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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.DCSupplemental**

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ImmunoHorizons

RESEARCH ARTICLE

Clinical and Translational Immunology

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 FcεRIγ 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 FcεRIγ (2 aa of the EC, the entire TM domain and the entire IC domain of FcεRIγ). The construct used by Kudo et al. (9) associated the EC domain of CD16 to the TM domain of CD8α and the IC domains of 4-1BB and CD3ζ. 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

Received for publication May 31, 2017. Accepted for publication June 23, 2017.

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This work was supported by the program FUI "Premium ADCC" and an institutional grant from INSERM.

Abbreviations used in this article: ADCC, Ab-dependent cellular cytotoxicity; CAR, chimeric Ag receptor; EC, extracellular domain; IC, intracytoplasmic; TM, transmembrane.

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https://doi.org/10.4049/immunohorizons.1700022

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 αβTCR T lymphocyte subsets (12). Both NK and T lymphocytes express the signaling chains CD3ζ and FcεRIγ, 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 FcεRIγ 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 FcεRIγ (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 FcεRIγ.

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 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 FCGR3A-158V genotype. Genotyping of the FCGR3A-158V/F polymorphism was kindly documented by Dr. V. Gouilleux-Gruart (UMR CNRS 6239, Tours, France) as previously described (17). The CD16L48V158 cDNA was amplified using the forward primer 5′-GGTGGATCCACCATGTGGCAGCTGCTCCTCCCA-3′ and the reverse primer 5′-GAGGAATTCTCATTTGTCTTGAGGGTCCTT-3′. The primers contain BamHI and EcoRI sites for the insertion of the PCR product to the pPB-UbC plasmid between the Ubc promoter and the bovine growth hormone polyadenylation signal. The human CD3ζ and FcεRIγ coding sequences were amplified from pCI-neo-CD3ζ and pCI-neo-FcεRIγ (kindly provided by R. Breathnach, INSERM U1232, Nantes, France). The CD3ζ was amplified using the forward primer 5′-GGTTGATCACCATG-AAGTGGAAGGCGCTT-3′ and the reverse primer 5′-GAGGAA-TTCTTAGCGAGGGGGCAGGGC-3′. The primers contain BclI and EcoRI sites for the insertion of the PCR product to the pPB-UbC plasmid. The FcεRIγ was amplified using the forward primer 5′-GGTGGATCCACCATGATTCCAGCAGTGGTC-3′ and the reverse primer 5′-GAGGAATTCCTACTGTGGTGGTTTCTC-3′. The primers contain BamHI and EcoRI sites for the insertion of the PCR product to the pPB-UbC plasmid.

All plasmids constructs were confirmed by 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 leukoagglutinin (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 (2.5×10^6 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

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(Lonza, 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 (2.5×10^6 cells) 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 (2.5×10^6) were mixed with 2 μg of pCMV-hyPBase and 10 μg of pPB-UbC-hCD16 ± 2 μg of pPB-UbC-CD3Z or FcεRIγ. PBMCs were electroporated with Human T cell nucleofector kit VPA-1002 (Lonza) 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 ~5×10^4 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, 0.2×10^6 cells were incubated for 15 min at room temperature in the dark with mAbs in PBS 0.1% human albumin in a final volume of 30 μl. After staining, cells were washed twice with PBS 0.1% human albumin and analyzed with a FACSCalibur instrument and CellQuest software (BD Biosciences). For FACSAria III cell sorting, cells were washed with cold PBS 0.1% human albumin and incubated with mAbs for 1 h at 4°C. After two washes with cold PBS 0.1% human albumin, cells were resuspended in cold PBS-EDTA 2% FCS, 70 μm filtered, and kept on ice until sorting.

ADCC assay

Cytotoxic activity was assessed using a standard ^{51}Cr release assay. Target cells were labeled with 75 μCi (2.77 MBq) ^{51}Cr (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 a 4-h incubation at 37°C, 25 μl of supernatant was removed from each well, mixed with 100 μl scintillation fluid, and ^{51}Cr 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

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

The gene coding CD16 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, CD16 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 CD16 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 CD16 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).

