**INTRODUCTION**

Pediatric B-lineage acute lymphoblastic leukemia (ALL) is the most common childhood malignancy and the leading cause of cancer-related death during childhood (1). Improvement of chemotherapy protocols as well as application of alloimmune stem cell transplantation (SCT) in relapsed or refractory patients has improved outcome tremendously. Graft manipulation strategies have evolved from CD34-positive selection over CD3/CD19 depletion, leaving CD3/CD19-depleted T cells potent effector cells not only during prolonged periods of time periods. Combination of CD19 antibodies with primary as well as expanded γδ T cells exhibiting a promising approach, which may enhance clinical outcome of patients with pediatric B-lineage ALL and requires clinical evaluation.

**Keywords:** γδ T cell expansion, ADCC, tumor immunotherapy, therapeutic antibodies, xCELLigence system

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**ORIGINAL RESEARCH ARTICLE**

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**Title:** γδ T cell-mediated antibody-dependent cellular cytotoxicity with CD19 antibodies assessed by an impedance-based label-free real-time cytotoxicity assay

**Authors:** Ursula Jördis Eva Seidel1, Fabian Vogt2,3, Ludger Grosse-Hovest2,4, Gundram Jung2,3, Rupert Handgretinger1,3 and Peter Lang1,3*

1 Department of General Paediatrics, Oncology/Haematology, University Children’s Hospital Tübingen, Tübingen, Germany
2 Department of Immunology, Interfaculty Institute for Cell Biology, University of Tübingen, Tübingen, Germany
3 Partner Site Tübingen, German Cancer Consortium (DKTK), German Cancer Research Center (DKFZ), Tübingen, Germany
4 SYNIMMUNE GmbH, Tübingen, Germany

**Abstract:** γδ T cells are not MHC restricted, elicit cytotoxicity against various malignancies, are present in early post-transplant phases in novel stem cell transplantation strategies and have been shown to mediate antibody-dependent cellular cytotoxicity (ADCC) with monoclonal antibodies (mAbs). These features make γδ T cells promising effector cells for antibody-based immunotherapy in pediatric patients with B-lineage acute lymphoblastic leukemia (ALL). To evaluate combination of human γδ T cells with CD19 antibodies for immunotherapy of B-lineage ALL, γδ T cells were expanded after a GMP-compliant protocol and ADCC of both primary and expanded γδ T cells with an Fc-optimized CD19 antibody (4G7SDIE) and a bi-specific antibody with the specificities CD19 and CD16 (N19-C16) was evaluated in CD107a-degranulation assays and intracellular cytokine staining. CD107a, TNFα, and IFNγ expression of primary γδ T cells were significantly increased and correlated with CD16-expression of γδ T cells. γδ T cells highly expressed CD107a after expansion and no further increased expression by 4G7SDIE and N19-C16 was measured. Cytotoxicity of purified expanded γδ T cells targeting CD19-expanding cells was assessed in both europium-TDA release and in an impedance-based label-free method (using the xCELLigence system) measuring γδ T cell lysis in real-time. Albeit in the 2 h end-point europium-TDA release assay no increased lysis was observed, in real-time xCELLigence assays both significant antibody-independent cytotoxicity and ADCC of γδ T cells were observed. The xCELLigence system outperformed the end-point europium-TDA release assay in sensitivity and allows drawing of conclusions to lysis kinetics of γδ T cells over prolonged periods of time periods. Combination of CD19 antibodies with primary as well as expanded γδ T cells exhibits a promising approach, which may enhance clinical outcome of patients with pediatric B-lineage ALL and requires clinical evaluation.

**Keywords:** γδ T cell expansion, ADCC, tumor immunotherapy, therapeutic antibodies, xCELLigence system
γδ T cell-mediated cytotoxicity with bi-specific anti-γδ T cell construct but present a label-free impedance-based method, facilitating the detection of γδ T cells mediating ADCC with an Fc-optimized CD19 antibody and Trastuzumab (18, 19).

