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Submitted on 18 Jul 2017

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ImmunoHorizons 2017, 1 (5) 63-70
doi: https://doi.org/10.4049/immunohorizons.1700022
http://www.immunohorizons.org/content/1/5/63

This information is current as of July 17, 2017.

Supplementary Material
http://www.immunohorizons.org/content/suppl/2017/07/14/1.5.63.DCSupplementary

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Transfection of FcγRIIIa (CD16) Alone Can Be Sufficient To Enable Human αβTCR T Lymphocytes To Mediate Antibody-Dependent Cellular Cytotoxicity

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ABSTRACT
To combine the immune potential of T cells and Ab therapy, we and others have previously shown that T cells transduction with a fusion receptor that binds the Fc portion of human Ig enable them to mediate Ab-dependent cellular cytotoxicity (ADCC). The fusion receptors previously described included the FcγRIIIa (CD16) receptor coupled to different chains intended to translate the signal. In this work, we questioned whether the transfection of CD16 alone into T human lymphocytes and NK cells could be sufficient for CD16 expression and function, or whether the cotransfection of a transducing chain was mandatory. Our results demonstrated that: 1) transfection of CD16 alone into a human NK cell line and primary T cells can be sufficient for CD16 expression and function; 2) cotransfection of CD3ζ or FcγRI increased CD16 expression; 3) yet this increased CD16 expression increased the ADCC score only for trastuzumab, not for rituximab or cetuximab; and 4) compared with that of peripheral NK cells, ADCC scores by autologous CD16-transfected T cells ranked differently according to the opsonized target cell. Together, these results showed that neither the use of a fusion receptor nor the cotransfection of a transducing chain is mandatory to transfer the ADCC function to human lymphocytes. Thus, depending on the effector/Ab/target combination considered, transfection of CD16 alone can be sufficient to enable T cells to mediate ADCC. In the context of immunotherapy, such a strategy is by nature safer than the use of a chimeric receptor, and is freely available. ImmunoHorizons, 2017, 1:63–70.

INTRODUCTION
Because of the well-known immune potential of transfused T lymphocytes and the accumulating evidence of the role of the Ab-dependent cellular cytotoxicity (ADCC) pathway in the clinical efficacy of several therapeutic Abs (1–7), we proposed several years ago to arm T cells with a receptor that enabled them to mediate ADCC (8). We demonstrated that after transduction with a CD16/γ fusion gene the T cells expressed this Fc receptor and became able to kill target cells through ADCC. This concept, which has been adopted and confirmed by others (9, 10), has recently been the subject of a preliminary clinical evaluation by Campana’s group (11). In all cases, ADCC capacity was transferred to T lymphocytes after transduction of different fusion genes, where the extracellular domain (EC) of CD16 was covalently associated with different transmembrane (TM) and intracytoplasmic (IC) domains in charge of the signal transduction. The construct we used comprised the EC domain of CD16 linked to FcγRIγ (2 aa of the EC, the entire TM and the entire IC domains). The construct used by Kudo et al. (9) associated the EC domain of CD16 with CD3ζ (2 aa of the EC, the entire TM domain and the entire IC domain of FcγRIγ). The construct used by Ochi et al. (10) associated the EC domain of CD16 with CD3ζ (2 aa of the EC domain, the entire TM and the entire IC domains). All of the constructs mentioned earlier...
were expressed by T cells after transduction and enabled the transduced T cells to mediate ADCC. These artificial Fc receptors are thus chimeric proteins and have in common with chimeric Ag receptors (CARs) the structural particularity that the portion of the receptor bearing the ligand specificity (the Fc or the Ag) was covalently linked to the portion of the receptor in charge of the signal transduction. Although such chimerical design was one option to build receptors such as CARs, which do not exist in nature, it had a priori no reason to be necessary for the expression of a receptor such as CD16, whose expression, as we have recently shown, is natural for some αβ TCR T lymphocyte subsets (12). Both NK and T lymphocytes express the signaling chains CD3ζ and FcRIγ, and both chains allow efficient expression of CD16 at the cell surface through interaction with their TM segments (13). Thus, transfection of CD16 alone can theoretically lead to CD16 expression at the cell surface, provided that the number of endogenous CD3ζ or FcRIγ available for an association is sufficient. If not, or if CD16 expression after transfection of CD16 alone appeared not to be sufficient to permit ADCC, then cotransfection with CD3ζ or FcRIγ (without the need to design a fusion protein) may allow to increase CD16 expression. The experiments presented were initiated to address these different possibilities. To this end, we analyzed CD16 expression and ADCC function first by the human NK-92 cell line (because of its NK lineage) and next by human T cells. CD16 expression and ADCC were assessed after either transfection of CD16 alone or after cotransfection of CD16 and the transducing chains CD3ζ or FcRIγ.