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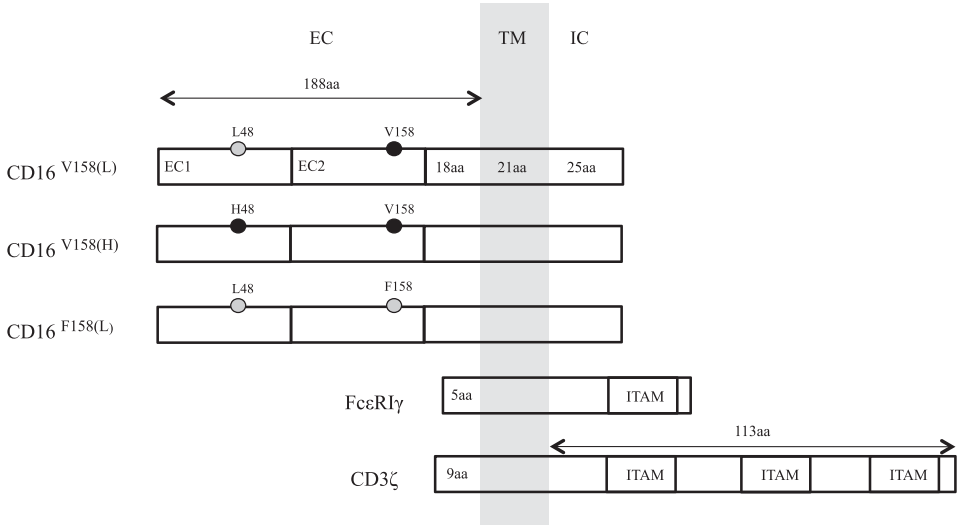


FIGURE 1. Schematic representation of the different chains transfected.
The CD16L48V158 coding sequence was obtained from the PBMC of a healthy donor. The CD16L48V158 and CD16H48V158 sequences were kindly provided by M. Ohresser. No influence of these CD16 polymorphisms was observed on the CD16 expression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

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.

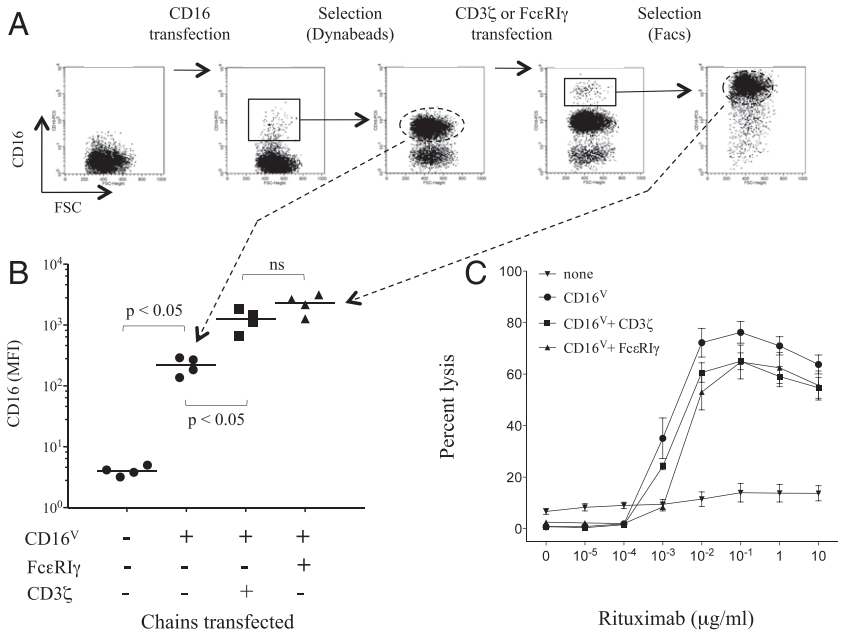


FIGURE 2. CD16 expression and function after transfection of NK-92 with CD16 alone or together with a transducing chain.
(**A**) Transfection/selection procedure for the NK-92 cell line. (**B**) CD16 expression by selected NK-92 after transfection with the indicated chains. For each NK-92 cell line, 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 ^{51}Cr 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.