Besides second generation mAbs as chimerized antibody Rituximab and humanized antibody Trastuzumab, several third-generation antibodies have been developed in order to further enhance ADCC in vivo and, thus, improving clinical efficacy (20). The main approaches to optimize FcyRIIIa binding by enhancing the affinity of mAbs developed in recent years, were molecular modifications in the Fc domain of mAbs leading to amino acid substitutions (21–23), modifying Fc-linked glycosylation (24–26) and replacement of the reactive Fc portion by a binding domain for CD16 (27). For treatment of acute myeloid leukemia (AML) several of these third-generation constructs are currently under pre-clinical and early clinical investigation and have been shown to mediate higher ADCC than their unmodified counterparts (28–30).

The standard techniques to determine the antibody-independent cytotoxicity (AIC) and ADCC in vitro include 51 chromium release assays, Europium-TDA assays, [(3)H] thymidine incorporation assays, MTT assays, and flow cytometry-based CD107a-degranulation assays (31–35). However, those methods share various limitations including the labeling of cells and that they can only be readily performed as end-point assays, thereby lacking the information required for kinetic studies (36). Recent studies reported on the deployment of a novel label-free electrical impedance-based assay allowing the dynamic detection of AIC and ADCC and suggest several advantages compared to other established killing assays. This technique, based on the continuous assessment of electrical impedance, has been validated for the assessment of NK cell AIC and ADCC and antigen-specific T-cell-mediated cytotoxicity and deployed for the assessment of γδ T cell-mediated cytotoxicity with bi-specific antibodies binding CD3 and Vγ9 on γδ T cells, respectively (36–38). Impedance to an electric current is increased by the isolating properties of the cell body, when adherent tumor cells attach to electrodes on the bottom of multi-well plates. Killing of these tumor cells results in detachment or disintegration, reducing the electrical impedance that can be measured by the xCELLigence system (36).

Here, we not only show that primary as well as expanded γδ T cells mediate ADCC with an Fc-optimized CD19 antibody and a CD19–CD16 bi-specific construct but present a label-free impedance-based method, facilitating the detection of γδ T cell lysis kinetics over prolonged periods of time.

### MATERIAL AND METHODS

**CELLS AND CULTURE CONDITIONS**

PBMC from leukocytes of thrombaphereses of healthy blood donors and leukemic blasts were isolated by density gradient centrifugation using Biocoll Separating Solution (Biochrom, Berlin, Germany). Healthy donor samples were kindly provided by the Institute for Clinical and Experimental Transfusion Medicine at Tübingen University after obtaining written informed consent. Primary leukemic blasts were obtained from a patient with common-ALL. Over 90% of bone marrow cells were positive for CD10/CD34/CD19 as determined by flow cytometry. PBMC, leukemic blasts, and pediatric B-lineage ALL cell line SEM (ACC 546, DSMZ, Braunschweig, Germany) were cultured in IMDM (Lonza, Basel, Switzerland), breast adenocarcinoma cell line MCF-7 (ACC 115), and B-lineage ALL cell line NALM-6 (ACC 128) were kept in EMEM and RPMI 1640 (Biochrom), respectively. All media were supplemented with 10% fetal calf serum or pooled human AB serum (Invitrogen, Karlsruhe, Germany), 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, and 2 mM L-glutamine (all reagents Biochrom).

**MCF-7 TRANSFEKTANT**

Full-length cDNA of human CD19 (GenBank no. BC006338.2) was purchased from source Bioscience (Berlin, Germany) and cloned into the expression vector pGEM-1. MCF-7 cells were transfected by electroporation (230 V, 975 µF) and CD19 expressing clones were selected and sorted by flow cytometric analysis. Stable CD19 expression of transfected MCF-7 (termed MCF-7CD19tm) was verified over course of culture. Medium of selected clones was supplemented with 1 mg/ml G418-BSC sulfate (Biochrom).

