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Flexible and sensitive method to functionally validate tumor-specific receptors via activation of NFAT

Niels Schaft, Birgit Lankiewicz, Jan Willem Gratama, Reinder L.H. Bolhuis, Reno Debets*

Department of Medical Oncology, Laboratory of Medical and Tumor Immunology, Erasmus MC-Daniel den Hoed, Groene Hilledijk 301, 3075 EA Rotterdam, The Netherlands

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Abstract

Tumor-specific receptors may provide effective tools for anti-tumor immunogene therapy. However, the functional analysis of primary human T cells engrafted with tumor-specific receptors is laborious and emphasizes the need for a fast and sensitive method to validate such receptors. To this end, we have set up a Jurkat T cell-based reporter gene assay, and tested receptors with various formats, i.e., receptors based on either a monoclonal antibody (mAb), a full-length T cell receptor (fl-TCR)αβ or a chimeric (ch-)TCRαβ, and various antigen specificities for their ability to mediate tumor-specific activation of nuclear factor of activated T cells (NFAT). The mAb-based receptor specifically mediates NFAT activation after stimulation with tumor antigen-positive target cells. The observed receptor-mediated NFAT responses were validated by the use of ligand- and receptor-specific mAbs, as well as cyclosporin A (CsA) and a dominant negative mutant of NFAT. Furthermore, anti-TCR mAbs, peptide-loaded tumor cells and antigen-positive tumor cells all resulted in specific NFAT activation in TCR/CD8 co-transduced Jurkat T cells, irrespective of the TCR format used. Importantly, receptor-mediated NFAT responses parallel tumor-specific cytolysis and TNFα production of receptor-transduced primary human T lymphocytes. In fact, inhibition of NFAT activation compromises the immune responses of primary human T lymphocytes, pointing to a central involvement of NFAT in anti-tumor T cell responses. Taken together, receptor-mediated activation of NFAT constitutes a representative measure of anti-tumor T cell responses, and the genetically modified Jurkat T cells provide a flexible and sensitive tool with which to select rapidly tumor-specific (chimeric) receptors for immunogene therapy.

Keywords: NFAT; Reporter gene assay; T cell retargeting; Tumor-specific receptors

1. Introduction

Transfer of genes encoding tumor-specific receptors into human T lymphocytes is a promising strategy to induce antigen-specific immunity. Tumor-specific receptors used to functionally redirect human T cells exist in various single- or two-chain receptor formats, including mAb, full-length TCRαβ, as well as chimeric

Abbreviations: BCS, bovine calf serum; CsA, cyclosporin A; dnNFATmut, dominant negative NFAT mutant; fl, full length; GaM, goat-anti-mouse; mAb, monoclonal antibody; MFI, mean fluorescence intensity; mIg, mouse gamma globulin; NFAT, nuclear factor of activated T cells; PMA, Phorbol 12-myristate 13-acetate; RCC, renal cell carcinoma; RLU, relative light units; sc, single chain; tc, two chain; TCR, T cell receptor; wt, wild type.

* Corresponding author. Tel.: +31-10-4391718; fax: +31-10-4391005.

E-mail address: j.debets@erasmusmc.nl (R. Debets).

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TCRαβ-based receptors (Eshhar et al., 1993; Weijtens et al., 1998; Clay et al., 1999; Cooper et al., 2000; Willemsen et al., 2000; Kessels et al., 2001). T lymphocytes equipped with tumor-specific receptors, with or without an MHC-restriction element, are not only able to recognize the relevant antigen, but also produce cytokines and kill tumor target cells upon antigenic stimulation both in vitro and in vivo (Eshhar et al., 1993; Hwu et al., 1995; Weijtens et al., 1998; Clay et al., 1999; McGuinness et al., 1999; Cooper et al., 2000; Willemsen et al., 2000; Kessels et al., 2001). Individual receptor components, such as the transmembrane and intracellular domains, greatly affect expression and/or function of these receptors in human T lymphocytes (Eshhar et al., 1993; Patel et al., 1999). The design and validation of mAb- or TCR-based receptors that permit optimal antigen-specific T cell activation are, therefore, of critical importance to the clinical implementation of receptor genes in the treatment of cancers. To date, the validation of tumor-specific receptors is labor-intensive and time-consuming since primary human T lymphocytes have to be transduced, enriched for chimeric receptor expression and subsequently tested for antigen-specific function in vitro, with anti-tumor cell cytotoxicity and cytokine production being the accepted read outs.

In this paper, we studied whether receptor-mediated activation of NFAT would constitute an easy-to-use and reliable alternative to validate such receptor genes. Reporter genes under the control of NFAT are considered valid tools to measure T cell activation (Fiering et al., 1990; Karttunen and Shastri, 1991; Sanderson and Shastri, 1994) with the human Jurkat T cell line often used as a model cell line to study antigen-specific responses mediated by introduced full-length TCRαβ chains (Cole et al., 1995; Calogero et al., 2000; Aarnoudse et al., 2002). Nevertheless, TCR-mediated activation of NFAT in CD4-positive Jurkat T cells equipped with exogenous TCRαβ chains and, in some cases, the co-receptor CD8α could only be detected in response to peptide-loaded target cells but not tumor-antigen positive target cells (Cole et al., 1995; Calogero et al., 2000; Aarnoudse et al., 2002). Here, we introduce a flexible and sensitive method that detects receptor-mediated activation of NFAT, irrespective of whether a mAb-based receptor, full-length TCRαβ chains or TCRαβ chains fused to CD3ζ are used in response to native tumor target cells. The NFAT reporter gene assay described relies on Jurkat E6.1 T cells retrovirally transduced with tumor-specific receptors and, in the case of TCR-based receptors, CD8α co-receptor, and allows for co-stimulation which is especially relevant for TCR-mediated NFAT activation. The specificity of the receptor-mediated responses was confirmed by blocking the activation of NFAT via ligand- or receptor-specific antibodies or specific inhibitors of NFAT activation, such as CsA and a dominant negative NFAT mutant (Chow et al., 1999). It is important to note that NFAT responses in Jurkat T cells clearly reflect the tumor-specific cytolysis and TNFα production by primary human T cells transduced with the identical receptor genes. In fact, blocking experiments with CsA show that anti-tumor responses of primary human T cells depend on NFAT activation.