MATERIALS AND METHODS

PiggyBac transposon vector and piggyBac transposase-expression vector

The hyperactive piggyBac transposase plasmid (pCMV-hyPBase) and the PB-Transposon plasmid (pPB-UbC) have been described previously (14–16) and were provided by Wellcome Trust Sanger Institute (Hinxton, U.K.). The PB-Transposon plasmid is transcriptionally regulated by the human ubiquitin Ubc promoter, which is very active in conferring expression of exogenous genes after transient transfection of the appropriate expression vectors in various cell lines (M. Schorpp and P. Angel, unpublished observations).

Plasmids construction

Schematic representation of the different chains used for transfection is indicated in Fig. 1: the FCGR3A L48F158 (CD16L48F158) cDNA and the H48V158 (CD16H48V158) were obtained from pcDNA3.1/FcγRIIIa-L48F158-blasticidin and pcDNA3.1/FcγRIIIa-H48V158-blasticidin plasmid (kindly provided by M. Ohresser, UMR CNRS 6239, Tours, France). The FCGR3A L48V158 (CD16L48V158) coding sequence was amplified by RT-PCR from RNA isolated from PBMCs of a healthy donor with restriction digestion and DNA sequencing (Plateforme séquençage génotypage, Nantes, France).

Blood donor, cell lines, and cell culture

PBMCs were obtained from healthy donors after informed consent. NK cells were purified from PBMCs using the Human NK Cell Enrichment Kit from STEMCELL Technology (Grenoble, France) according to the supplier’s instructions. The following cell lines were used: BK01/12, a locally obtained EBV-transformed B lymphoblastoid cell line (18); Raji, an EBV+ cell line derived from a patient with a Burkitt’s lymphoma (19); BT-474, a HER2+ breast cancer cell line (ATCC HTB-20; ATCC, Rockville, MD); and the LN-18 glioblastoma cell line (ATCC CRL-2610). BK01/12, Raji, and the NK-92 cell lines were cultured in RPMI 1640 medium (Life Technologies, Cergy Pontoise, France) supplemented with 10% FCS (PAA Laboratories, Les Mureaux, France), 2 mM l-glutamine (Life Technologies), penicillin (100 IU/ml; Life Technologies), and streptomycin (0.1 µg/ml; Life Technologies). For NK-92, the culture medium was supplemented with IL-2 (100 IU/ml) (Novartis Pharma SAS, Rueil-Malmaison, France). The BT-474 and the LN-18 cell lines were cultured in DMEM medium (Life Technologies) supplemented with 10% heat-inactivated FCS (PAA Laboratories, Velizy Villacoublay, France), 2 mM l-glutamine, penicillin (100 IU/ml), and streptomycin (0.1 µg/ml). PBMCs were cultured in RPMI 1640 medium supplemented with 8% pooled human serum, 2 mM l-glutamine, penicillin (100 IU/ml) and streptomycin (0.1 µg/ml), and IL-2 (300 IU/ml). T cells were stimulated with irradiated (35 Gy) pooled allogenic feeder cells, 1 µg/ml leuкоagglutinin (PHA-L) (L4144; Sigma-Aldrich, Lille-Lezennes, France), and IL-2 (300 IU/ml). For ADCC assays, transfected T lymphocytes were tested at least 2–3 wk after the stimulation.

