Journal of Immunological Methods 430 (2016) 10–20

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

Assessment of costimulation and coinhibition in a triple parameter T cell reporter line: Simultaneous measurement of NF-κB, NFAT and AP-1

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ARTICLE INFO

Article history:
Received 8 June 2015
Received in revised form 21 December 2015
Accepted 13 January 2016
Available online 15 January 2016

Keywords:
T-cell activation
Costimulation
NF-κB
NFAT
AP-1
Reporter-assay

ABSTRACT

Engagement of the T cell receptor complex reprograms T cells for proliferation, cytokine production and differentiation towards effector cells. This process depends on activating costimulatory signals and is counteracted by coinhibitory molecules. Three transcription factors, namely NF-κB, NFAT and AP-1, have a major role in inducing the transcriptional program that is required for T cell activation and differentiation. Here we describe the generation of a triple parameter reporter based on the human Jurkat T cell line, where response elements for NF-κB, NFAT and AP-1 drive the expression of the fluorescent proteins CFP, eGFP and mCherry, respectively. The emission spectra of these proteins allow simultaneous assessment of NF-κB, NFAT and AP-1 activity in response to stimulation. Ligation of the TCR complex induced moderate reporter activity, which was strongly enhanced upon coengagement of the costimulatory receptors CD2 or CD28. Moreover, we have generated and tested triple parameter reporter cells that harbor costimulatory and inhibitory receptors not endogenously expressed in the Jurkat cells. In these experiments we could show that engagement of the costimulatory molecule 4-1BB enhances NF-κB and AP-1 activity, whereas coinhibition via PD-1 or BTLA strongly reduced the activation of NF-κB and NFAT. Engagement of BTLA significantly inhibited AP-1, whereas PD-1 had little effect on the activation of this transcription factor. Our triple parameter reporter T cell line is an excellent tool to assess the effect of costimulatory and coinhibitory receptors on NF-κB, NFAT and AP-1 activity and has a wide range of applications beyond the evaluation of costimulatory pathways.

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1. Introduction

T cell responses play a key role in adaptive immunity and it is therefore of crucial importance to understand how external signals modulate the intracellular T cell signaling machinery. Engagement of the TCR–CD3 complex and the coreceptors CD4 and CD8 are regarded as the main trigger to turn on T cells. Activation and nuclear translocation of transcription factors are a major downstream consequence of TCR signaling. These molecular events eventually lead to reprogramming of T cells for proliferation and differentiation in order to acquire a specific effector phenotype. The three transcription factors NF-κB, NFAT and AP-1 play an important role in T cell activation processes (Crabtree and Clipstone, 1994). NF-κB is a dimer composed of the NF-κB family members RELA, RELB, cREL, p100/52 and p105/p50. Together, they form homo- or heterodimers, usually of RELA and p50 or p52, inducing transcription of NF-κB dependent genes for survival, proliferation and cytokine production (Li and Verma, 2002). The NFAT family consists of five members with a REL-homology region for DNA binding and is widely expressed in different cell types. Four of the five NFAT proteins are regulated by intracellular Ca^{2+} signaling. NFAT activation upon TCR signaling, autoregulation or cytokine receptor signaling results in translocation into the nucleus and expression of cytokines such as IL-2 (Macian, 2005). In the nucleus, NFAT cooperates with other transcription factors, mainly AP-1 (Jain et al., 1992), a dimeric protein which is expressed in many cell types and differentially composed of the subfamilies c-Jun, Fos, Maf and ATF, with c-Jun being the most potent activator of transcription among these molecules. Stimuli such as TCR triggering activate AP-1 thereby promoting differentiation, survival and proliferation of cells (Shaulian and Karin, 2002).

Abbreviations: ANOVA, analysis of variance; APC, allophycocyanin; CsA, Cyclosporin A; CAR, chimeric antigen receptor; CFP, cyan fluorescent protein; gMFI, geometric mean of fluorescence intensity; MP, minimal promoter; NIP45, NFAT-interacting protein; RE, response element; TCS, T cell stimulator cells; TPR, triple parameter reporter.

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http://dx.doi.org/10.1016/j.jim.2016.01.007
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Accessory signals generated in T cells during their interaction with cells that present their cognate antigen can potently modulate TCR-signals and thus enhance or attenuate the activity of NF-κB, NFAT and AP-1. So-called second signals, which are generated by the interaction of the T cell-expressed costimulatory receptors with their corresponding membrane-bound ligands, are arguably the most important among these accessory signals. Receptor–ligand pairs belonging to different molecule families have been categorized to this ever-growing group of molecules (Chen and Flies, 2013; Leitner et al., 2010a; Watts, 2005). The individual role of many costimulatory receptors in cellular activation processes including their impact on the transcriptional program of T cells is currently not fully understood.

Studies on transformed T cell lines have greatly contributed to our understanding of T cell signaling and T cell costimulatory signals in particular. The human leukemic T cell line Jurkat is probably the most widely employed model system to study T cell signaling (Abraham and Weiss, 2004). Jurkat-based reporter systems are commonly used to explore the impact of external stimuli on the activation of transcription factors such as NF-κB, NFAT and AP-1. Most of the reporter lines only account for one, maximally two signaling pathways simultaneously (Magness et al., 2004; Noguchi et al., 2008), and thus in most cases the use of different reporters is necessary to get insight into the capacity of agents to trigger or inhibit distinct activation programs (Ratzinger et al., 2014). Here we report on the generation of a Jurkat-based reporter system that enables to measure simultaneously and independently the activity of NF-κB, NFAT and AP-1. Moreover, we have assessed the utility of this reporter line to study the impact of costimulatory and coinhibitory pathways on the activation of these transcription factors.

2. Materials and methods

2.1. Antibodies, cell culture and flow cytometry

Bw5147 (short designation within this work Bw) and the Jurkat T cell line JE6.1 were cultured as described previously (Pfistershammer et al., 2006; Derdak et al., 2006).

To confirm surface expression of the indicated molecules expressed on T cell stimulator cells (TCS) and Jurkat reporter cell lines, the following PE-conjugated mAbs were used: CD58 (TS2/9), CD80 (2D10), PD-L1 (29E.2A3), 4-1BBL (5F4), HVEM (TR2), PD-1 (EH12.2H7), 4-1BB (4B4-1), BTLA (MIH26) and appropriate isotype control, all purchased from BioLegend (San Diego, CA). Expression of a membrane-bound anti-CD3-antibody-fragment on the surface of the T cell stimulator cells was detected via DyLight-649-conjugated goat-anti-mouse IgG (H + L) antibody (Jackson ImmunoResearch, West Grove, PA) as described previously (Leitner et al., 2010b). In coculture experiments where reporter cells were stimulated with T cell stimulator cells, a mouse (m)CD45.2-APC (clone 104, BioLegend) was used to exclude the T cell stimulator cells from the FACS analyses. Flow cytometric analysis was performed using a LSRFORTESSA flow cytometer (Becton Dickinson Immunocytometry System, CA) supported by FACS Diva software. Data was analyzed using the FlowJo software (version 10.0.6., Tree Star, Ashland, OR). Fluorescence intensity is shown on a standard logarithmic scale. Functional grade CD28 (clone 28.2, BioLegend), functional grade CD2 (clone TS1/8, BioLegend), functional grade PD-L1 (5 μg/ml final concentration, clone 29E.2 A3, BioLegend), polyclonal HVEM (8 μg/ml final concentration, R&D Systems, Minneapolis, MN), CD3 (OKT3, Ortho Pharmaceutical Corp., Raritan, NJ), CD63 (clone 11C9, produced in our laboratory) or CD28 Superagonist (2 μg/ml final concentration, ANC28.1/5D10, Ancell Bayport, MN) antibodies were used to stimulate reporter cells.