**ANTIBODIES AND FLOW CYTOMETRY**

Initial chimerization and Fc-optimization of χ4G7 and 4G7SDIE, respectively, was described previously (28). For a good manufacturing practice (GMP)-compliant production, antibody genes, codon-usage optimized for CHO expression, were inserted into the expression vector pGEM-1 and used for serum-free transfection of CHO cells. The antibody 4G7SDIE was then produced by transfected CHO cells and purified in GMP-compliant clean rooms using disposable technology including a 100-l biowave reactor (Sartorius, Goettingen, Germany) for fermentation and an AKTA ready system for purification by protein A, ion exchange, and hydrophobic interaction chromatography (MabSelect SuRe and Capto-Adhere columns, GE Healthcare, Munich, Germany). N9-C16 (CD19–CD16) and N9-CU (CD19–CD3) were generated in a bi-specific format, termed Fabsc. This bi-specific format contains the CD19 antibody (clone 4G7) as a Fab fragment, which is linked by an Fc-attenuated CH2 domain to a C-terminal single-chain Fv fragments derived from the CD16 antibody (clone 3G8) and the CD3 antibody (clone UCHT1), respectively (Dubren et al., manuscript submitted). The constructs were produced by transfected Sp2/0 cells and purified by affinity chromatography with κ-select (GE Healthcare).

CD107a-APC, CD56-PE/Cy7, CD45-APC/Cy7, CD3-PercP, IFNy-BV711, TNFa-PB, CD16-AF700, CD4-BV421, CD8-APC/Cy7, and isotype control antibodies were purchased from...
Biolegend (San Diego, CA, USA). TCRγδ-FITC, CD19-PE, CD56-PE, and anti-human CD19 antibody (clone 4G7) were purchased from BD Biosciences (Heidelberg, Germany) and LIVE/DEAD Fixable Aqua and Yellow Dead Stain Kits from Invitrogen. All antibodies were incubated with cells for 20 min at 4°C. Quantitative analysis was performed with QIFIKIT (Dako, Hamburg, Germany) according to the manufacturers’ recommendations. Cells were analyzed with a FACSCalibur or a LSRII and sorted with a FACSSJazz (BD Biosciences).

**CD107a-DEGRANULATION ASSAY**

Percentage of γδ T cells (CD3^+ , TCRγδ^+ ) was determined by flow cytometry and donors with γδ T cells >1.5% were selected for CD107a assays. Equal cell numbers of PBMC and NALM-6 or SEM were incubated with 1 μg/ml 4G7SDIE or N19-C16, 2 μM GolgiStop (BD Biosciences), 10 μg/ml Breifeldin A (Sigma, St. Louis, MO), and CD107a-APC overnight at 37°C, 5% CO₂ in supplemented IMDM. Subsequently, PBMC were stained for surface and intracellular markers and analyzed by flow cytometry.

**EXPANSION OF γδ T CELLS**

PBMC were seeded at 1.5 × 10^6 per well in 24-well plates and cultured in supplemented IMDM containing 100 IU/ml of recombinant human IL-2 (rhIL-2) (Novartis, Basel, Switzerland) and 400 nM zoledronate (Hexal, Holzkirchen, Germany). Every 2–3 days medium containing 100 IU/ml rhIL-2 and 400 nM zoledronate was added. After 12–14 days of culture expanded populations were positively selected using a Hapten-modified anti TCR-γδ antibody and FITC-conjugated anti-Hapten MicroBeads with the autoMACS system (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of the isolated populations was determined by flow cytometric analysis and isolated cells were incubated with 400 IU/ml rhIL-2 overnight prior to functional assays. After 24 h isolated γδ T cells had lost their FITC-labeling and restored TCRγδ surface expression (data not shown).