Thus, receptor-mediated activation of NFAT constitutes a representative measure of anti-tumor T cell responses, with the Jurkat T cell-based NFAT reporter gene assay allowing for rapid testing and selection of tumor-specific (chimeric) receptors for immunogene therapy of cancers and viral infections.

2. Materials and methods

2.1. Cells and reagents

The Jurkat T cell clone E6.1, the G250^{neg} renal cell carcinoma (RCC)-derived SKRC-17 clone 1 and the G250 cDNA-transfected SKRC-17 clone 4 (both kindly provided by E. Oosterwijk, Nijmegen, The Netherlands) were cultured with RPMI 1640 medium supplemented with 200 nM L-glutamine, 10% bovine calf serum (BCS, Hyclone, Logan, UT) and the antibiotics streptomycin (100 μg/ml) and penicillin (100 U/ml). The human amphotropic packaging cell line Phoenix, the endogenously G250^{pos} RCC-derived A75 cell line (generated in our laboratory by Weijtens et al., 1996), the gp100^{pos}/HLA-A2^{pos} melanoma cell line FM3 (kind gift from G. Adema, Nijmegen, the Netherlands) and the TAP-deficient HLA-A2^{pos} TxB hybrid T2 cells were grown in DMEM (Gibco BRL, Paisley, Scotland, UK) supplemented with 10% BCS and antibiotics. The gp100^{neg}/HLA-A2^{pos} melanoma cell line BLM and BLM cells transfected with human gp100-encoding cDNA (BLMgp100) were cultured as described previously (Bakker et al., 1994). Expression

of the G250 or gp100 tumor antigens on these cell lines was verified by flow cytometry. The mAbs used in this study were: G250 mAb; anti-idiotypic mAb NUH-82 (both from E. Oosterwijk, Nijmegen, The Netherlands); PE-conjugated goat-anti-mouse (GaM^{PE}) Ig mAb (ITK, Uithoorn, The Netherlands); anti-TCRVβ mAb; anti-TCRVβ14 mAb; PE-conjugated anti-TCRVβ14 mAb (TCR mAbs all from Beckman Coulter, Marseille, France); FITC-conjugated anti-CD8α mAb (Becton Dickinson Biosciences, San Jose, CA); mouse gamma globulin (mIg; Jackson Immuno Research Laboratories, West Grove, PA); and anti-CD28 mAb (clone 15E8; CLB, Amsterdam, The Netherlands). Other reagents used in this study were: RetroNectin (human fibronectin fragments CH-296; Takara Shuzo, Otsu, Japan); PMA (Phorbol 12-myristate 13-acetate; Sigma, Zwijndrecht, The Netherlands); ionomycin (Calbiochem, La Jolla, CA); Cyclosporin A (CsA; Sandoz Pharmaceuticals, NJ); INFγ; IL-1β (both from PeproTech, NJ); gp100 wild-type (wt) peptide (YLEPGPVTA); irrelevant HLA-A2-binding EBV peptide (GLCTLVAML); and a dominant negative mutant of NFAT (dnNFATmut; kindly provided by R.J. Davis, Worcester, MA (Chow et al., 1999).

2.2. Construction of chimeric receptor genes

The chimeric antibody-based single-chain (sc) FvG250:γ receptor was constructed as described elsewhere (Weijtens et al., 1998). Briefly, the scFvG250:γ receptor comprises the variable domains of the human Ig heavy chain and κ light chain of the G250 mAb connected by a flexible linker. The antigen-binding part is coupled to a few amino acids of the constant domain of the κ light chain, the transmembrane domain of the human CD4 molecule and the signaling domain of Fc(ε)RIγ (i.e., scFvG250:γ). The chimeric antibody-based scFvG250:γ receptor was subsequently cloned into the retroviral vector pSTITCH (Weijtens et al., 1998). The full-length (fl-)296 TCRαβ consisted of the complete TCR α and β chain, whereas the chimeric two-chain (tc-)296 TCRαβ:ζ consisted of the extracellular domains of the TCR α and β chain, each linked to the human CD3ζ molecule (Willemsen et al., 2000). TCR α and β DNAs were obtained by PCR using cDNA of the CTL 296 clone (described by Zarour et al., 1996) as template DNA. Specific primer sequences to amplify the TCR domains will be provided upon request. The TCR genes were subsequently cloned into the retroviral vector pBullet containing a heterologous signal peptide (Willemsen et al., 2000).

2.3. Retroviral gene transduction of (chimeric) receptors into Jurkat T cells

Jurkat T cells were retrovirally transduced according to a protocol optimized for primary human T lymphocytes as described by Lamers et al. (2002). In short, 24-well culture plates were coated with RetroNectin and pretreated with receptor-positive retroviral particles, derived from receptor-positive Phoenix cells. Next, 10^6 Jurkat T cells per well were centrifuged in fresh retrovirus containing supernatant and cultured for 4–5 h at 37 °C/5% CO_2. Cells were allowed to recover in culture medium overnight prior to a second transduction cycle, after which cells were harvested and transferred to T25 culture flasks. After sufficient numbers were obtained, cells were analyzed for receptor expression by flow cytometry and used in the NFAT reporter gene assay. The fl-296 TCRαβ and tc-296 TCRαβ:ζ genes were introduced into CD8-transduced Jurkat T cells.