2.2. Design of reporter constructs

A self-inactivating retroviral vector encoding a NF-κB-eGFP reporter construct (pSIRV-NF-κB-eGFP) was described previously (Platzer et al., 2004). The construct contains a response element (RE) for NF-κB, a minimal promoter (MP) and eGFP as reporter gene. The construct was modified through removal of a fragment corresponding to the 5' end of a luciferase gene. For this, a part of the minimal promoter and eGFP was PCR-amplified with primers omitting the partial luciferase sequence. EcoRI and NotI restriction sites were added to the ends of the primers. The PCR product was cloned into the reporter vector and the integrity of the construct was confirmed by DNA sequencing.

For the construction of a modular system, the cassette containing the response element (RE) for NF-κB, minimal promoter and eGFP was modified. Using the original construct as a template and multiple overlapping primers, PCR products containing the NF-κB, the minimal promoter (MP) and eGFP were generated and fused by overlap extension PCR. The PCR primers contained unique restriction sites to generate a cassette (BamHI–NF-κB-RE–EcoRI–MP–HindIII–eGFP–NotI) where the individual parts could easily be replaced. The integrity of the cassette was confirmed by DNA sequencing.

Cyan fluorescent protein (CFP) was cloned from pCAG-CFP (Matsuda and Cepko, 2004) (pCAG-CFP was a gift from Connie Cepko, Addgene plasmid # 11179) using primers containing HindIII and NotI restriction sites. Subsequently, eGFP in the NF-κB-eGFP-reporter was replaced with CFP to obtain an NF-κB-CFP reporter.

For the NFAT-eGFP reporter, a NFAT reporter construct (Schichl et al., 2009) (a kind gift from R. de Martin) was PCR-amplified using a primer pair with flanking BamHI and EcoRI restriction sites to obtain the NFAT-RE. Subsequently, the NF-κB-RE in the modular NF-κB-eGFP reporter construct was replaced with the NFAT-RE.

For the AP-1-mCherry reporter, the AP-1 RE consisting of three repeats of two alternative AP-1 binding sites (tgactcag, tgagtcag (Angel et al., 1987)) with flanking BamHI and EcoRI restriction sites, and an alternate minimal promoter with EcoRI and HindIII sites were amplified individually using overlapping PCR Primers. The mCherry cDNA was cloned from the pLV-mCherry plasmid (a gift from Pantelis Tsoulfas, Addgene plasmid # 36084). To obtain the AP-1-mCherry reporter, the minimal promoter (described by Vodjdani et al. (Vodjdani et al., 1988)) and eGFP from the modular NF-κB-eGFP cassette were replaced with the alternate minimal promoter and mCherry. The integrity of all inserts was verified by DNA sequencing.

2.3. Generation of triple parameter T cell reporter cell lines and T cell stimulator cells

In a first step an ecotropic receptor (Jorgl et al., 2007) was introduced into JE6.1 cells to increase transduction efficiency. Subsequently, JE6 cells were transduced simultaneously with NF-κB-CFP, NFAT-eGFP and AP-1-mCherry constructs using a previously described retroviral transduction protocol (Steinberger et al., 2004). Single cell clones were established from the transduced cell pool and screened for low-absent expression of reporter genes in a resting state and strong upregulation of eGFP, mCherry and CFP upon stimulation with PMA/Ionomycin. A cell clone fulfilling these criteria was selected and termed triple parameter reporter (short designation in this work: TPR cells).

Sequences encoding PD-1, 4-1BB and BTLA cloned from a human T cell cDNA library (Popow et al., 2013) were introduced in the retroviral expression vector pCJK2 and integrity of the resultant constructs was confirmed by DNA sequencing. Subsequently, the TPR cells were retrovirally transduced with these constructs, and single cell clones expressing human 4-1BB, PD-1 or BTLA were established.

For the knockdown experiments, oligonucleotides for shRNA-sequences from the RNAi Consortium for NF-κB1 (accession numbers TRCN0000006518, TRCN0000006520), NFATc3 (accession numbers TRCN0000014529, TRCN0000014529) and AP-1 (accession numbers TRCN0000016004, TRCN0000016007) were introduced in the lentiviral vector Tet-pLKO-puro (Wiederschain et al., 2009) (a gift from Dimitri Wiederschain, Addgene plasmid # 21915). The integrity of the constructs was confirmed by DNA sequencing, followed by lentiviral transduction of the TPR cells with these plasmids. Transduced cells were

selected with 1 μg/ml Puromycin (Clontech, Mountain View, CA). Expression of shRNA was induced by adding 100 ng/ml Doxycycline for 48 h (Sigma Aldrich) and knockdown of mRNA expression was confirmed by real-time PCR. Real-time PCR-Data were normalized to the mRNA levels measured in the cells expressing control shRNA.

T cell stimulator cells (short designation: TCS) have been described previously (Leitner et al., 2010b). In brief, TCS are Bw5147 cells (murine thymoma cell line) that have been engineered to stably express an anti-human CD3 single chain fragment anchored to the cell membrane via a human CD14 stem (CD5L-OKT3scFv-CD14) and thus can activate human T cells upon coculture. These cells were retrovirally transduced to express high levels of human CD58, CD80, PD-L1, 4-1BBL or HVEM as described previously. Cells expressing no human costimulatory molecule were used as control TCS (TCS-ctrl). Surface expression of membrane-bound anti-CD3 and the costimulatory ligands was confirmed by FACS analysis (Leitner et al., 2010b; Kober et al., 2008).

2.4. Stimulation experiments and reporter assays

Triple parameter reporter cells (5×10^4/well) were either cocultured with T cell stimulator cells (5×10^3/well) or stimulated with plate-bound antibodies in 96-well flat bottom plates for 24 h, unless indicated otherwise. For plate-bound assays CD3 antibodies (final concentration as indicated) were coated at 4 °C overnight in conjunction with either isotype control, CD28, CD2 or CD63 mAb (each at a final concentration of 2 μg/ml), as described in detail (Leitner et al., 2009).