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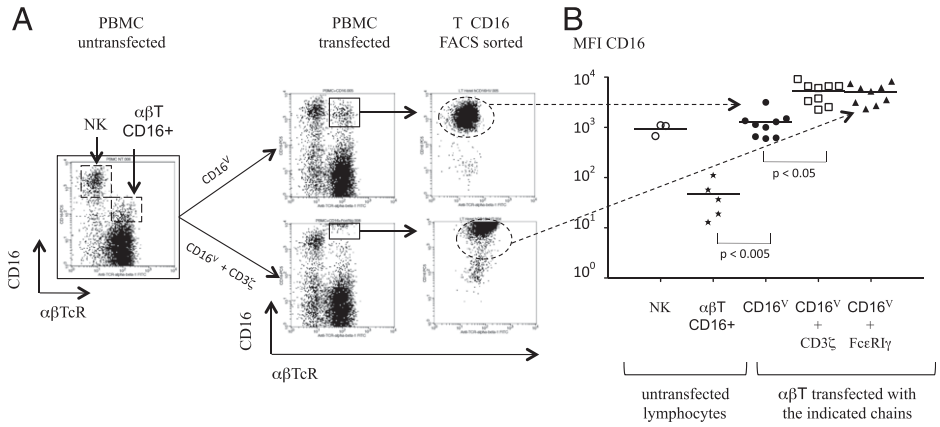


FIGURE 3. Transfection/selection procedure for T cells and CD16 expression after transfection.

(**A**) The windows in the dot plots spot the different lymphocytes subsets in which CD16 fluorescence is shown in (**B**). For the increased expression of CD3ζ and FcεRIγ after transfection, see Supplemental Fig. 1. (B) For each lymphocyte population, symbols represent CD16 mean fluorescence tested between days 20 and 41 after transfection. For the transfected population, three measures were performed within the transfected total PBMCs and six after purification of the CD16 subset.

CD16 expression by T lymphocytes after transfection of CD16V alone, CD16V and CD3ζ, or CD16V and FcεRIγ

Next, CD16 expression after transfection of CD16 alone or after transfection of CD16V and CD3ζ or FcεRIγ was assessed on human peripheral T lymphocytes. Note that in this case, because of applicability in a clinical context, FcεRIγ 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 $\alpha\beta TCR^{-}$ $CD16^{+}$ NK population (Fig. 3B). Also in the same way as for NK-92, cotransfection of CD16 with CD3ζ or FcεRIγ 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 FcεRIγ 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 + FcεRIγ ($p = 0.02$ for CD16 alone versus CD16 + CD3ζ, $p = 0.02$ for CD16 alone versus CD16 + FcεRIγ, and $p = 0.98$ for CD16 + CD3ζ versus CD16 + FcεRIγ). 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 FcεRIγ ($p = 0.04$ for CD16 alone versus CD16 + CD3ζ, $p = 0.05$ for CD16 alone versus CD16 + FcεRIγ, and $p = 0.97$ for CD16 + CD3ζ versus CD16 + FcεRIγ) (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 + FcεRIγ, and $p = 0.23$ for CD16 + CD3ζ versus CD16 + FcεRIγ). 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 $HER\text{-}2^{+}$ breast cancer cell line BT-474 or the cetuximab-coated LN-18 (an $EGFR^{+}$ glioblastoma cell line), but the differences were

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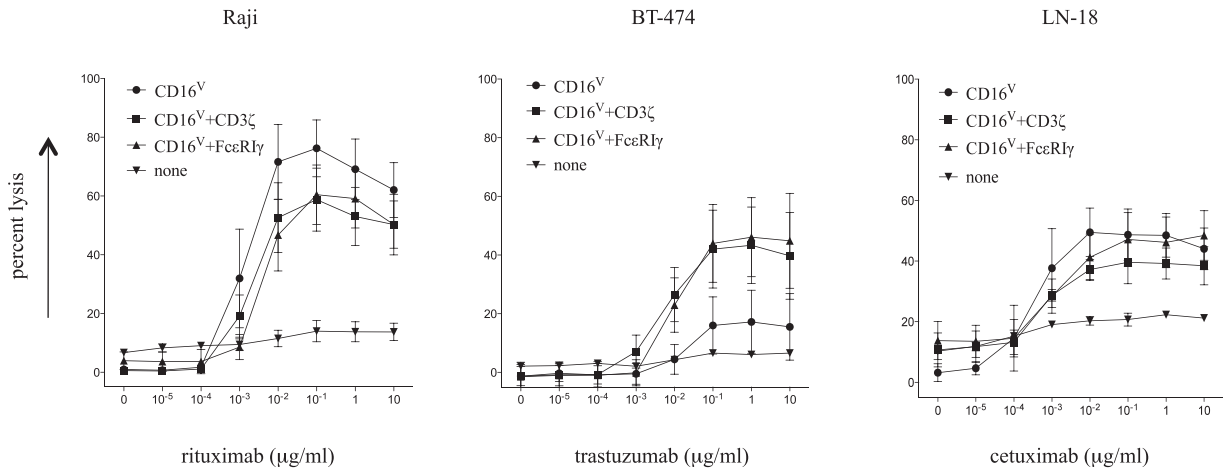