**REAL-TIME CYTOTOXICITY ASSAY (xCELLIGENCE) ASSAY**

The cytolytic potential of expanded and isolated γδ T cells was analyzed in a real-time cytotoxicity assay with an xCELLIGENCE RTCA SP instrument (ACEA Biosciences, San Diego, CA, USA). In each well 5 × 10^3 MCF-7-CD19tm cells were seeded. After 20–24 h expanded γδ T cells and 1 μg/ml 4G7SDIE and N19-C16 were added, respectively. Cell viability was monitored every 15 min for 48 h. Cell indexes (CIs) were normalized to CI of the time-point when γδ T cells were added and specific lysis was calculated in relation to the control cells lacking any effector γδ T cells.

**EUROPIUM-TDA CYTOTOXICITY ASSAY**

The cytolytic activity of expanded and isolated γδ T cells was analyzed in a 2 h-DELFIA EuTDA cytotoxicity assay (PerkinElmer, Waltham, MA, USA) according to the manufacturers recommendations and as described previously (39). Briefly, cryopreserved primary B-lineage ALL blasts were labeled with the fluorescence enhancing ligand BATDA for 60 min at 37°C. After five wash cycles 5 × 10^3 target cells per well were seeded and γδ T cells and 1 μg/ml 4G7SDIE and N19-C16 were added, respectively. After coculture of 2 h, 20 μl of supernatant was mixed with 200 μl DELFIA Europium Solution and after 15 min fluorescence of Europium-TDA chelates was quantified using a VICTOR multi label reader (Wallac, Turku, Finland). Specific lysis was calculated as follows: % specific lysis = (experimental TDA release – spontaneous TDA release)/(maximum TDA release – spontaneous TDA release) × 100.

**RESULTS**

**EXPANSION OF HUMAN CD16^+ γδ T CELLS**

Human γδ T cells, obtained from leukapheresis products from six healthy volunteers, were expanded after a GMP-compliant protocol using 400 nM zoledronate and 100 IU/ml rhIL-2. After 12–14 days of culture γδ T cells were enriched from 2.87% ± 1.48% of PBMC prior to expansion to 42.35% ± 10.85% of cultured cells. A 1.27-fold ± 0.49 expansion of total cells and a 22.55-fold ± 1.91 expansion of γδ T cells were observed. Percentage of CD16^+ γδ T cells reached 26.55% ± 6.02 after expansion (Table 1). As described previously, expanded γδ T cells exhibited an continuum of CD16-expression (data not shown) (17, 18).

**INDIRECT DETECTION OF CYTOTOXICITY OF PRIMARY γδ T CELLS AND CD19-SPECIFIC ANTIBODY CONSTRUCTS AGAINST CD19 LEUKEMIC CELL LINES**

Due to low percentages of primary γδ T cells in PBMC samples (Table 1), isolation of sufficient numbers of γδ T cells in order to perform cytotoxicity assays may be challenging. Hence, an assay detecting the activation of γδ T cells in PBMC samples deploying the degranulation marker CD107a as well as simultaneously measuring cytokine production by intracellular cytokine staining (ICS) for IFNγ and TNFα was established. PBMC samples were incubated with pediatric B-cell precursor leukemia cell lines NALM-6 or SEM and CD19 antibody 4G7SDIE or N19-C16 constructs. CD107a, IFNγ, and TNFα expression of CD3^+ TCRγδ^+ γδ T cells were determined using flow cytometry. γδ T cells incubated with NALM-6 or SEM and γδ T cells incubated with irrelevant control antibody and NALM-6 or SEM, respectively, displayed low expression of CD107a, IFNγ, and TNFα (Figure 1). However, Fc-optimized CD19 antibody 4G7SDIE enhanced CD107a and TNFα expression of γδ T cells, when incubated with target cell lines, significantly (Figure 1A). IFNγ expression was significantly enhanced as well, though percentages of positive cells remained low. Without adding target cell lines, γδ T cells incubated with 4G7SDIE did not express significantly enhanced levels of CD107a, IFNγ, and TNFα (Figure 1A). The chimerized, unmodified counterpart of 4G7SDIE, χ4G7, did not increase CD107a, IFNγ, and TNFα expression of γδ T cells when incubated with target cell lines NALM-6 (Figure 1B). Bi-specific CD19–CD16 antibody construct N19-C16 induced a significantly enhanced CD107a, IFNγ as well
as TNFα expression of γδ T cells, when incubated with target cell lines, as well (Figure 1C).

