Nature 2001;413:732–8.
23. Soria I, Myhre P, Horton V, et al. Effect of food on the pharmacokinetics and bioavailability of oral imiquimod relative to a subcutaneous dose. Int J Clin Pharmacol Ther 2000;38:476–81.
24. Kabelitz D, Wesch D, Pitters E, Zoller M. Characterization of tumor reactivity of human Vγ9Vδ2γδ T cells *in vitro* and in SCID mice *in vivo*. J Immunol 2004;173: 6767–76.
25. Kabelitz D, Wesselborg S. Life and death of a super-antigen-reactive human CD4+ T cell clone: staphylococcal enterotoxins induce death by apoptosis but simultaneously trigger a proliferative response in the presence of HLA-DR+ antigen-presenting cells. Int Immunol 1992;4:1381–8.
26. Janssen O, Wesselborg S, Heckl-Ostreicher B, et al. T cell receptor/CD3-signaling induces death by apoptosis in human T cell receptor γδ+ T cells. J Immunol 1991; 146:35–9.
27. Caccamo N, Meraviglia S, Cicero G, et al. Aminobisphosphonates as new weapons for γδ T cell-based immunotherapy of cancer. Curr Med Chem 2008;15:1147–53.
28. Burjanadze M, Condomines M, Reme T, et al. *In vitro* expansion of γ δ T cells with anti-myeloma cell activity by Phosphostim and IL-2 in patients with multiple myeloma. Br J Haematol 2007;139:206–16.
29. Viey E, Lucas C, Romagne F, Escudier B, Chouaib S, Caignard A. Chemokine receptors expression and migration potential of tumor-infiltrating and peripheral-expanded Vγ9Vδ2 T cells from renal cell carcinoma patients. J Immunother 2008;31:313–23.
30. Bennouna J, Bompas E, Neidhardt EM, et al. Phase-I study of Innacell γδ, an autologous cell-therapy product highly enriched in γ9δ2 T lymphocytes, in combination with IL-2, in patients with metastatic renal cell carcinoma. Cancer Immunol Immunother 2008;57: 1599–609.
31. Bouet-Toussaint F, Cabillic F, Toutirais O, et al. Vγ9Vδ2 T cell-mediated recognition of human solid tumors. Potential for immunotherapy of hepatocellular and colorectal carcinomas. Cancer Immunol Immunother 2008;57:531–9.
32. Marten A, Lilienfeld-Toal M, Buchler MW, Schmidt J. Soluble MIC is elevated in the serum of patients with pancreatic carcinoma diminishing γδ T cell cytotoxicity. Int J Cancer 2006;119:2359–65.
33. Pries R, Hogrefe L, Xie L, et al. Induction of c-Myc-dependent cell proliferation through Toll-like receptor 3 in head and neck cancer. Int J Mol Med 2008;21: 209–15.
34. Kistowska M, Rossy E, Sansano S, et al. Dysregulation of the host mevalonate pathway during early bacterial infection activates human TCR γδ cells. Eur J Immunol 2008;38:2200–9.
35. Salaun B, Coste I, Rissoan MC, Lebecque SJ, Renno T. TLR3 can directly trigger apoptosis in human cancer cells. J Immunol 2006;176:4894–901.
36. Schon MP, Schon M. Immune modulation and apoptosis induction: two sides of the antitumoral activity of imiquimod. Apoptosis 2004;9:291–8.
37. Inglefield JR, Larson CJ, Gibson SJ, Lebrec H, Miller RL. Apoptotic responses in squamous carcinoma and epithelial cells to small-molecule Toll-like receptor agonists evaluated with automated cytometry. J Biomol Screen 2006;11:575–85.
38. Smith EB, Schwartz M, Kawamoto H, et al. Antitumor effects of imidazoquinolines in urothelial cell carcinoma of the bladder. J Urol 2007;177:2347–51.
39. Salaun B, Lebecque S, Matikainen S, Rimoldi D, Romero P. Toll-like receptor 3 expressed by melanoma cells as a target for therapy? Clin Cancer Res 2007;13: 4565–74.
40. Bohnhorst J, Rasmussen T, Moen SH, et al. Toll-like receptors mediate proliferation and survival of multiple myeloma cells. Leukemia 2006;20:1138–44.
41. Jego G, Bataille R, Geffroy-Luseau A, Descamps G, Pellat-Deceunynck C. Pathogen-associated molecular patterns are growth and survival factors for human myeloma cells through Toll-like receptors. Leukemia 2006; 20:1130–7.
42. Kelly MG, Alvero AB, Chen R, et al. TLR-4 signaling promotes tumor growth and paclitaxel chemoresistance in ovarian cancer. Cancer Res 2006;66:3859–68.
43. Skinner R. Role of topical therapies in the management of cutaneous disease. J Cutan Med Surg 2004;3: 22–31.
44. Jasani B, Navabi H, Adams M. Ampligen: a potential Toll-like 3 receptor adjuvant for immunotherapy of cancer. Vaccine 2009;27:3401–4.