2.4. Cytofluorometric analysis of retrovirally transduced Jurkat T cells

Transduced Jurkat T cells were analyzed for transgene expression by flow cytometry using either the anti-idiotypic NUH-82 mAb for the scFvG250:γ receptor or PE-conjugated anti-TCRVβ14 mAb for the fl-296 TCRαβ and tc-296 TCRαβ:ζ. FITC-conjugated anti-CD8α mAb was used to analyze expression of the CD8α molecule. For immunostaining, $0.25–0.5 \times 10^6$ transduced Jurkat T cells were incubated with the primary mAbs on ice for 30 min (in the case of the NUH-82 mAb, this was followed by a wash step and a second incubation with GaM^{PE}), washed, fixed with 1% PFA and analyzed on a flowcytometer (Becton Dickinson). Jurkat T cells were subsequently enriched for receptor-positive cells via the primary mAbs mentioned and GaM-coated magnetic beads (Dynal, Oslo, Norway) or anti-PE MACS MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturers' instructions.

2.5. NFAT reporter gene assay

For reporter gene assays, conditions for transfection, lysis and measurement have been optimized, resulting in the following procedure (in part described by Debets et al., 2000): exponentially growing receptor-transduced Jurkat T cells ($4-5 \times 10^6$) were transfected by electroporation (300 V, 1920 μF, 129 Ω) with 5 μg of both the NFAT–luciferase construct (containing four NFAT binding sites; Stratagene, La Jolla, CA) and β-galactosidase construct. Twenty hours posttransfection, Jurkat T cells were transferred to round-bottom 96-well plates (Costar, Corning, NY) at 2×10^5 cells/well and were stimulated for 6 h with mAbs or target cells (at 10^5 cells/well) in RPMI 1640 medium supplemented with 1% BCS at 37 °C/5% CO_2. The scFvG250:γ^{pos} Jurkat T cells were stimulated with the following RCC-derived tumor target cells: SKRC-17 clone 1, SKRC-17 clone 4, and the A75 cell line. The RCC specificity of NFAT activation in scFvG250:γ^{pos} Jurkat T cells was tested via blocking experiments with the G250 mAb (at 25 μg/ml final) or the NUH82 mAb (at 40 μg/ml final) added at the start of co-cultivation. The fl-296 TCRαβ^{pos} and tc-296 TCRαβ:ζ^{pos} Jurkat T cells were stimulated with anti-TCR mAbs, peptide-loaded T2 target cells, and the melanoma cell lines FM3, BLM and BLMgp100. For mAb stimulations, TCR-positive Jurkat T cells were added to wells precoated with anti-TCRVβ8 mAb, anti-TCRVβ14 mAb or control mIg (at 0.1 μg/well final). T2 cells were peptide-loaded for 30 min at 37 °C/5% CO_2 with 1 μM of the gp100 wt peptide or irrelevant HLA-A2-binding EBV peptide prior to their use in NFAT reporter gene assays. FM3, BLM and BLMgp 100 cells were preincubated O/N with INFγ (10 ng/ml) and IL-1β (30 ng/ml), and co-cultivation of these melanoma cells with TCR-transduced Jurkat T cells was performed in the presence of anti-CD28 mAb (2 μg/ml). Stimulations with PMA (10 ng/ml) and ionomycin (1 μM) served as positive controls. For NFAT inhibition experiments, CsA (at 100 nM final) was added to the Jurkat T cells 30 min prior to the 6 h co-culture experiments. In some experiments, a dominant negative mutant of NFAT (10 μg) was co-electroporated with the NFAT reporter construct and the β-galactosidase construct into Jurkat T cells prior to stimulation experiments. Amounts of DNA were kept constant by adding pcDNA3.1 (backbone vector of dnNFATmut). After stimulation, cells were collected, lysed with cell lysis buffer (Promega, Madison, WI), and luciferase and β-galactosidase activities were assessed using chemiluminescent substrates according to the manufacturer's instructions (Mediators, Vienna, Austria). Samples were analyzed in a 96 well plate luminometer (Mediators), and luciferase activities were normalized on the basis of β-galactosidase activities and expressed (in RLU) relative to nonstimulated conditions (medium only: set to 1.0).

2.6. Cytotoxicity assay

Primary human T cells expressing the same receptors as tested in the NFAT reporter gene assay were assayed in a standard 6 h ^{51}Cr-release assay (Lamers et al., 1991) using the target cells described above. To this end, primary human T cells were retrovirally transduced with the scFvG250:γ, fl-296 TCRαβ or tc-296 TCRαβ:ζ receptors resulting in receptor expression levels of over 45%. The significance of NFAT activation to receptor-mediated cytotoxicity was also directly assayed by adding CsA (100 nM final concentration, or as indicated) to the T lymphocyte effector cells 30 min prior to cytotoxicity assays.

2.7. TNFα production

Receptor-positive primary human T lymphocytes were also tested for antigen-specific TNFα production as described (Willemsen et al., 2000). To inhibit NFAT activation, CsA was added to the T lymphocyte effector cells as described above. Supernatants were harvested and levels of TNFα were measured by standard ELISA (CLB, Amsterdam, the Netherlands) according to the manufacturer's instructions.