In some experiments, PMA, Ionomycin (each 100 nM, Sigma Aldrich) or Cyclosporin A (CsA, Sandimmun®, Switzerland, used at indicated final concentrations) were added.

Following 24 h of stimulation, cells were harvested and cocultures of reporter cells with TCS were stained with an APC-labeled anti-murine CD45.2 mAb to exclude the APC-positive cells (TCS) from the analysis. Subsequently, expression of eGFP, CFP and mCherry were measured by flow cytometry. Mean and standard deviation of the geometric mean of fluorescence intensity (gMFI) of the whole population of viable reporter cells identified according to their forward and side-scatter properties were determined from duplicate wells. Transcription factor activity upon activation was calculated by normalizing to the gMFI of the unstimulated control (fold induction).

For the knockdown experiment, the fold induction of the reporter fluorophore in the target shRNA expressing cells was normalized to the fold induction measured in the cells containing the control shRNA.

2.5. Microscopy

Unstimulated TPR cells and TPR cells stimulated for 24 h with PMA and Ionomycin (each 100 nM final concentration) were washed and directly recorded in PBS at 25 °C. Image acquisition was performed with a Leica DMI4000B microscope (Leica Microsystems; Wetzlar, Germany) equipped with a 100× immersion objective (Leica HCX PL Apo 100×, NA 1.47) and an Andor iXon Ultra-8871 EM-CCD camera (Andor Technologies; Belfast, UK), controlled by the Leica Application Suite Advanced Fluorescence software (version AF6000LX). mCherry excitation and emission light filtering was done using TRITC configuration of Leica Quad-Sedat filter system (set 11532564). To avoid crosstalk of CFP and eGFP, the Leica CFP/YFP FRET filter system (set 11522073) was used, with eGFP being recorded in YFP configuration. Image analysis was performed with ImageJ open source software (Version 1.48, National Institute of Health, Washington, DC).

2.6. Statistics

Means from duplicate wells and +/− SD are shown. Using GraphPad Prism (Version 5, GraphPad Software, Inc., La Jolla, CA), data were analyzed by two-sided paired t-test, one-way analysis of variance (ANOVA) followed by Turkey posthoc tests or two-way ANOVA followed by Bonferroni post tests (ns not significant, *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001).

3. Results

3.1. Generation of a triple parameter reporter T cell line

The transcription factors NF-κB, NFAT and AP-1 play essential roles in the activation of T cells. Our goal was to establish a reporter T cell line enabling the measurement of the activity of those three transcription factors simultaneously and to use it for the investigation of costimulatory and inhibitory molecules. In a first step, we generated a modular reporter cassette where transcription factor response elements, minimal promoter sequences and reporter genes can easily be exchanged. Constructs harboring different combinations of responsive elements and minimal promoters were introduced into the human T cell line Jurkat E6.1 and combinations that yielded optimal reporter gene induction were chosen for further use (data not shown). Subsequently, we generated reporter constructs where the selected responsive elements for NF-κB, NFAT and AP-1 were combined with sequences encoding CFP, eGFP and mCherry, respectively (Fig. 1 A). Jurkat cells were transduced with retroviral constructs containing these three reporters and a large panel of cell clones was established from the resulting cell pool. A single cell clone that showed high induction of all three reporter genes upon activation with PMA and Ionomycin was selected for further use (Fig. 1 B, C) and was designated triple parameter reporter (short designation within this work TPR cells).

In a next step, we analyzed the reporter activity in our TPR cells upon TCR-complex engagement in absence or presence of costimulatory signals to validate our system. For this, we used T cell stimulator cells (TCS) that carry a membrane-bound anti-CD3 antibody fragment and thus generate signal 1 in T cells (Leitner et al., 2010b). In addition to TCS that do not express costimulatory ligands (TCS-control) we used TCS expressing the costimulatory ligands human CD80 (TCS-CD80) or CD58 (TCS-CD58) to trigger the costimulatory receptors CD28 and CD2, respectively (Fig 1 D). Coculture of the reporter cells with control TCS resulted in a moderate expression of all three reporter genes, whereas TCS expressing CD80 or CD58 strongly enhanced transcriptional activity of NF-κB, NFAT and AP-1 (Fig. 1 E, F).

Of note, while engagement of CD28 (via CD80) led to a significantly stronger NF-κB activation compared to CD2-CD58 stimulation, the difference in NFAT and AP-1 activation was not significant (Fig. 1 G). Combined, these results demonstrate that our TPR cell line is both a robust and precise tool to assess transcription factor activity by means of reporter gene expression upon delivery of signal 1. Moreover, a marked up-regulation of reporter proteins can be measured in the reporter cells when signal 2 is provided in addition to signal 1.

3.2. Kinetics of NF-κB, NFAT and AP-1 activation

We performed time course stimulation experiments to compare the kinetics of NF-κB, NFAT and AP-1 activity and to determine the time required for maximal activation. For strong costimulation we employed TCS expressing CD80. TPR cells were cocultivated with stimulator cells, harvested at different time points and subsequently analyzed by FACS. While AP-1 activity peaked already at the 8-h time point, NF-κB activity reached its maximum after 24 h. Activation was slowest for NFAT with the highest reporter expression measurable after 72 h. At this time point NF-κB and AP-1 activity was already declining (Fig. 2).

3.3. Effects of PMA, Ionomycin and Cyclosporin A

In a next set of experiments, we assessed the impact of PMA, Ionomycin and Cyclosporin A (CsA) on the TPR cells. The phorbol ester PMA is described to activate protein kinase C and consequently AP-1 and NF-κB (Khalaf et al., 2010). The ionophore Ionomycin leads to an increase in cytoplasmic Ca^{2+} levels, which in turn is required for the dephosphorylation of NFAT followed by its translocation into the nucleus (Ruff and Leach, 1995). Therefore, TPR cells were activated with either