**INDIRECT DETECTION OF CYTOTOXICITY OF EXPANDED γδ T CELLS AND CD19-SPECIFIC ANTIBODY CONSTRUCTS AGAINST CD19+ LEUKEMIC CELL LINES**

γδ T cells were expanded as described and the TCRγδ T-cell population was isolated. After cell recovery of 24 h, TCRγδ expression was restored and CD107α expression was analyzed. CD107α expression was observed when incubated with target cell line NALM-6, N19-C16, and 4G7SDIE when incubated with target cell line NALM-6. IFNγ expression was slightly enhanced when incubated with target cell line NALM-6.

**CORRELATION OF CD16-EXPRESSION BY PRIMARY γδ T CELLS WITH ADCC BY CD19-SPECIFIC ANTIBODY CONSTRUCTS**

CD16-expression of γδ T cells was not detected simultaneously in CD107α-degranulation assays due to technical limitations, which prohibited staining of CD16 when analyzing the CD19-specific antibody constructs. Hence, PBMC samples were stained for γδ T-cell markers CD3 and TCRγδ as well as CD16 prior to CD107α-degranulation assays with 4G7SDIE and N19-C16 and cell line NALM-6 as described above (Figure 2A). Mean percentage of CD16-positive γδ T cells was 38.93% ± 20.43. Expression of CD107α, TNFα, and IFNγ induced by target cell line only was deducted from expression levels reached by incubation with NALM-6 and CD19-specific antibody constructs. A positive correlation between CD107α, TNFα, and IFNγ-expression by 4G7SDIE (Figure 2B) or N19-C16 (Figure 2C) stimulated γδ T cells and CD16-positive γδ T cells was observed.

**ASSESSMENT OF CYTOTOXICITY OF EXPANDED γδ T CELLS IN A LABEL-FREE REAL-TIME ASSAY (XCELLIGENCE)**

In order to test the suitability of impedance-based measurement of cell viability for monitoring γδ T cell-mediated AIC and ADCC by CD19-specific antibody constructs, CD19-expressing, adherent cells were required. Adherent breast adenocarcinoma cell line MCF-7 was stably transfected with transmembrane CD19. A CD19+ clone, expressing CD19 comparable to CD19 surface expression levels displayed by pediatric B-lineage ALL blasts, was selected by cell sorting (Figure S1 in Supplementary Material).

MCF-7-C19 transfected cells of the selected clone (MCF-7-C19tm) were seeded on 96-well E-plates and after growing overnight expanded isolated and recovered γδ T cells were added in various effector to target ratios (E:T). Medium, control antibody, CD19-specific antibody constructs 4G7SDIE and N19-C16 were added at the same time-point, respectively. An E:T ratio-dependent cytosis of target cells could be reproducibly monitored as decreasing impedance values in real-time, whereas medium controls were not affected in their growing displayed by continuously increasing normalized CI values (Figures 4A,B). Depending on E:T ratios, normalized CI values of MCF-7-CD19tm incubated with expanded γδ T cells reached baseline, displaying a target cell lysis of 100%, after 24–48 h (Figures 4A,B). Addition of CD19-specific antibody constructs 4G7SDIE and N19-C16 greatly enhanced the reduction of normalized CIs of MCF-7-C19tm cells. Specific lysis of target cells was calculated for different time-points (Figures 5A,B). At E:T 20:1 specific lysis was increasing rapidly over time and reached 79% after 12 h whereas lysis with E:T 10:1 and E:T 5:1 was increasing less rapidly over time, reaching 44 and 17% after 12 h, respectively. Addition of CD19-specific antibody constructs 4G7SDIE and N19-C16 greatly enhanced the specific lysis of MCF-7-C19tm cells. Depending on the time-point to observe AIC and ADCC, differences between γδ T cells alone (AIC) and γδ T cells with CD19-specific antibody constructs (ADCC), were more or less pronounced (Figures 5A,B). Greatest differences of AIC and ADCC were at 4 h (20:1) and 8 h