3. Results

3.1. Expression of (chimeric) mAb- and TCR-based receptors on transduced Jurkat T cells

In order to employ the Jurkat T cell line E6.1 as a tool to functionally analyze tumor-specific receptors, we retrovirally transduced these cells with a chimeric mAb-based single-chain receptor specific for the renal cell carcinoma antigen G250 (scFvG250:γ) (Weijtens

et al., 1998), a full-length (fl-) 296 TCRαβ and a two-chain 296 TCR chimerized to human CD3ζ (tc-296 TCRαβ:ζ), both specific for the HLA-A2-presented gp100 melanoma antigen. Cell surface expression of the mAb- and TCR-based receptors was determined by flow cytometry using anti-G250 idiotypic and anti-TCRVβ14 mAbs, respectively. Transduction of Jurkat T cells, followed by selection for receptor-positive cells, resulted in expression levels of 92% (mean fluorescence intensity (MFI) = 432), 93% (MFI = 204) and 82% (MFI = 223) for the scFvG250:γ receptor, the fl-296 TCRαβ and the tc-296 TCRαβ:ζ, respectively (Fig. 1). Jurkat T cells used for the transduction of TCR-based receptors were co-transduced

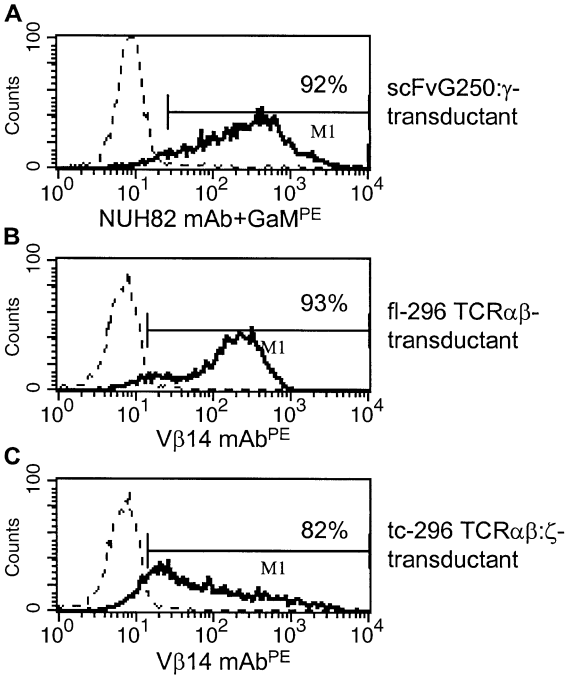

Fig. 1. Cell surface expression of mAb- and TCR-based receptors on Jurkat T cells. Jurkat T cells were transduced with the scFvG250:γ receptor, fl-296 TCRαβ or tc-296 TCRαβ:ζ, and subsequently enriched for receptor-positive cells. Receptor expression was analyzed by flow cytometry using the anti-idiotypic NUH82 mAb followed by GaM^{PE} mAb (for scFvG250:γ transductants: solid line in A), or with the PE-labeled anti-TCRVβ14 mAb (for fl-296 TCRαβ and tc-296 TCRαβ:ζ transductants: solid lines in B and C, respectively). Nontransduced Jurkat T cells were used as a negative control (A, B, C; dotted line). Marker M1 (< 5% positive staining) was set in the histogram of nontransduced Jurkat T cells, and the percentage of positively stained receptor-transductants relative to M1 is indicated.

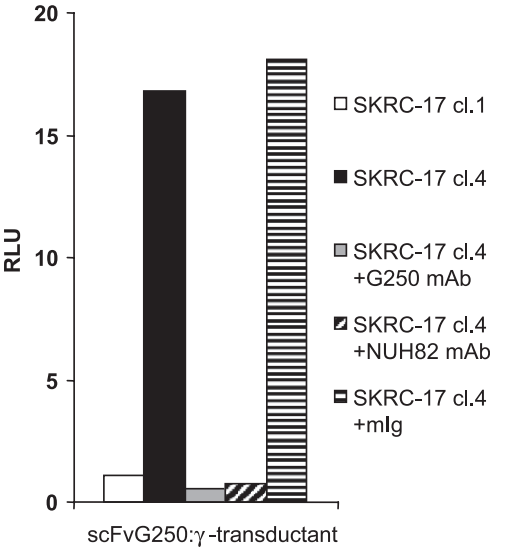

Fig. 2. The scFvG250:γ receptor specifically mediates activation of NFAT in response to G250^{pos} target cells. Jurkat T cells expressing the scFvG250:γ receptor were transfected with 5 μg of both the NFAT reporter and β-galactosidase constructs, and stimulated for 6 h with the following target cells: the G250^{neg} SKRC-17 clone 1 (white bar) and the G250^{pos} SKRC-17 clone 4, the latter target in the absence (black bar) or presence of G250 mAb (grey bar; 25 μg/ml), NUH82 mAb (diagonally striped bar; 40 μg/ml) or control mouse gamma globulin (mIg; horizontally striped bar; 40 μg/ml). Luciferase activities were determined in cell lysates, normalized for β-galactosidase activities, and expressed relative to a non-stimulated condition (i.e., medium only: RLU = 0.04 which is set to 1.0). Stimulation with PMA (10 ng/ml) and ionomycin (1 μM) was used as a positive control. Nontransduced Jurkat T cells did not respond to stimulation with any target cell (data not shown). Results of one (out of two) representative experiment are shown.

with the human CD8α gene (expression level 100%; MFI = 590), and TCR/CD8α-grafted Jurkat T cells specifically bound gp100/HLA-A2 tetramer complexes, whereas the binding of an irrelevant EBV/HLA-A2 complex was negligible (data not shown).

3.2. The scFvG250:γ receptor induces antigen-specific activation of NFAT in Jurkat T cells

Single-chain FvG250:γ-mediated activation of NFAT was analyzed by electroporation of the scFvG250:γ^{pos} Jurkat T cells with an NFAT–luciferase reporter construct, after which these cells were cocultivated for 6 h with G250^{neg} and G250^{pos} renal cell carcinoma cell lines, derived from carcinoma

biopsies. Antigen-induced activation of NFAT was seen when scFvG250:γ^{pos} Jurkat T cells were stimulated with the G250^{pos} cell line SKRC-17 cl.4 but not when stimulated with the G250^{neg} cell line SKRC-17 cl.1 (Fig. 2). Antigen-induced NFAT activation was also seen after stimulation with the G250^{pos} cell line A75 (data not shown). The antigen-specific activation of NFAT by scFvG250:γ^{pos} Jurkat T cells in response to the G250^{pos} cell line SKRC-17 cl.4 was completely inhibited by adding either G250 mAb or NUH-82 mAb, but not control mIg (Fig. 2). Nontransduced Jurkat T cells did not show any NFAT activation upon stimulation with target cells.