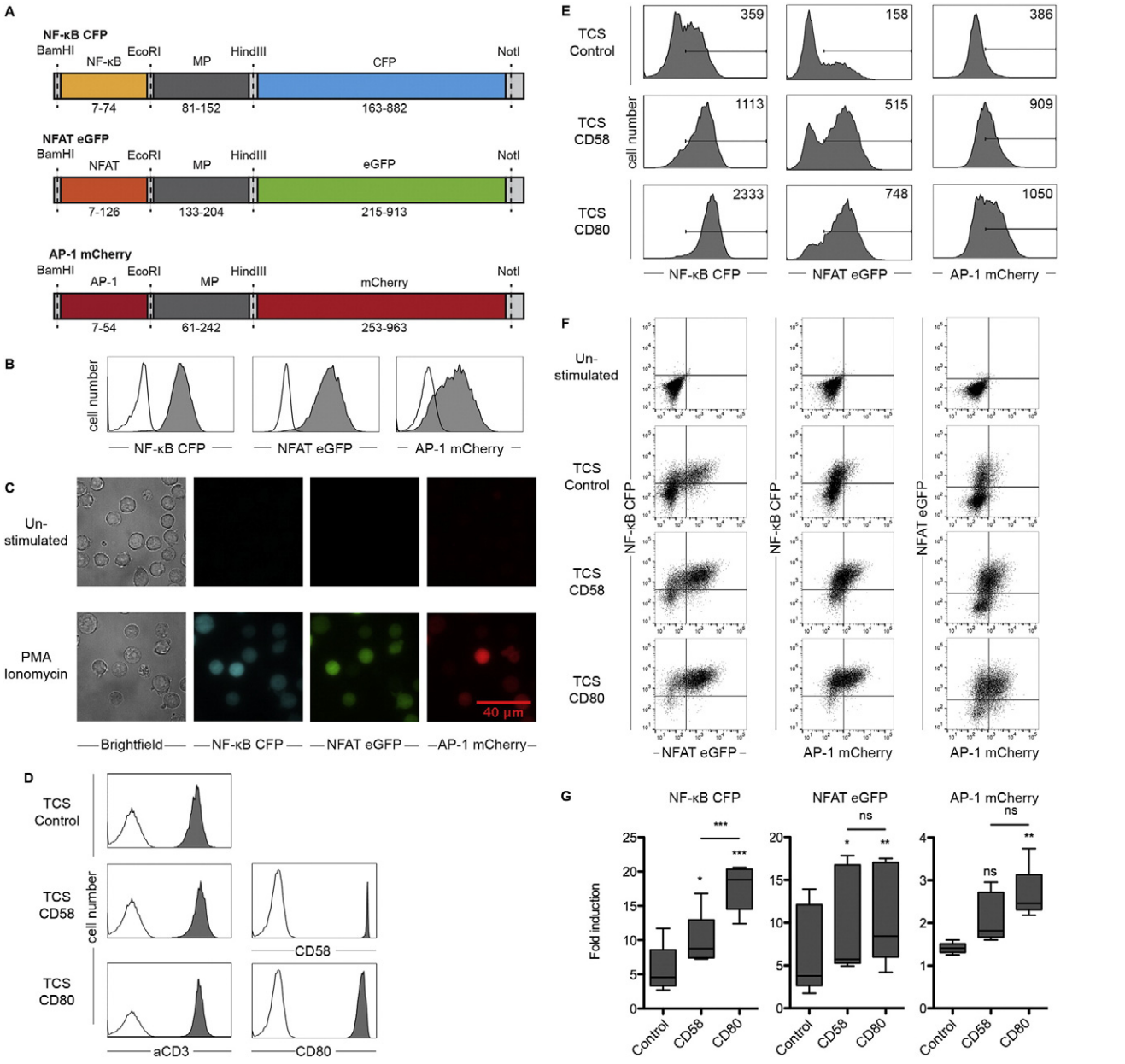

Fig. 1. Generation and characterization of the triple parameter reporter T cell line (TPR). A) Schematic illustration of reporter constructs encoding NF-κB CFP, NFAT eGFP and AP-1 mCherry with restriction enzyme recognition sites. MP: minimal promoter. B) Activation of TPR cells with PMA and Ionomycin to induce activation of NF-κB, NFAT and AP-1. Open histograms: unstimulated control; filled histograms: cells stimulated with PMA and Ionomycin. C) Fluorescent microscopy images of TPR cells activated with PMA and Ionomycin (lower panel). Unstimulated cells served as negative control (upper panel). D) FACS analysis of the expression of surface molecules on T cell stimulator cells (TCS). Open histograms: control cells; filled histograms: control TCS or TCS coexpressing CD58 or CD80. E) Histogram and F) dot plot representation of reporter assays to measure the effect of CD58 and CD80 on the transcription factor activity of NF-κB, NFAT and AP-1. TPR cells were cocultivated with TCS expressing CD58 or CD80 for 24 h. Activation of NF-κB, NFAT and AP-1 were measured by FACS analysis. Numbers show geometric mean of fluorescence intensity (gMFI). Experiment shown is representative for 5 independently performed experiments. G) Summary of the normalized fold induction of gMFI of the TPR cells compared to unstimulated cells from five independent experiments. Statistics by one-way ANOVA, followed by Tukey's multiple comparison post test (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ns: not significant).

PMA or Ionomycin alone, or with both compounds for 24 h, followed by FACS analysis of reporter gene expression. Treatment with PMA alone strongly induced the activation of NF-κB and AP-1 reporters, but did not induce NFAT activity as expected. Ionomycin alone did not change transcriptional activity of NF-κB, AP-1 and it was also ineffective in upregulation of NFAT activity. A strong upregulation of the NFAT reporter was only observed when Ionomycin was added in combination with PMA. This is in line with previous reports that demonstrate that additional stimuli are required to induce NFAT activity upon Ionomycin treatment of Jurkat cells (Boss et al., 1996; Northrop et al., 1993; Wu et al., 1995). In addition, Ionomycin further increased the PMA-induced upregulation of NF-κB and AP-1 reporters (Fig. 3 A). These results show that the response elements of NF-κB and AP-1 are specifically bound by the respective transcription factors upon addition of PMA.

The calcineurin inhibitor CsA is known to reduce the activation of NFAT, but also that of NF-κB (Flanagan et al., 1991; Frantz et al., 1994). The inhibitory effect of different CsA concentrations on the activation of NF-κB, NFAT and AP-1 was analyzed in the TPR cells. To assess whether CsA has a differential effect on the costimulatory pathways CD2-CD58 and CD28-CD80, TPR cells were cocultivated with TCS-CD58 or TCS-