FIGURE 4. ADCC by T lymphocytes after transfection.

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 + FcεRIγ, and none, n = 7, 5, 5, and 2 against Raji and n = 6, 4, 4, and 2 against BT-474 and LN-18.

not statistically significative (p = 0.09 and 0.13 for BT-474 and LN-18, respectively).

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 FcεRIγ 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

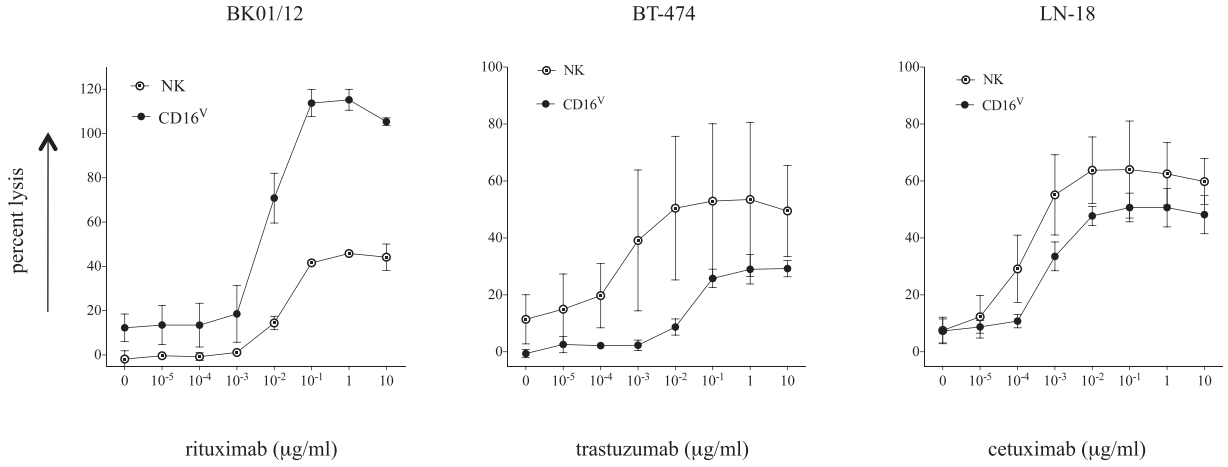


FIGURE 5. Comparison of ADCC by CD16V-transduced T lymphocytes and autologous NK cell.

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.

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ranking of ADCC performance according to the availability of the transducing chains (that is, CD16 alone < CD16 + CD3ζ or + FcεRIγ), this is not necessarily the case.

The number of elements of complexity that govern effector/target interactions in the case of ADCC are numerous: the Ag number on the surface of the target cell, the Fab affinity for this Ag, the Fc affinity for the FcR, the Ab concentration, the FcR number, nonspecific interactions involved in cellular contacts such as ICAM-1/LFA1, CD2/LFA3, and so on.

The effector/Ab/target interaction, which leads to the ADCC mechanism, is also more complex than that which leads to CTL activity after MHC-restricted T cell recognition. This additional complexity is due to the nature of the extracellular interactions. Indeed, in MHC-restricted T cell recognition, the fixed dimensions of the TCR and MHC molecules determine the spatial interactions of T cell and target cell, whereas in the case of ADCC, the effector/Ab/target interaction may be influenced also drastically by the location of the epitope that is recognized. For ADCC to occur, surface-bound Ab must be spatially oriented and organized in such a way as to allow for favorable Fc-FcR contact without steric hindrance from neighboring cell surface molecules, a concept of favorable Ab orientation and favorable organization put forward already 20 y ago by Christiaansen et al. (22). Notably, these different levels of complexity are also valid for the comparison between recognition by the TCR or by a CAR. Obviously, mechanistic explanation for the different behavior of each effector/Ab/target interaction would require specific studies because each combination appears singular and its outcome in terms of ADCC is conditioned by many other variables than the number of transducing chains available.