3.3. The fl-296 TCRαβ and tc-296 TCRαβ:ζ mediate activation of NFAT in response to native gp100 antigen

Receptor-mediated activation of NFAT was analyzed in the fl-296 TCRαβ^{pos}/CD8^{pos} and tc-296 TCRαβ:ζ^{pos}/CD8^{pos} Jurkat T cells after stimulation with anti-TCRVβ14 mAb as well as gp100^{pos}/HLA-A2^{pos} melanoma target cells. When stimulated with anti-TCRVβ14 mAb, activation of NFAT was seen in TCR-transduced Jurkat T cells, whereas control mIg was not able to induce NFAT activation in these cells (Fig. 3A). As a positive control, Jurkat T cells were stimulated with anti-TCRVβ8 mAb, which is specific for the endogenous TCRβ expressed on Jurkat T cells (Fig. 3A). As expected, nontransduced Jurkat T cells only showed NFAT activation upon stimulation with anti-TCRVβ8 mAb, confirming their ability to mediate activation of NFAT upon TCR triggering, but not with anti-TCRVβ14 mAb. When the TCR-transduced Jurkat T cells were co-cultured with T2 cells that were loaded with gp100 wild-type (wt) peptide, we observed an activation of NFAT, whereas T2 cells loaded with an irrelevant HLA-A2-binding peptide did not induce NFAT activation in these cells (Fig. 3B). More importantly, a clear activation of NFAT was

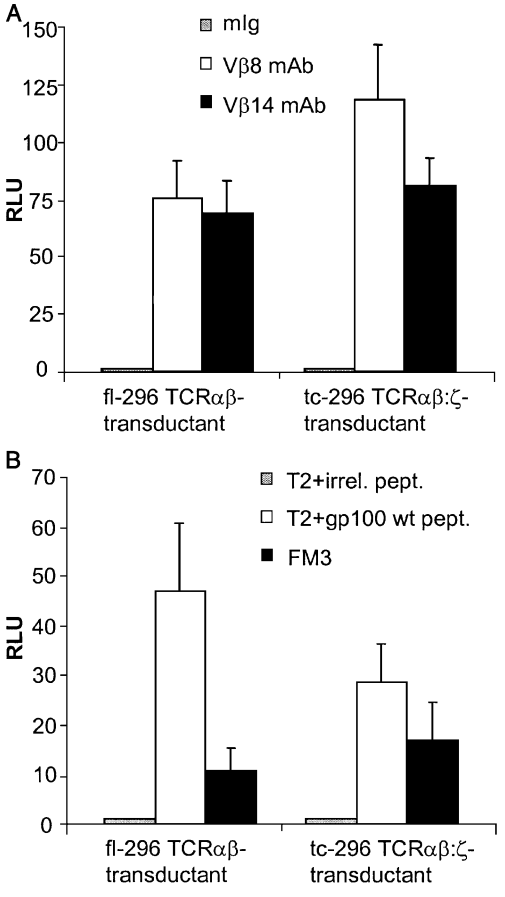

Fig. 3. The fl-296 TCRαβ and tc-296 TCRαβ:ζ mediate NFAT activation in response to antigen-positive melanoma cells. (A) Anti-TCR mAb induces receptor-mediated activation of NFAT in TCR-transduced Jurkat T cells. Jurkat T cells transduced with the fl-296 TCRαβ and the tc-296 TCRαβ:ζ were transfected with 5 μg of both NFAT reporter and β-galactosidase constructs, and subsequently stimulated for 6 h with anti-TCRVβ14 mAb (black bar), anti-TCRVβ8 mAb (open bar) or control mIg (grey bar) (0.1 μg/well final). Luciferase activities were determined, normalized for β-galactosidase activities and expressed relative to the control mIg response (fl-296 TCRαβ: RLU = 0.08; and tc-296 TCRαβ:ζ: RLU = 0.07, which are both set to 1.0). Nontransduced Jurkat T cells only responded to stimulation with anti-TCRVβ8 mAb (data not shown). Averages of three to five experiments are shown, with error bars indicating standard errors of the mean. (B) Peptide-loaded and native antigen-positive melanoma cells induce antigen-specific activation of NFAT in TCR-positive Jurkat T cells. TCR-transduced Jurkat T cells were transfected with the NFAT reporter construct, and subsequently co-cultured for 6 h with T2 cells loaded with either the gp100 wt peptide (open bars) or an irrelevant HLA-A2-binding peptide (grey bars), or with the gp100^{pos} FM3 melanoma cell line (black bars). Luciferase activities were determined and expressed relative to either T2 cells loaded with irrelevant peptide (fl-296 TCRαβ: RLU = 0.06; and tc-296 TCRαβ:ζ: RLU = 0.36, both set to 1.0) or medium only (fl-296 TCRαβ and tc-296 TCRαβ:ζ: RLU of both = 0.04 and set to 1.0). The high endogenous β-galactosidase activities of melanoma cells did not permit the normalization of luciferase activities for introduced β-galactosidase activities. Non-transduced Jurkat T cells did not respond to stimulation with any target cell (data not shown).

observed when TCR-transduced Jurkat T cells were cocultivated with the endogenously gp100^{pos}/HLA-A2^{pos} FM3 melanoma cell line (Fig. 3B). Nontransduced Jurkat T cells did not show any NFAT activation upon stimulation with peptide-pulsed T2 cells or FM3.