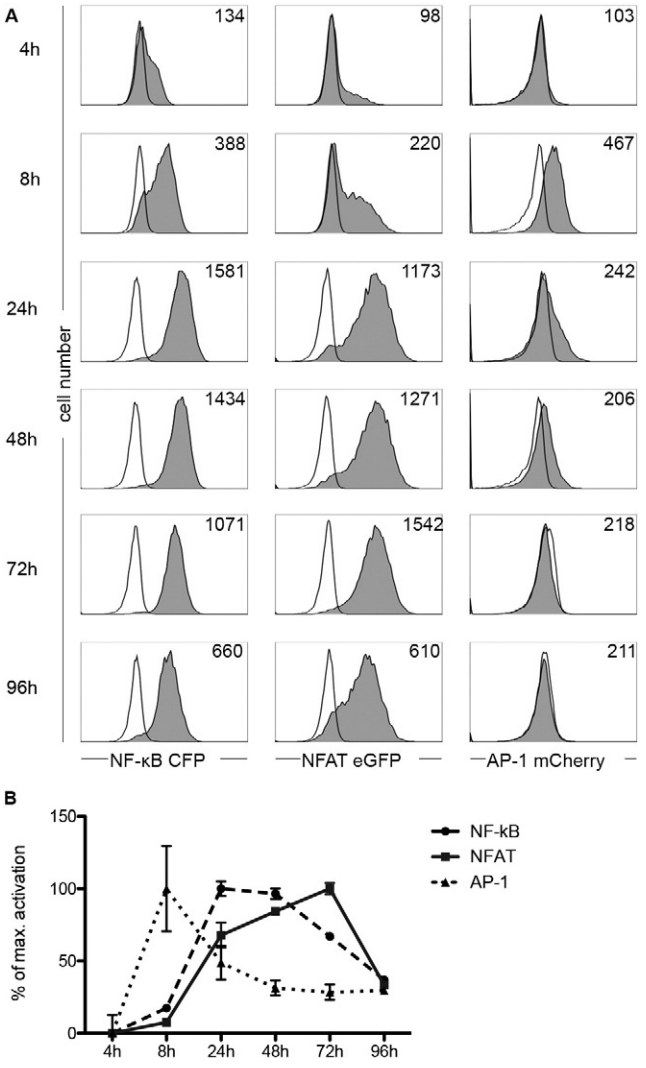

Fig. 2. Kinetics of the transcription factor activity of NF-κB, NFAT and AP-1. A) TPR cells were stimulated with TCS-CD80 for the indicated time-points. Activation of NF-κB, NFAT and AP-1 was analyzed by FACS. Open histograms: unstimulated TPR cells; filled histograms: TCS-CD80 activated TPR cells. Numbers show gMFI of TCS-CD80 activated TPR cells. B) Percentage of maximum activation of NF-κB, NFAT and AP-1. Fold induction of gMFI of the TPR cells was normalized to the highest fold induction of NF-κB, NFAT and AP-1 in this experiment, respectively.

CD80 to induce T cell activation, and CsA was added at the indicated concentrations for 24 h. In TCS-CD80 stimulated TPR cells, transcription factor activity of NF-κB and NFAT were significantly reduced in the presence of CsA at a final concentration of 200 nM. AP-1 activity was not significantly influenced by CsA (Fig. 3 B, C, Suppl. Fig. 1 A). When we compared the effect of CsA in reporter cells that were costimulated via CD2 or CD28, we observed that NF-κB is more sensitive to CsA in CD2- than in CD28 signaling. In contrast, CsA-mediated inhibition of NFAT activity was not differentially affected by costimulation via CD2 or CD28. (Fig. 3 D).

3.4. Specificity of the responsive elements in the TPR cells

In order to further assess the specificity of the reporter constructs in our TPR cells we used lentiviral doxycycline-inducible shRNA vectors to knockdown NF-κB1, NFATc3 or c-Fos. Two constructs were generated for each target gene and were introduced into the TPR cells by lentiviral transduction. Following expansion and puromycin selection of transduced cells, shRNA expression was induced by adding doxycycline, and 48 h later the mRNA expression was assessed by qPCR. Gene silencing was achieved for each target gene with at least one of the constructs (Fig. 4 A, C, E). In parallel, the TPR cells were activated using CD80 expressing TCS and following 24 h of coculturing the reporter-gene expression was assessed by flow cytometry. Silencing of NF-kB1, NFATc3 and c-Fos resulted in a significant and specific reduction of the targeted transcription factors (Fig. 4 B, D, F).

3.5. Stimulation of TPR cells with mAbs

Agonistic antibodies to the TCR–CD3 complex and to costimulatory receptors are widely used to activate T cells and T cell lines. Consequently we analyzed the response of the TPR cells to CD3 antibodies and antibodies to costimulatory receptors coimmobilized on ELISA-plates. In addition to stimulating mAbs to CD2 and CD28, we used a CD63 antibody that was previously described to potently costimulate cytokine production and proliferation in primary human T cells (Pfistershammer et al., 2004). CD3 antibodies alone induced a modest upregulation of NF-κB, NFAT and AP-1 reporter genes only when immobilized at a high concentration (1 μg/ml). The presence of stimulating antibodies to CD28 or CD2 strongly costimulated the NF-κB, NFAT and AP-1 activity at all CD3 concentrations tested. Interestingly, the CD63 antibody (mAb 11C9) was as effective in inducing NF-κB, NFAT and AP-1 reporter activity as antibodies reactive against CD28 or CD2 (Fig. 5 A–C).

Superagonistic CD28 antibodies have the capability to induce T cell activation in absence of TCR-signals (Waibler et al., 2008). We tested the capacity of a soluble CD28 superagonistic mAb to stimulate the TPR cells. For comparison a stimulating CD3 mAb was also used. Both agents induced similar levels of NF-κB and AP-1 activation but compared to the CD3 mAb, the capacity of the superagonistic CD28 mAb to induce NFAT activity was significantly lower (Fig. 5 D).

3.6. Effect of 4-1BB costimulation on NF-κB, NFAT and AP-1 activity

Jurkat T cells are devoid of several costimulatory and coinhibitory receptors that play important roles in enhancing or attenuating responses in primary T cells. We thus addressed whether our TPR cell system can be utilized to study costimulators normally not expressed on Jurkat T cells. One such molecule is 4-1BB, which acts as major costimulatory receptor in CD4 and CD8 T cells (Kober et al., 2008; DeBenedette et al., 1997). We expressed 4-1BB in our TPR cells and used T cell stimulator cells expressing 4-1BBL to trigger this receptor on the resultant reporters (Fig. 6 A). Stimulation of TPR-4-1BB in presence of 4-1BBL significantly enhanced NF-κB and AP-1 activation, whereas NFAT activity was even reduced. In addition, TCS-CD80 induced higher AP-1 activation than TCS-4-1BBL indicating that CD80 is a stronger costimulator than 4-1BBL (Fig. 6 B–C, Suppl. Fig. 1 B). The presence of 4-1BBL on the TCS did not costimulate control TPR cells not expressing 4-1BB (Suppl. Fig. 2 A).

3.7. Effect of PD-1 and BTLA signals on NF-κB, NFAT and AP-1 activity

In addition to activating costimulatory molecules, the response of T cells is shaped by coinhibitory receptors that generate inhibitory intracellular signals when engaged by their cognate ligands. We created TPR cells expressing the well-established coinhibitory molecules PD-1 or BTLA to quantitate their effect on NF-κB, NFAT and AP-1 activity. TCS expressing high levels of PD-L1 or HVEM were used to trigger PD-1 or BTLA, respectively, in the reporter cells (Fig. 7 A, B). Coculture experiments revealed that TPR-PD-1 cells exhibited strongly reduced NF-κB and NFAT activity when stimulated with TCS expressing PD-L1 compared to control TCS. (Fig. 7 C, E, Suppl. Fig. 1 D) Similar results were obtained for TPR-BTLA stimulated in presence of HVEM (Fig 7 D, F, Suppl. Fig. 1 C). However, we observed differences in the capacity of PD-1 and BTLA to downmodulate the activity of the transcription factor AP-1: Whereas PD-1 engagement resulted only in a slight non-significant inhibition, the presence of HVEM reduced AP-1-reporter activity in BTLA-expressing