### Table 1 | Expansion of human γδ T cells.

| Donor | γδ T cells prior to expansion (%) | Days of expansion | γδ T cells after expansion (%) | CD16+ γδ T cells after expansion (%) | Fold-expansion of total cells | Fold-expansion of γδ T cells cells |
|-------|----------------------------------|-----------------|-------------------------------|-------------------------------------|-----------------------------|----------------------------------|
| 1     | 2.24                             | 13              | 35.9                          | 22.70                               | 0.93                        | 14.84                            |
| 2     | 1.60                             | 13              | 43.8                          | 30.26                               | 1.12                        | 30.66                            |
| 3     | 4.51                             | 14              | 60.6                          | 35.10                               | 2.13                        | 28.59                            |
| 4     | 1.20                             | 13              | 33.8                          | 28.90                               | 0.88                        | 24.84                            |
| 5     | 5.18                             | 13              | 32.0                          | 24.00                               | 1.01                        | 6.22                             |
| 6     | 2.50                             | 12              | 48.0                          | 18.32                               | 1.57                        | 30.13                            |
| Mean (SD) | 2.87 (±1.61) | 13 (±0.63)     | 42.35 (±10.85)               | 26.55 (±6.02)                       | 1.27 (±0.49)               | 22.55 (±9.91)                    |
FIGURE 1 | Indirect assessment of primary and expanded γδ T cell-mediated AIC and ADCC in CD107a-degranulation assays and intracellular cytokine stainings (ICS).

(Continued)
We observed a positive correlation between percentages of CD16-γδ T cells and percentage of CD107a+ T cells, further enhanced by CD19 antibody constructs. In contrast to Fc-γδ T cells, primary expanded γδ T cells direct cytotoxicity assays seem favorable. For Rituximab, a suitable method for analyzing ADCC capacity of untouched, primary γδ T cells was observed when target cell lines NALM-6 or SEM were incubated with or without target cell lines NALM-6 or SEM and 1 µg/ml control antibody or 1 µg/ml 4G7SDIE. Five different donors of 5 independent experiments are shown. (B) γδ T cells were incubated with or without target cell lines NALM-6 or SEM and 1 µg/ml control antibody or 1 µg/ml 4G7SDIE. One representative experiment, of three independent experiments performed, is shown. (C) γδ T cells were incubated with or without (light gray bars) target cell lines NALM-6 (black bars) or SEM (gray bars) and without, 1 µg/ml control antibody or 1 µg/ml N19-C16. Experiments were performed in triplicates. (D) Expanded, isolated, and recovered γδ T cells were incubated with or without (light gray bars) target cell lines NALM-6 (black bars) or SEM (gray bars) and without, 1 µg/ml N19-C16 or 1 µg/ml 4G7SDIE. Experiments were performed in triplicates. Error bars represent the standard deviation (SD).

**DISCUSSION**

Albeit tremendous improve in outcome of pediatric patients with B-lineage ALL over the last decades, prognosis for primary refractory or relapsed patients remains poor. Since immunotherapeutic therapy with chimeric antigen receptor-modified T cells with specificity for CD19 have been successful in pediatric patients with B-lineage ALL, further immunotherapeutic strategies are emerging (40, 41).