NK-92 transfection and selection

NK-92 cells (2.5 × 106 cells) were mixed with 5 µg of pPB-UbC-hCD16 (V158 or F158) and 10 µg of pCMV-hyPBase and then electroporated with the Cell Line Nucleofector Kit R VCA-1001

https://doi.org/10.4049/immunohorizons.1700022
(Lonzà, Levallois-Perret, France) using the program A-024. From 3 wk after transfection, NK-92 CD16+ cells were stained with mouse anti-CD16 (clone 3G8; Immunotech, Marseille, France) and immunoselected using anti-mouse IgG-coated beads (Dynabeads M-280; Dynal AS, Oslo, Norway), according to the supplier’s instructions. Next, NK-92 CD16+ cells (2.5 × 10⁶) were mixed with 5 μg of pPB-Ubc-CD3ζ or FcεRIγ and 10 μg of pCMV-hyPBase for the second transfection. After the second transfection, NK-92 with the highest CD16 expression were cell sorted using a FACSaria III (BD Biosciences).

**T cells transfection and selection**

PBMCs were obtained from blood donors at the Etablissement Français du Sang with informed consent (blood products transfer agreement relating to biomedical research protocol 97/5-B – DAF 03/4868) and were isolated by Ficoll-Hypaque centrifugation (Eurobio, les Ulis, France). PBMCs (2.5 × 10⁶) were mixed with 2 μg of pCMV-hyPBase and 10 μg of pPB-Ubc-hCD16 ± 2 μg of pPB-Ubc-CD3ζ or FcεRIγ. PBMCs were electroporated with Human T cell nucleofector kit VPA-1002 (Lonzà) and the program U-14. The next day, transfected lymphocytes were stimulated with irradiated (35 Gray) pooled allogenic feeder cells, 1 μg/ml leukoagglutinin (PHA-L), and IL-2 (300 IU/ml). Twenty-two days after electroporation, transfected cells were stained with anti–αβTCR-FITC (MCA2815F; AbD Serotec, Kidlington, U.K.) and anti–CD16-PE (IM1238; Beckman Coulter), and ~5 × 10⁴ αβTCR+ CD16+ cells were sorted using a FACSaria III (BD Biosciences) and stimulated.

**Flow cytometry**

The following mAbs and their isotype controls were used: anti–CD16-PE-Cy5 clone 3G8 (A07767; Beckman Coulter) and anti–αβTCR-FITC (MCA2815F). For staining, 0.2 ml leukoagglutinin (PHA-L), and IL-2 (300 IU/ml). Twenty-two days after electroporation, transfected cells were stained with anti–αβTCR-FITC (MCA2815F; AbD Serotec, Kidlington, U.K.) and anti–CD16-PE (IM1238; Beckman Coulter), and ~5 × 10⁴ αβTCR+ CD16+ cells were sorted using a FACSaria III (BD Biosciences) and stimulated.

**ADCC assay**

Cytotoxic activity was assessed using a standard ⁵¹Cr release assay. Target cells were labeled with 75 μCi (2.77 MBq) ⁵¹Cr (PerkinElmer, Courtaboeuf, France) for 1 h at 37°C, washed four times with culture medium, and then plated at an E:T cell ratio of 10:1 in a 96-well, flat-bottom plate. The anti-CD20 mAb rituximab (Roche, Neuilly, France), the anti-Her2/neu mAb trastuzumab (Roche), and the anti-EGFR mAb cetuximab (Merck, Lyon, France) were used for ADCC assays at the indicated concentrations. After 4 h incubation at 37°C, 25 μl of supernatant was removed from each well, mixed with 100 μl scintillation fluid, and ⁵¹Cr activity was counted in a scintillation counter (MicroBeta; PerkinElmer). Each test was performed in triplicate. The results are expressed as the percentage of lysis, which is calculated according to the following equation: (experimental release – spontaneous release)/(maximal release – spontaneous release) × 100, where experimental release represents the mean cpm for the target cells in the presence of effector cells, spontaneous release represents the mean cpm for target cells incubated without effector cells, and maximal release represents the mean cpm for target cells incubated with 1% Triton X-100 (Sigma).