Whatever the signification of the quantitative difference observed, the data presented demonstrated that transfection of the unmodified CD16 chain alone into T lymphocytes allowed CD16 cell surface expression to a level sufficient for the T cell to express its lytic potential, even though it was not maximal in the case of trastuzumab. Thus, whatever the particular complex relationships between the effector, the Ab, and the target, these results showed that CD16 expression after it has been transfected alone may be sufficient to achieve ADCC, depending on the Ag targeted.

For the purpose of improving a patient's ADCC potential, we have previously considered the possibility of arming T cells with a receptor that would enable them to mediate ADCC (8). To this end, we first built on the previous literature and relied on the transfection of a CD16/FcεRIγ fusion gene whose expression has been shown to elicit an intracellular response after transfection into the Jurkat cell line (23). Once we had demonstrated that human $CD4^+$ and $CD8^+$ T lymphocytes displayed stable expression of the CD16 recombinant receptor at their surface and became able to mediate ADCC, we initiated a series of assays to optimize the system. At the same time, we described a population of $CD16^+$ memory αβTCR T lymphocytes, present in healthy individuals and increased during lymphocytosis, which happen to be able to perform ADCC at a level comparable with that of the autologous NK cells (12). Yet, despite some attempts to manipulate this particular T cell subset in the perspective of adoptive therapy, we have failed so far to find a reproducible strategy to obtain sufficient numbers of $CD16^+$ T cells to consider clinical applications with a reasonable level of feasibility. This was mainly due to the highly variable number of $CD16^+$ αβTCR T cells from one donor to another, and also to the transient nature of CD16 expression after in vitro activation (21). The earlier-mentioned observation that CD16 expression and ADCC function were natural for some subsets of human αβTCR T cells led us to consider the possibility that a minimal modification (transfection of CD16 alone in the present case) would enable αβTCR T cells to perform ADCC. The data presented demonstrated that this is an option.

In the context of genetically engineered T cells, innumerous solutions for T cell modifications can be considered today. For example, a CAR encompasses an ectodomain (most commonly an ScFv), a hinge region (the connecting region between the ectodomain and the TM domain, with different length and flexibility), the TM domain, and the endodomain that transmit activation and costimulatory signal to the T cell. All these regions can potentially affect the behavior of the modified T cells. In addition, as stated earlier, because the localization at the cell surface of an Ag recognized by an Ab can be highly variable compared with that of an MHC complex, ADCC and CAR recognition are expected to be much more affected by the position of the Ag than TCR recognition. Because of these multiple elements of complexity, notwithstanding the technology used for gene transfer and the clinical context in which the modified T cells are intended to be used, there are no general rules today to design the most appropriate construct for a particular application.

On a mechanistic point of view one can state that the $CD16^+$ T cell populations presented in this article are closer to nature than T cells that have been modified using a fusion protein. How such CD16-expressing T cells would compare with other $CD16^+$-modified T cells in terms of usefulness and safety could be assessed only for a particular target Ag, in a particular clinical context, and finally could only be established in clinical trials. Data presented in this study, which suggest this possibility, represent the first step toward this objective.

DISCLOSURES

The authors have no financial conflicts of interest.