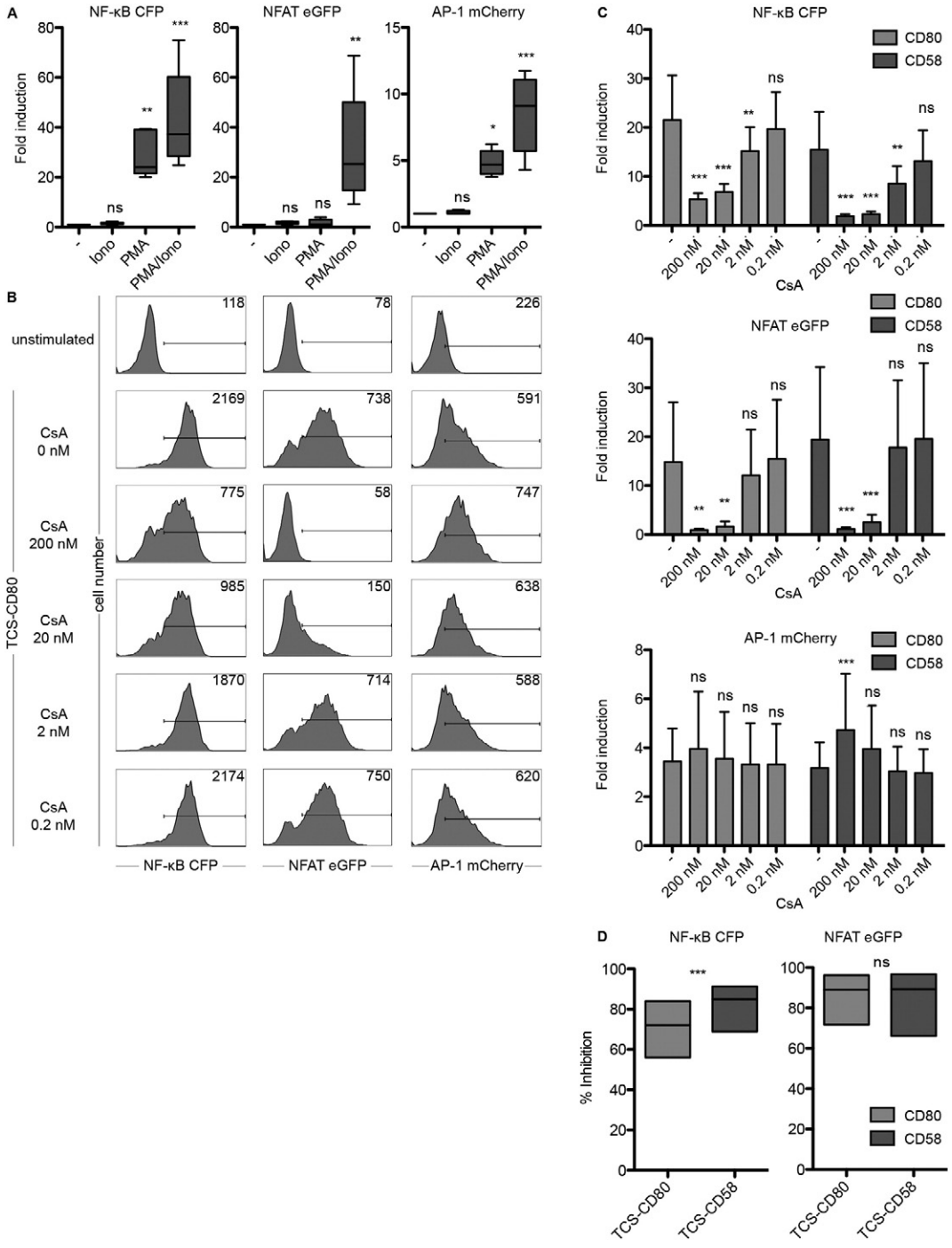

Fig. 3. Effect of PMA, Ionomycin and Cyclosporin A on the transcription factor activity of NF-κB, NFAT and AP-1. A) Reporter assay to measure the effect of PMA and Ionomycin on the transcription factor activity of NF-κB, NFAT and AP-1. TPR cells were stimulated with PMA, Ionomycin or PMA with Ionomycin (each at a final concentration of 100 nM) for 24 h. Summary of the normalized fold induction of gMFI of the TPR cells compared to unstimulated cells from five independent experiments of NF-κB, NFAT and AP-1 is shown. Statistics by one-way ANOVA, followed by Tukey's multiple comparison post test (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ns: not significant). B) Effect of different concentrations of Cyclosporin A on NF-κB, NFAT and AP-1 during stimulation with TCS-CD80. Numbers show gMFI. Experiment shown is representative for six independently performed experiments. C) Comparison of the effect of Cyclosporin A on signaling via CD2 and CD28 on NF-κB, NFAT and AP-1. Different concentrations of Cyclosporin A were used for TPR cells cocultivated with TCS-CD58 or TCS-CD80. Mean and standard deviation (SD) of the normalized fold induction of gMFI of the TPR cells compared to unstimulated cells from six independent experiments of NF-κB, NFAT and AP-1 are shown. Statistics by one-way ANOVA, followed by Tukey's multiple comparison post test (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ns: not significant). D) Comparison of the inhibitory effect of 200 μM Cyclosporin A on NF-κB and NFAT via CD2 and CD28. Inhibition is expressed as decrease of reporter induction in TPR cells stimulated with TCS-CD80 or TCS-CD58 upon addition of CsA. NF-κB ***P = 0.0003 and NFAT ns P = 8990 (two-tailed paired t-test), TCS expressing CD80 compared to TCS expressing CD58.

TPR cells significantly. Antibodies to PD-L1 or HVEM fully blocked the inhibitory effects of these molecules on the TCS. PD-1 and BTLA are not endogenously expressed on Jurkat cells and consequently control TCS and PDL1 or HVEM expressing TCS induced similar level of NF-κB, NFAT and AP-1 activity in the control TPR cells (Suppl. Fig. 2 B, C).