**Statistical analysis**

To compare groups for statistically significant differences, we analyzed data with the Mann–Whitney test for the mean fluorescences and the two-way ANOVA test for the ADCC curves; analyses were performed using GraphPad Prism version 5.00 (GraphPad Software, La Jolla, CA) on a Mac OSX version 1.6.8.

**RESULTS**

**CD6 expression by NK-92 after transfection with CD16 alone, CD16 and CD3ζ, or CD16 and FcεRIγ**

The gene coding CD6 displays a functional allelic dimorphism generating allotypes with either a phenylalanine (F) or a valine (V) residue at amino acid position 158. The latter form (CD16V) has a better affinity for IgG1 and IgG3 than the former (CD16F) (20). The results presented were obtained with the CD16V form, referred to as CD16 in this article (Fig. 1). For the first set of assays, CD6 expression after transfection of CD16 alone or with FcεRIγ or CD3ζ was assessed on the NK-92 cell line. NK-92 was used because it belongs to the human NK lineage, the main subset naturally expressing CD16 among PBLs. NK-92 cells were first transfected with CD16, immunoselected using the anti-CD16 mAb 3G8 and Dynabeads, and then further transfected with FcεRIγ or CD3ζ (Fig. 2A). As it is shown on Fig. 2B, the CD16 receptor was readily and stably expressed (the four points represent four determinations through 2 mo of culture) by the NK-92 cells after transfection (mean fluorescence 4 ± 1 for the control versus 504 ± 197 for CD16 alone). The CD6 expression by the NK-92 cell line after transfection of CD16 alone was confirmed through six independent transfections (Supplemental Table I). When CD16-transfected NK-92 cells were also transfected with CD3ζ or FcεRIγ, a further increase in CD6 expression was observed in both cases (mean fluorescences: 504 ± 197, 1964 ± 586, 2871 ± 808 for CD16, CD16 + CD3ζ, and CD16 + FcεRIγ respectively; Fig. 2A, 2B).

**ADCC activity by NK-92 after transfection**

The four NK-92 lines whose CD16 expression level is depicted in Fig. 2B were tested for ADCC activity against the CD20+ B lymphoblastoid cell line BK01/12 in the presence of variable doses of the anti-CD20 mAb rituximab. The three NK-92 lines that received CD16 alone, or with CD3ζ or FcεRIγ, showed comparable levels of ADCC activity at all rituximab concentrations (Fig. 2C).
Thus, although the cotransfection of FcεRIγ or CD3ζ increased the level of expression of CD16 at the cell surface, this did not translate into an increased score of ADCC, which suggested that in that particular effector/Ab/target interaction, the signal provided by the number of CD16 available after transfection of CD16 alone was sufficient for the NK-92 to exert all its cytotoxic potential. This may not necessarily always be the case, as shown later with transfected T cells.

**FIGURE 1. Schematic representation of the different chains transfected.**
The CD16L48V158 coding sequence was obtained from the PBMC of a healthy donor. The CD16L48V158 and CD16H48V158 sequences were kindly provided by M. Ohresser. No influence of these CD16 polymorphisms was observed on the CD16 expression (Supplemental Table I) and the CD16H48V158 chain was used throughout the study.

**FIGURE 2. CD16 expression and function after transfection of NK-92 with CD16 alone or together with a transducing chain.**
(A) Transfection-selection procedure for the NK-92 cell line. (B) CD16 expression by selected NK-92 after transfection with the indicated chains. For each NK-92 cell line, symbols represent CD16 mean fluorescence at different time points after selection through 2 mo of culture. Increased expression of CD3ζ and FcεRIγ are presented in Supplemental Fig. 1. (C) NK-92 ADCC activity was assessed using a 4-h 51Cr release assay against the CD20+ B lymphoblastoid cell line BK01/12 in the presence of variable doses of the anti-CD20 mAb rituximab. Symbols represent mean ± SD of three independent experiments; no significant difference was observed among the three populations.
CD16 expression by T lymphocytes after transfection of CD16V alone, CD16V and CD3ζ, or CD16V and FcRIγ