ACKNOWLEDGMENTS

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

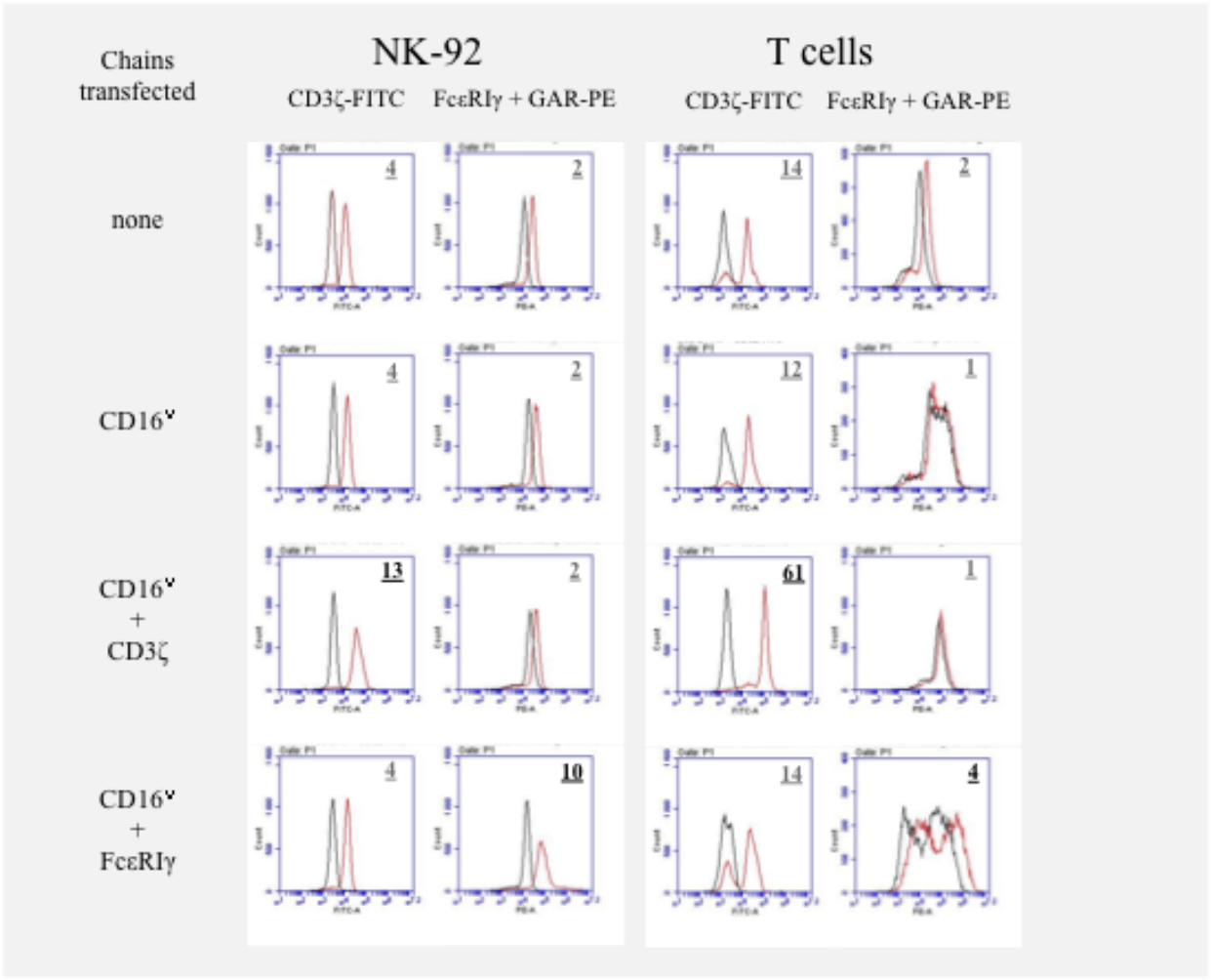
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Supplemental Figure 1 : CD3ζ and FcεRIγ intracytoplasmic staining for the NK-92 cell line and human T cells before and after transfection. Values underlined indicate the RFIs (geometric mean for the fluorescence of the chain / geometric mean for the 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.

Experiment n°	Cells transfected	CD16 allotype 48/158	% CD16+ after		ADCC[a] assay n°		
			transfection	selection	1	2	3
1	NK-92	H/V	2	89	42	62	45
		L/V	2	96	68	69	86
		L/F	2	78	56	68	54
2	NK-92	H/V	7	99	82	97	94
		L/V	6	99	86	107	91
		L/F	11	99	86	99	87
3	PBMC	H/V	-	99	56	70	76
4	PBMC	H/V	-	99	65	60	-
		L/V	-	99	81	73	-
		L/F	-	98	69	46	-
			-				
5	PBMC	H/V	-	99	56	62	84
		L/V	-	99	81	79	99
		L/F	-	87	51	61	73

[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