3.4. Validation of the NFAT reporter assay with Jurkat T cells

We further validated the NFAT reporter assay using specific inhibitors of NFAT activation. First, blocking experiments were performed with CsA, a specific inhibitor of calcineurin and consequently of nuclear translocation and activation of NFAT. CsA (at 100 nM final) was added to the Jurkat T cells 30 min prior to the 6 h co-culture experiments. CsA completely inhibited the activation of NFAT triggered by the G250^{pos} cell line SKRC-17 cl.4 (Fig. 4A). Next, a dominant negative mutant of NFAT was used (Chow et al., 1999), which competes with endogenous NFAT for binding to calcineurin and thereby selectively inhibits NFAT-mediated gene expression. The dn-NFATmut (10 μg) was co-electroporated with the NFAT reporter and the β-galactosidase constructs into scFvG250:γ^{pos} Jurkat T cells prior to co-cultivation experiments. The dnNFAT mutant also completely inhibited the G250 antigen-induced activation of NFAT (Fig. 4B).

3.5. Antigen-specific activation of NFAT in Jurkat T cells parallels antigen-specific immune responses of primary human T lymphocytes

To investigate whether receptor-mediated NFAT activation is a representative measure of anti-tumor responses of human T cells, we compared NFAT activation in Jurkat T cells to immune responses of primary human T cells, such as cytotoxicity and cytokine production, following antigen-specific stimulation. Data obtained with scFvG250:γ^{pos} primary

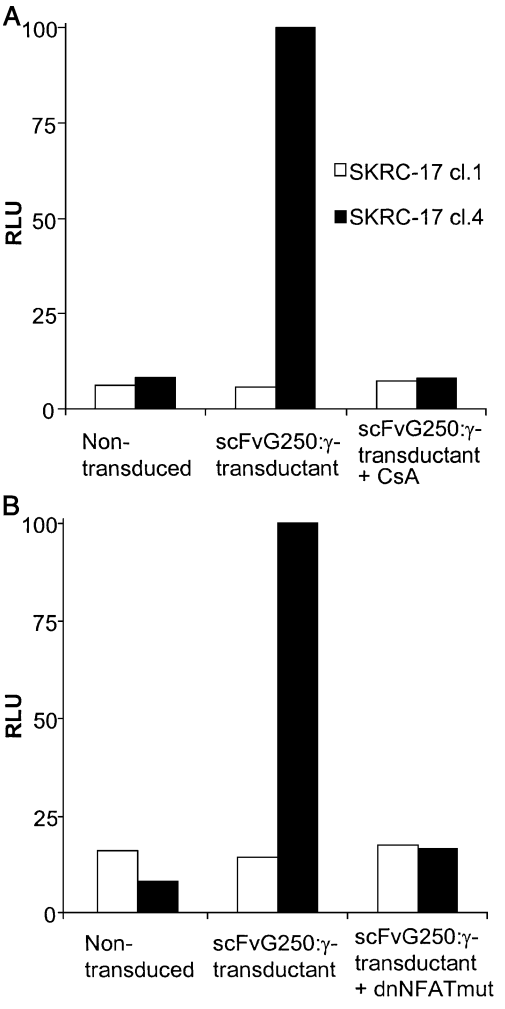

Fig. 4. Antigen-specific responses in Jurkat T cells are inhibited by specific inhibitors of NFAT. (A) Inhibition of antigen-specific activation of NFAT in Jurkat T cells by CsA. scFvG250:γ-transduced Jurkat T cells were transfected with 5 μg of both NFAT reporter and β-galactosidase constructs, and subsequently co-cultured for 6 h with the G250^{neg} SKRC-17 clone 1 (open bars) or the G250^{pos} SKRC-17 clone 4 (black bars), both in the absence or presence of CsA (100 nM). Luciferase activities were determined, normalized for β-galactosidase activities and expressed relative to the SKRC-17 cl.4 signal obtained without CsA (RLU relative to medium only = 7.0; set to 100%). Stimulation with PMA and ionomycin was used as a positive control (data not shown). Nontransduced Jurkat T cells did not respond to stimulation with any target cell. Results of one (out of four) representative experiment are shown. (B) Inhibition of antigen-specific activation of NFAT in Jurkat T cells by a dominant negative mutant. Jurkat T cells transduced with the chimeric scFvG250:γ receptor were transfected with 5 μg of both NFAT reporter and β-galactosidase constructs and 10 μg of dnNFATmut construct, with amounts of DNA kept constant by adding pcDNA3.1 (backbone vector of dnNFATmut). Subsequently, cells were co-cultured for 6 h with the same target cells as described in the legend to (A), after which luciferase activities were determined, normalized and expressed relative to the SKRC-17 cl.4 signal obtained without dnNFATmut.

human T lymphocytes is summarized in Table 1A. Single-chain FvG250:γ^{pos} T lymphocytes were cytolytic and produced TNFα when co-cultured with G250^{pos} but not G250^{neg} target cells. In addition to NFAT activation, the G250 mAb also inhibited the antigen-induced cytolysis and TNFα production by scFvG250:γ-transduced primary human T lymphocytes. Data obtained with fl-296 TCRαβ^{pos} and tc-

Table 1
Antigen-specific activation of NFAT parallels antigen-specific immune responses of primary human T lymphocytes

(A) scFvG250:γ

Target cells	CTX[a] (% ^{51}Cr release)	TNFα production[b] (pg/ml)	NFAT activity[c] (RLU)	
			Exp. 1	Exp. 2
SKRC-17 cl.1	1	0	1.1	0.4
SKRC-17 cl.1 + CsA	0	2		0.5
SKRC-17 cl.4	45	361	16.8	7.0
SKRC-17 cl.4 + G250 mAb	2	0	0.6	
SKRC-17 cl.4 + CsA	27	8		0.6