Due to their features including non-MHC restriction, cytotoxicity against hematological malignancies and capacity for ADCC, CD19-expressing target cell line was generated. In consideration of that the generated cell line MCF-7-C19tm expressed comparable CD19 surface levels to leukemic blasts and commonly used cell lines, we hypothesize that results obtained in this assay allow conclusions regarding ADCC by CD19 antibody constructs in response to pro-inflammatory cytokines IL-2, IL-15, and IL-21 (43, 44). Enhancing CD16-expression of γδ T cells in vivo and in clinical large-scale expansion protocols, respectively, may be approaches to advance combined immunotherapy with therapeutic antibodies. Examining the effect of these cytokines on γδ T cell-mediated ADCC would be particularly interesting as it has been shown that NK cell-mediated ADCC is enhanced by IL-2, IL-15, and IL-21 as well (7, 45, 46).

CD107α-degranulation assays measure the cytolytic capacity of effector cells but lack detection of direct cytolysis of target cells. However, considering the low frequencies of primary γδ T cells, isolation of sufficient numbers of γδ T cells for direct cytolysis assays may be impracticable. Furthermore, analysis of ADCC of untouched primary γδ T cells in direct cytolysis assays, requiring isolation of cells, is currently challenging as, to our knowledge, no cell isolation kits are commercially available, which allow isolation of γδ T cells without depleting CD16+ γδ T cells. γδ T cells were expanded after a protocol, using reagents in pharmaceutical quality, making a possible clinical translation achievable. AIC and ADCC of expanded, isolated and recovered γδ T cells was analyzed in 2 h-europium-TDA assays. No lysis of leukemic blasts by expanded γδ T cells alone and with 4G7SDIE and N19-C16 was measured, respectively. When expanded γδ T cells were strongly activated by a CD19–CD3-recruiting bi-specific antibody construct (N19-CU), strongly enhanced lysis was observed. Unlike CD16, which was expressed by 26.55% ± 6.02 of the expanded γδ T cells, CD3 is expressed by every γδ T cell. Hence, activation of a significantly greater number of γδ T cell can be achieved with antibodies recruiting CD3-positive cells rather than CD16-positive cells. This end-point assay is feasible for detecting cytotoxicity of expanded γδ T cells against leukemic blasts. However, potent and rapid activation of expanded γδ T cells seems to be required. AIC and ADCC of expanded γδ T cells may be obliterated due to the short end-point of the assay, which is limited by time-dependent increase of spontaneous release of TDA by labeled target cells. Furthermore, no conclusions to lysis kinetics can be drawn.

Due to the mentioned limitations of the end-point assay, AIC and ADCC of γδ T cells were detected in real-time cytolysis assays (xCELLigence). One drawback of this method is the requirement of adherent target cells. Since primary leukemic blasts as well as common leukemic cell lines are suspension cells, a CD19-expressing target cell line was generated. In consideration of that the generated cell line MCF-7-C19tm expressed comparable CD19 surface levels to leukemic blasts and commonly used cell lines, we hypothesize that results obtained in this assay allow conclusions regarding ADCC by CD19 antibody constructs.
FIGURE 2 | Correlation of CD16-expression by γδ T cells and degranulation and cytokine production of antibody-stimulated primary γδ T cells.

(Continued)
CD16-expression of γδ T cells was assessed by flow cytometric analyses prior to functional assays. Gating hierarchy was: lymphocytes, single cells, viable cells, CD3<sup>+</sup>TCR<sup>γδ</sup>, TCR<sup>γδ</sup>CD16<sup>+</sup>. Gates were defined based on marker-negative populations within the viable cells. Three representative donors are shown. Same gating strategy was used for all samples (A). PBMC were incubated with equal cell numbers of target cell lines NALM-6 and antibodies for 16 h. γδ T cell degranulation (CD107a) and cytokine production (TNFα and IFNγ) were detected on viable, single CD3<sup>+</sup>TCR<sup>γδ</sup> lymphocytes by extracellular and intracellular staining and flow cytometric analysis. Expression of CD107a, TNFα, and IFNγ induced by NALM-6 only was deducted from expression levels reached by incubation with NALM-6 and 4G7SDIE or N19-C16. PBMC were incubated with target cell line NALM-6 and medium, 1 µg/ml 4G7SDIE (B) and 1 µg/ml N19-C16 (C), respectively. Statistical significance was assessed using Pearson's correlation. Experiments were performed in triplicates.