4. Discussion

T cell activation and differentiation are processes governed by a tightly regulated transcriptional network. It is well established that activation of NF-κB, NFAT and AP-1 drives proliferation and cytokine

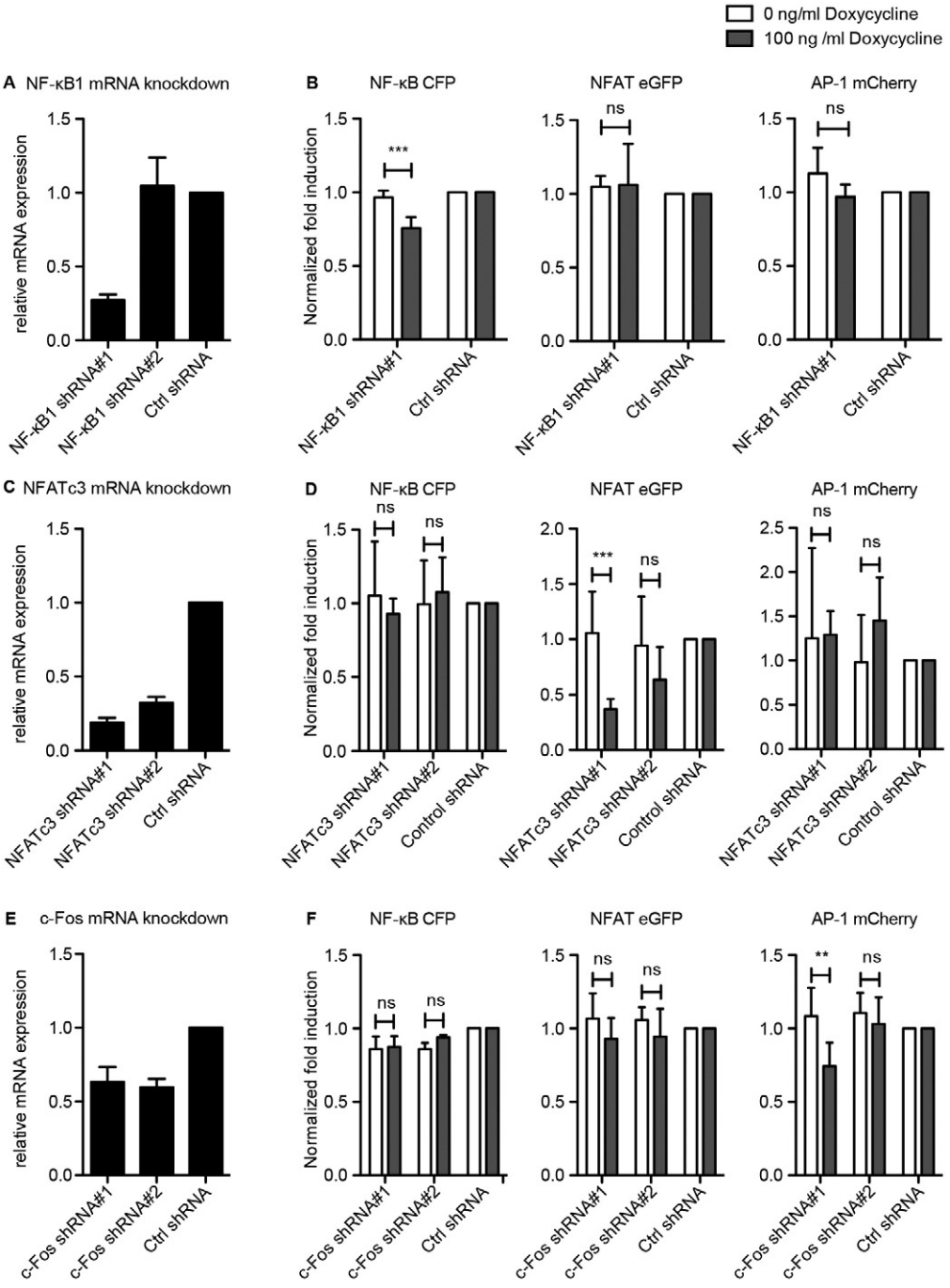

Fig. 4. Knockdown of NF-κB1, NFATc3 or c-Fos and analysis of its effect on the transcription factor activity of NF-κB, NFAT and AP-1. A) Relative NF-κB1 mRNA expression in TPR cells expressing NF-κB1 shRNA#1 or NF-κB1 shRNA#2 normalized to TPR cells expressing Ctrl shRNA. B) Reporter assay to investigate the effect of NF-κB1 knockdown on the transcription factor activity of NF-κB, NFAT and AP-1. ShRNA was induced with 100 ng/ml doxycycline. TPR cells expressing NF-κB1 shRNA#1 or Ctrl shRNA were cocultivated with TCS-CD80. Summary of the normalized fold induction of gMFI of NF-κB, NFAT and AP-1 reporter expression from five independent experiments is shown. Statistics by two-way ANOVA, followed by Bonferroni post tests (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ns P > 0.05). C) Relative NFATc3 mRNA expression in TPR cells expressing NFATc3 shRNA#1 or NFATc3 shRNA#2 normalized to TPR cells expressing Ctrl shRNA. D) Reporter assay to investigate the effect of NFATc3 knockdown on the transcription factor activity of NF-κB, NFAT and AP-1. ShRNA expression was induced with 100 ng/ml doxycycline. TPR cells expressing NFATc3 shRNA#1, NFATc3 shRNA#2 or Ctrl shRNA were cocultivated with TCS-CD80. Summary of the normalized fold induction of gMFI of NF-κB, NFAT and AP-1 reporter expression from seven independent experiments is shown. Statistics by two-way ANOVA, followed by Bonferroni post tests (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ns P > 0.05). E) Relative c-Fos mRNA expression in TPR cells expressing c-Fos shRNA#1 or c-Fos shRNA#2 normalized to TPR cells expressing Ctrl shRNA. F) Reporter assay to investigate the effect of c-Fos knockdown on the transcription factor activity of NF-κB, NFAT and AP-1. ShRNA expression was induced with 100 ng/ml doxycycline. TPR cells expressing c-Fos shRNA#1, c-Fos shRNA#2 or Ctrl shRNA were cocultivated with TCS-CD80. Summary of the normalized fold induction of gMFI of NF-κB, NFAT and AP-1 reporter expression from four independent experiments is shown. Statistics by two-way ANOVA, followed by Bonferroni post tests (**P ≤ 0.01; ***P ≤ 0.001; ns P > 0.05).

production in T cells. The Jurkat T cell line is a widely used model to study T cell signaling and the activation of transcription factors in response to external stimuli (Abraham and Weiss, 2004). The availability of fluorescent proteins with non-overlapping emission spectra prompted us to develop a triple parameter Jurkat reporter, where response elements for NF-κB, NFAT and AP-1 were coupled to CFP, mCherry and GFP, respectively. We could demonstrate that reporter gene induction is both a function of the strength and the quality of