(B) fl-296 TCRαβ

Target cells	CTX[a] (% ^{51}Cr release)	TNFα production[b] (pg/ml)	NFAT activity[c] (RLU)
T2 + irrel. pept.	0	0	1.7
T2 + gp100 wt pept.	93	665	22.0
T2 + gp100 wt pept. + CsA	81	3	0.7
BLM	1	0	1.1
BLMgp100	35	945	2.2
BLMgp100 + CsA	18	0	0.6
FM3	30	243	16.0
FM3 + CsA	19	0	0.5

(C) tc-296 TCRαβ:ζ

Target cells	CTX[a] (% ^{51}Cr release)	TNFα production[b] (pg/ml)	NFAT activity[c] (RLU)
T2 + irrel. pept.	16	0	5.3
T2 + gp100 wt pept.	85	283	140.0
T2 + gp100 wt pept. + CsA	71	0	1.4
BLM	15	0	1.9
BLMgp100	25	145	9.4
BLMgp100 + CsA	12	0	1.1
FM3	36	25	32.0
FM3 + CsA	24	0	1.3

[a] Percentages of ^{51}Cr release from various target cells when cocultivated with primary human T lymphocytes expressing the scFvG250:γ receptor (A), fl-296 TCRαβ (B) and tc-296 TCRαβ:ζ (C). Single-chain FvG250:γ-positive T lymphocytes were used at an effector-to-target cell (E/T) ratio of 5:1. The fl-296 TCRαβ^{pos} and tc-296 TCRαβζ^{pos} T lymphocytes were co-cultured with peptide-loaded T2 target cells at an E/T ratio of 7.5:1, whereas TCR-transduced T lymphocytes were co-cultured with the melanoma cell lines BLM, BLMgp100 and FM3 at an E/T ratio of 30:1. T2 cells were peptide-loaded for 30 min at 37 °C/5% CO_2 prior to their use in cytotoxicity assays. Final concentrations of CsA, G250 mAb and peptides are 100nM, 25 μg/ml and 1 μg/ml, respectively. Abbreviations used: irrel. pept. = irrelevant peptide; gp100 wt pept. = gp100 wild-type peptide.

[b] TNFα production (determined by ELISA; expressed in pg/ml) by receptor-positive primary human T lymphocytes that were co-cultured with the same target cells as described in (A) in the absence or presence of G250 mAb or CsA. E/T ratio used was 3:1.

[c] NFAT activity (in RLU) of receptor-positive Jurkat T cells that were co-cultured with the target cells as described in (A) in the absence or presence of the G250 mAb or CsA. BLM, BLMgp100 and FM3 cells were pretreated with INFγ and IL-1β, and used in combination with anti-CD28 mAb to stimulate TCR-transduced Jurkat T cells. Luciferase activities were determined in cell lysates, normalized for β-galactosidase activities, and expressed relative to a nonstimulated condition (i.e., medium only). See Section 2 for details.

296 TCRαβ:ζ^{pos} primary human T lymphocytes is summarized in Table 1B and C, respectively. Again, the fl-296 TCRαβ^{pos} and tc-296 TCRαβ:ζ^{pos} T lymphocytes showed cytolytic activity and produced TNFα when co-cultured with gp100 peptide-loaded T2 cells and gp100^{pos}/HLA-A2^{pos} melanoma cells but not gp100^{neg} target cells. To assay directly the significance of NFAT activation to antigen-specific responses of primary human T lymphocytes, we added CsA to the T lymphocyte effector cells 30 min prior to cytotoxicity and TNFα production assays. CsA inhibited 35–50% of the antigen-specific cytotoxicity of primary human T lymphocytes expressing either mAb- or TCR-based tumor-specific receptors, whereas CsA completely inhibited the antigen-specific TNFα production in these T cell transductants (Table 1).

4. Discussion

We set up an NFAT reporter gene assay based on Jurkat T cells to functionally test tumor-specific receptors in a fast and sensitive manner, as opposed to the standard but more labor-intensive testing of tumor cell cytotoxicity and cytokine production of receptor-transduced primary human T lymphocytes. The NFAT reporter gene assay in Jurkat T cells is validated along the following lines: (i) receptor-positive Jurkat T cells are easy to obtain via retroviral transduction and, when necessary, to enrich via MACS; (ii) mAb-based as well as TCR-based anti-tumor receptors, having different (chimeric) receptor formats, mediate antigen-specific NFAT activation; (iii) various stimuli such as anti-receptor mAbs and tumor target cells that are either loaded with peptides or natively express the antigen or peptide/MHC complex of interest trigger an NFAT response; (iv) ligand- or receptor-specific antibodies as well as the immunosuppressor CsA and a dominant negative NFAT mutant block antigen-specific NFAT activation; and importantly (v) receptor-mediated NFAT responses parallel tumor-specific cytolysis and TNFα production of receptor-transduced primary human T lymphocytes, and the immune responses of receptor-engrafted primary human T lymphocytes are, in fact, compromised by inhibition of NFAT activation.