Significant lysis of target cell line MCF-7-CD19tm by expanded γδ T cells was observed and increased over time. CD19 antibody constructs 4G7SDIE and N19-C16 greatly enhanced the specific lysis of MCF-7-CD19tm cells by expanded γδ T cells. Notably, maximal cytotoxicity of expanded γδ T cells was delayed beyond 24 h and time-points of reaching maximal cytolysis varied for expanded γδ T cells alone (AIC) and expanded γδ T cells with CD19 antibody constructs (ADCC). Depending on the time-point considering AIC and ADCC, differences between γδ T cells alone (AIC) and...
γδ T cells with CD19 antibody constructs (ADCC), were more or less pronounced. These observations underline the importance of prolonged incubation times in cytotoxicity assays with expanded γδ T cells and monitoring lysis kinetics by real-time measurement of AIC and ADCC.

In summary, we show that untouched primary as well as expanded γδ T cells mediate ADCC with CD19 antibody constructs against various targets. Thus, combination of the presented antibody constructs with primary as well as expanded γδ T cells exhibit promising immunotherapeutic approaches that require clinical evaluation. Notably, assays assessing AIC and ADCC of γδ T cells have some limitations and should be chosen deliberately. The assessment of CD107a and cytokine expression is a feasible method for assessing cytotoxicity of untouched primary γδ T cells rather than expanded γδ T cells. Europium-TDA release assays are feasible for expanded γδ T cells but AIC and ADCC of γδ T cells can be obliterated due to the limited end-point of the assay and no firm conclusions to lysis kinetics may be drawn. The xCELLigence system allows to measure lysis kinetics of γδ T cells over prolonged periods of time and enables the detection of both AIC and ADCC of expanded γδ T cells against adherent target cells.
FIGURE 5 |Time-dependent cytolytic activity of expanded γδ T cells in xCELLigence assays. (A,B) MCF-7-CD19tm were seeded into 96-well E-plates at equal densities of 5000 cells per well. After cell attachment and expansion, expanded, isolated, and recovered γδ T cells at different E:T ratios (20:1–5:1) and antibodies were added at time-point \(t_0\). Impedance at well bottoms was measured every 15 min for >12 h and normalized to baseline impedance values with medium only. The cytolytic activity at different time-points was calculated as the percentage of specific lysis \(= \frac{\text{normalized CI}_{\text{no effector cells}} - \text{normalized CI}_{\text{γδ T cells (with antibody)}}}{\text{normalized CI}_{\text{no effector cells}}} \times 100\). One representative experiment, of four independent experiments performed, is shown. (A) γδ T cells (gray line), γδ T cells with 1 µg/ml control antibody (light gray line) and γδ T cells with 1 µg/ml CD19-specific Fc-optimized mAb 4G7SDIE (black line) were added, respectively. (B) γδ T cells (gray line), γδ T cells with 1 µg/ml control antibody (light gray line), and γδ T cells with 1 µg/ml CD19–CD16-bi-specific antibody construct N19-C16 (black line) were added, respectively.

AUTHOR CONTRIBUTIONS
Ursula Jördis Eva Seidel contributed to the conception and design of the work, development of methodology, acquisition, analysis and interpretation of data, and to the drafting of this article. Fabian Vogt contributed to the development of methodology and interpretation of data and critical revision of this article. Ludger Grosse-Hovest contributed to the development of methodology, acquisition, analysis and interpretation of data, and critical revision of this article. Gundram Jung contributed to the interpretation of data and critical revision of this article. Rupert Handgretinger contributed to the conception and design of the work, interpretation of data, and critically revised this article for the final approval of the submitted version. Peter Lang contributed to the conception and design of the work, interpretation of data, and critical revision of this article.

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SUPPLEMENTARY MATERIAL
The Supplementary Material can be found online at http://www.frontiersin.org/Journal/10.3389/fimmu.2014.00618/abstract

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