Next, CD16 expression after transfection of CD16 alone or after transfection of CD16V and CD3ζ or FcRIγ was assessed on human peripheral T lymphocytes. Note that in this case, because of applicability in a clinical context, FcRIγ and CD3ζ were cotransfected with CD16 (Fig. 3A), in contrast with the case of NK-92 for which the two genes were transfected sequentially. In the same way as for the NK-92, transfection of human T cells with CD16 alone was sufficient to induce CD16 expression at the cell surface through seven independent experiments (Fig. 3A, upper panel, Supplemental Table I). Notably, the level of CD16 expression by αβTCR T lymphocytes after transfection of CD16 alone was close to the level of CD16 expression by the αβTCR+CD16+ NK population (Fig. 3B). Also in the same way as for NK-92, cotransfection of CD16 with CD3ζ or FcRIγ increased CD16 expression at the cell surface (Fig. 3). In addition, CD16 expression at the cell surface was stable as shown in Fig. 3B, where the nine individual points represent the CD16 mean fluorescence tested at different time points through 1–4 wk after the stimulation (three measures were performed within the transfected PBMCs and six after purification of the CD16-transfected populations).

ADCC activity by human αβTCR T lymphocytes after transfection

T lymphocytes expressing CD16 after transfection with CD16 alone or after transfection with CD16 and CD3ζ or FcRIγ were assessed for ADCC activity in the presence of three different therapeutic mAbs (rituximab, trastuzumab, and cetuximab) coated on target cells harboring the cognate Ag: Raji (CD20), BT-474 (HER2), and LN-18 (EGFR), respectively (Fig. 4).

For Raji + rituximab, the ADCC score by the transfected T cell populations with CD16 alone was significantly superior to that observed with the T cell transfected with CD16 + CD3ζ or CD16 + FcRIγ (p = 0.02 for CD16 alone versus CD16 + CD3ζ, p = 0.02 for CD16 alone versus CD16 + FcRIγ, and p = 0.98 for CD16 + CD3ζ versus CD16 + FcRIγ). For BT-474 + trastuzumab, the reverse situation was observed: the ADCC score of T lymphocytes transfected with CD16 alone appeared inferior to that of T lymphocytes cotransfected with CD16 and CD3ζ or CD16 and FcRIγ (p = 0.04 for CD16 alone versus CD16 + CD3ζ, p = 0.05 for CD16 alone versus CD16 + FcRIγ, and p = 0.97 for CD16 + CD3ζ versus CD16 + FcRIγ) (Fig. 4). And finally, for LN-18 + cetuximab, the ADCC scores by the three transfected T cell populations were almost superimposable at all mAb concentrations tested (p = 0.28 for CD16 alone versus CD16 + CD3ζ, p = 0.93 for CD16 alone versus CD16 + FcRIγ, and p = 0.23 for CD16 + CD3ζ versus CD16 + FcRIγ). Note that ADCC scores by untransduced T cells showed some increase with increasing mAb concentrations (from 6.7 to 13.7 against Raji, 2.1 to 6.6 for BT-474, and 10.3 to 21.2 for LN-18). This corresponds most probably to a real “background ADCC” activity because of the few activated T cells that are able to express naturally some level of CD16 (12, 21).

Comparison of ADCC activity by CD16V-transfected T cells and autologous NK cells (V/V)

Finally, ADCC by T lymphocytes transfected with CD16 alone was compared with that of autologous purified NK cells (the donor being V158 homozygous). Results shown in Fig. 5 indicated that ADCC scores by NK cells or CD16-transduced T cells were different according to the opsonized target cells. In the example presented, CD16-transfected lymphocytes had superior activity compared with NK cells when tested against the rituximab-coated target EBV-LCL BK01/12 (p = 0.01). The reverse was apparently observed when they were tested against the trastuzumab-coated HER-2+ breast cancer cell line BT-474 or the cetuximab-coated LN-18 (an EGFR+ glioblastoma cell line), but the differences were
DISCUSSION

The results presented in this report demonstrated that transfection of the gene encoding CD16 can be sufficient to enable human T lymphocytes to express CD16 at the cell surface and to perform ADCC. In this way, the ADCC function can be conferred to T lymphocytes with a minimal genetic modification. In particular, there is no need to design a chimeric receptor where the ligand binding domain is covalently associated with the transducing chain.