Transduction of Jurkat T cells with retroviral vectors harboring mAb- or TCR-based receptor gene(s) using phoenix-derived viruses and retronectin as a substrate on average resulted in levels of expression of 30%, being slightly less but in good agreement with results obtained with primary human T lymphocytes (Lamers et al., 2002). Receptor-positive Jurkat T cells can easily be enriched resulting in levels of expression of over 80% (Fig. 1) and avoiding the need for cloning and antibiotic selection. Moreover, TCR/CD8 co-transduction resulted in specific binding of gp100/HLA-A2 tetramers, showing preservation of ligand binding affinity of TCR chains following gene transfer in immortalized T cells. Antigen-specific function of the introduced receptors was evident from their ability to mediate NFAT activation. A clear NFAT activation was observed in Jurkat T cells transduced with either the G250-specific mAb-based chimeric scFvG250:γ receptor or the gp100/HLA-A2-specific TCR-based 296 receptors (i.e., fl-296 TCRαβ or tc-296 TCRαβ:ζ) when stimulated with the G250^{pos} target cell (Fig. 2), and anti-TCR mAb or gp100 peptide-loaded target cells (Fig. 3A and B), respectively. More importantly, TCR-transduced Jurkat T cells show NFAT activation in response to melanoma cells presenting endogenously processed gp100 peptide (Fig. 3B).

The observed native response of TCR-reconstituted Jurkat T cells is in contrast to previous reports showing that TCR-reconstituted Jurkat T cells only respond to peptide-loaded target cells but not to tumor cells (Cole et al., 1995; Calogero et al., 2000; Aarnoudse et al., 2002). We ascribe the detection of native antigen responses mediated by TCR-based receptors to the following. First, the retroviral transduction protocol we employed results in high expression levels of receptor and CD8α on Jurkat T cells that can then be easily enriched. High expression levels of the introduced receptor as well as CD8α facilitate antigen-specific T cell responses (Nel, 2002). The lack of CD8α expression on Jurkat T cells is in fact suggested to explain the inability of Jurkat T cells transfected with MART-1-specific TCR to produce IL-2 in response to MART-1 positive tumor cells (Cole et al., 1995). Noteworthy in this respect is our finding that TCR-expressing Jurkat T cells with a low expression level of CD8α do not respond to gp100 positive melanoma cells (data not shown), in line with

the observation by Aarnoudse et al. (2002) that additional introduction of CD8α in Jurkat T cells increased the weak TCR-mediated response to melanoma cells. Second, in-house flow cytometry experiments show that the Jurkat E6.1 T cell clone, in sharp contrast to other Jurkat T cell clones often used for TCR-reconstitution experiments such as the TCRβ^{neg} Jurkat RT3-T3.5 T cell clone (Calogero et al., 2000; Liu et al., 2000; Aarnoudse et al., 2002), expresses high levels of endogenously expressed CD2, CD5, CD11α, CD18 and CD28 molecules (data not shown). The expression of such co-stimulatory and adhesion molecules is particularly important when the expression level of tumor antigens on target cells is low as is the case for endogenously expressed peptide/MHC molecules on melanoma cells. In fact, the use of Jurkat E6.1 T cells allowed us to provide these cells a co-stimulatory signal via an anti-CD28 mAb when testing TCR-mediated NFAT activation in response to tumor cells, by analogy to the stimulation conditions used by Liu et al. (2000) to study TCR-mediated IL-2 production in Jurkat E6.1 T cells in response to native p53-positive tumor cells. Third, cytokine-stimulation of tumor target cells prior to our NFAT activation assay, to increase the expression of MHC class I and adhesion molecules on tumor cells, further enhances antigen-specific responses. Finally, the NFAT reporter gene assay has been optimized for various parameters with the number of response elements present in the NFAT reporter construct being important to the assay's sensitivity and facilitating the detection of antigen-specific NFAT activation.

The NFAT responses in receptor-transduced Jurkat T cells are in parallel with the antigen-specific immune responses (i.e., cytotoxicity and cytokine production) of primary human T lymphocytes transduced with identical receptor gene(s) (Table 1). In addition, the sensitivity of antigen-specific cytotoxicity and TNFα of primary human T lymphocytes to CsA provides direct evidence of the contribution of NFAT activation to these immune responses. CsA inhibited 30–50% and 100% of the antigen-specific cytolytic and TNFα responses of primary human T cells transductants, respectively, irrespective of the receptor format used (Table 1). Inhibition of cytotoxicity of TCR-transduced T lymphocytes in response to peptide-loaded target cells was less efficient (Table 1B and C), most likely due to the fact that a peptide stimulus is nonphysiological for TCR-mediated cytotoxicity. Our observation that at least 50% of the cytotoxic response of primary human T cells are not affected by CsA is most likely due to the existence of two major pathways to induce cytotoxicity: (1) the FasL pathway, with an NFAT dependence of the transcriptional activation of FasL in cytotoxic T cells (Xiao et al., 1999), and (2) the granzyme/perforin release pathway, which is NFAT independent (Kagi et al., 1996).

The observation that tumor-specific receptors coupled to the intracellular signaling domains of Fc(ε)RIγ (scFvG250:γ) or CD3ζ (tc-296 TCRαβ:ζ) mediate NFAT activation points to the usefulness of the described NFAT reporter gene assay to test the function of immune receptors containing various building blocks. By analogy to the Fc(ε)RIγ-cross-linking on mast cells, receptors containing a γ domain probably mediate antigen-specific NFAT activation as a result of activation of the protein kinases Lyn and Syk followed by calcium mobilization and activation of MAP kinases (Hutchinson and McCloskey, 1995; Jouvin et al., 1995), whereas CD3ζ-containing receptors are able to recruit and activate ZAP-70, which propagates intracellular signaling resulting in the activation of NFAT and other transcription factors (Clements et al., 1999).

Taken together, receptor-mediated activation of NFAT is a representative measure of anti-tumor T cell responses and the genetically modified Jurkat T cells described in this paper provide a flexible and sensitive tool for the analysis and selection of chimeric receptors for immunogene therapy. In addition, the genetically engineered Jurkat T cells enable studies on whether and how (chimeric) immune-receptors (and other receptors, such as homing receptors or adhesion molecules, for that matter) interact with other T cell molecules, and provide an approach to the study of intracellular signaling pathways.

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