When CD3ζ or FcγRIγ were cotransfected with CD16, CD16 expression was increased at the cell surface. This increased expression of CD16 did not necessarily translate into an increased ADCC performance. In fact, compared with T cells transfected with CD16 and a transducing chain, the T cells transfected with CD16 alone performed better against Raji + rituximab, less well against BT-474 + trastuzumab, and not differently against LN-18 + cetuximab. Thus, although intuitively one would anticipate a

FIGURE 4. ADCC by T lymphocytes after transfection.
Transfected T cells were tested against the following target cells: the CD20+ EBV-LCL (Raji), the HER2+ breast cancer cell line BT-474, and the EGFR+ glioblastoma cell line LN-18 in the presence of increasing concentrations of the anti-CD20 mAb rituximab, the anti-HER2 mAb trastuzumab, and the anti-EGFR mAb cetuximab, respectively. Results are expressed as percentage of specific lysis (E:T ratio 10:1). Symbols represent the mean ± SD of independent experiments: for CD16V alone, CD16V + CD3ζ, CD16V + FcγRIγ, and none, n = 7, 5, 5, and 2 against Raji and n = 6, 4, 4, and 2 against BT-474 and LN-18.

FIGURE 5. Comparison of ADCC by CD16V-transduced T lymphocytes and autologous NK cell.
CD16V-transfected T cells and autologous NK cells (V/V) were tested against the following target cells: the CD20+ EBV-LCL (BK01/12), the HER2+ breast cancer cell line BT-474, and the EGFR+ glioblastoma cell line LN-18 in the presence of increasing concentrations of the anti-CD20 mAb rituximab, the anti-HER2 mAb trastuzumab, and the anti-EGFR mAb cetuximab, respectively. Results are expressed as percentage of specific lysis (E:T ratio 10:1, mean of triplicate). Symbols represent the mean ± SD of two (BK01/12) and three (BT-474 and LN-18) independent experiments.
The authors have no financial conflicts of interest.

ACKNOWLEDGMENTS

We thank the Cytometry Facility Cytocell from Nantes for expert technical assistance.

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Supplemental Figure 1: CD3ζ and FcεRIγ intracytoplasmic staining for the NK-92 cell line and human T cells before and after transfection. Values underlined indicate the RFI (geometric mean for the fluorescence of the chain / geometric mean for the fluorescence of the control isotype). T lymphocytes were fixed with PFA 2%, permeabilized (permeabilization buffer, e-Biosciences, CA, USA) and stained with anti-CD3ζ-FITC (clone 6B10.2; BioLegend Californie, USA) and anti-FcεRIγ (Rabbit polyclonal, Genetex, Californie, USA) for 20 minutes at 4°C. T cells stained with anti-FcεRIγ were then stained with PE-Goat anti-Rabbit IgG (BeckmanCoulter) for 30 minutes at 4°C. After incubation, cells are washed twice and analysed on Facs AcuriC6+. 
**Supplemental Table 1**: CD16 expression and ADCC by NK-92 or T lymphocytes after transfection of CD16 alone (allotype H/V, L/V or L/F). After transfection, CD16+ T lymphocytes were FACS sorted as shown in figure 3.

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<th>Experiment n°</th>
<th>Cells transfected</th>
<th>CD16 allotype</th>
<th>% CD16+ after transfection</th>
<th>% CD16+ after selection</th>
<th>ADCC(^a) assay n°</th>
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\(^a\) ADCC: values represent percent specific lysis against the CD20+ EBV B-cell line BK01/12 for experiment n° 1, 2, 4 and 5, or RAJI for 3. Effector to target ratio = 10/1. rituximab = 1 µg/ml