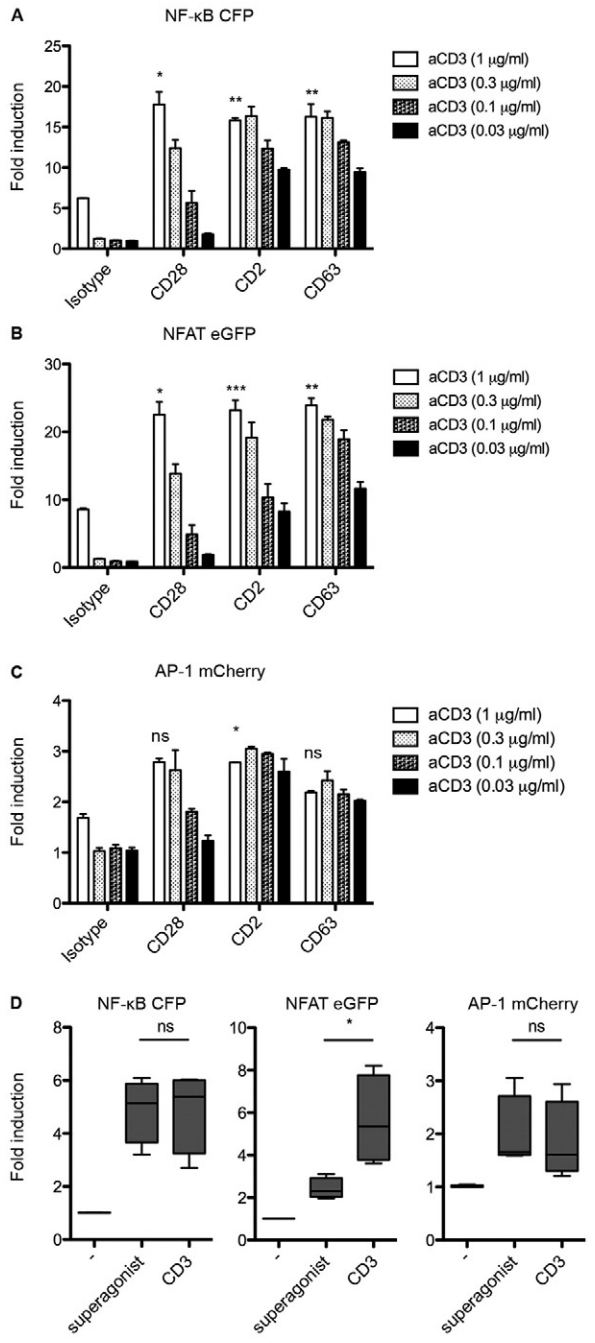

Fig. 5. A) Effect of CD28, CD2 and CD63 antibodies on the transcription factor activity of NF-κB, B) NFAT and C) AP-1. Different concentrations of aCD3 were used in combination with isotype, CD28, CD2 and CD63 antibodies to stimulate the TPR cells. Experiment shown is representative for six independently performed experiments. Mean and SD are shown. Statistics of TPR cells stimulated with 1 μg/ml aCD3 by one-way ANOVA, followed by Tukey's multiple comparison post test (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ns: not significant). D) Effect of superagonistic CD28 antibody on NF-κB, NFAT and AP-1. Summary of the normalized fold induction of gMFI of the TPR cells compared to unstimulated cells from four independent experiments. Statistics by one-way ANOVA, followed by Tukey's multiple comparison post test (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ns P > 0.05).

stimuli applied. Well-characterized agents were used to validate our system: PMA potently stimulated NF-κB and AP-1 reporters and induction of the NFAT reporter was dependent on the presence of the calcium ionophore Ionomycin. In agreement with published data we found that only in the presence of PMA the addition of Ionomycin led to the full activation of the NFAT reporter (Boss et al., 1996; Northrop et al., 1993; Wu et al., 1995). In line with well-established effects of CsA on NFAT and NF-κB activity we observed that this calcineurin inhibitor strongly inhibited NFAT reporter activity whereas NF-κB was affected to a much lesser extent (Flanagan et al., 1991; Frantz et al., 1994). By contrast the immunosuppressive agent Rapamycin, which acts on the mTor pathway (Ballou and Lin, 2008) had no significant effect on the reporter activity (data not shown). This was also observed with single reporter cells carrying responsive elements for NFAT, NF-κB or AP-1 controlling the expression of eGFP (Ratzinger et al., 2014). In addition, the specificity of the constructs used in our reporter cells was corroborated by shRNA-mediated knockdown of NFATc3, NF-κB and c-Fos expression.

We have previously devised a system termed T cell stimulators (TCS) to activate human T cells in the presence of costimulatory ligands of choice. Here we have used the TCS to study the effects of costimulatory signals on the NF-κB, NFAT and AP-1 reporter gene expression. We show that engagement of the primary costimulatory receptor CD28 by CD80 potently enhances induction of all three reporters. CD2 signals triggered through CD58 expressing TCS have previously been shown to generate potent activating signals in human T cells (Leitner et al., 2010b; Leitner et al., 2015) and in line with these results strong reporter gene expression was induced by this costimulatory pathway. However CD2 signals are less potent than CD28 signals and the lower stimulatory capacity of this costimulatory pathway was reflected by a significantly reduced upregulation of NF-κB reporter activity. T cells receiving CD28 signals are less sensitive to CsA inhibition (Hess and Bright, 1991; June et al., 1987) and we have previously shown that the proliferation of primary human T cells stimulated in the presence of CD80 is less sensitive to CsA inhibition than T cells stimulated by CD58 (Leitner et al., 2011). In our reporter assays we found that CsA treatment differentially affected NF-κB activity but not NFAT activity in CD28 and CD2 costimulated TPR cells. Thus, it is possible that the lower sensitivity of CD28-costimulated T cells towards CsA is a result of a reduced capacity of CsA to inhibit NF-κB in presence of CD28 signals.

Immobilized agonistic or soluble mAb are widely used to trigger costimulatory receptors on T cells and they are essential tools for functional studies on surface receptors for which no natural ligands have been described. We have previously reported on a mAb designated 11C9 that is equally potent as agonistic CD28 mAb to costimulate proliferation and cytokine production in anti-CD3 activated primary human T cells (Pfistershammer et al., 2004). The antigen targeted by this mAb was subsequently identified as CD63 and we have found that the potent costimulatory properties are a unique feature of clone 11C9 as they were not observed with other CD63 mAb. Here we have compared this mAb with CD2 and CD28 mAb regarding its capability to costimulate reporter gene expression in our TPR cells when coimmobilized with anti-CD3 mAb. In line with our previous results that describe potent costimulation via clone 11C9 we have observed that triggering CD63 can enhance NF-κB, NFAT and AP-1 activity as efficient as ligation of the costimulatory receptors CD2 or CD28 with agonistic antibodies. These experiments indicate that the TPR cells are equally suited to detect signals generated upon interaction of natural ligands or agonistic antibodies with costimulatory receptors expressed on the Jurkat T cell line.

However Jurkat cells express a limited set of costimulatory receptors and it was of interest to determine whether cosignaling molecules are functional when expressed on the TPR cells. To this end we have expressed activating (4-1BB) as well as inhibitory (PD-1 and BTLA) cosignaling molecules on the TPR cells and used T cell stimulator cells expressing their ligands to trigger these receptors along with the TCR-complex. We found that 4-1BB signals strongly upregulated NF-κB and AP-1 activity. In contrast, NFAT transcription factor activity decreased upon signaling via 4-1BB. This may be due to the fact that 4-1BB signaling is mediated by TRAF2, which in turn is described to inhibit NFAT-mediated transcription via NFAT-interacting protein (NIP45) (Lieberson et al., 2001; Saoulli et al., 1998). It is generally accepted that CD28 is a

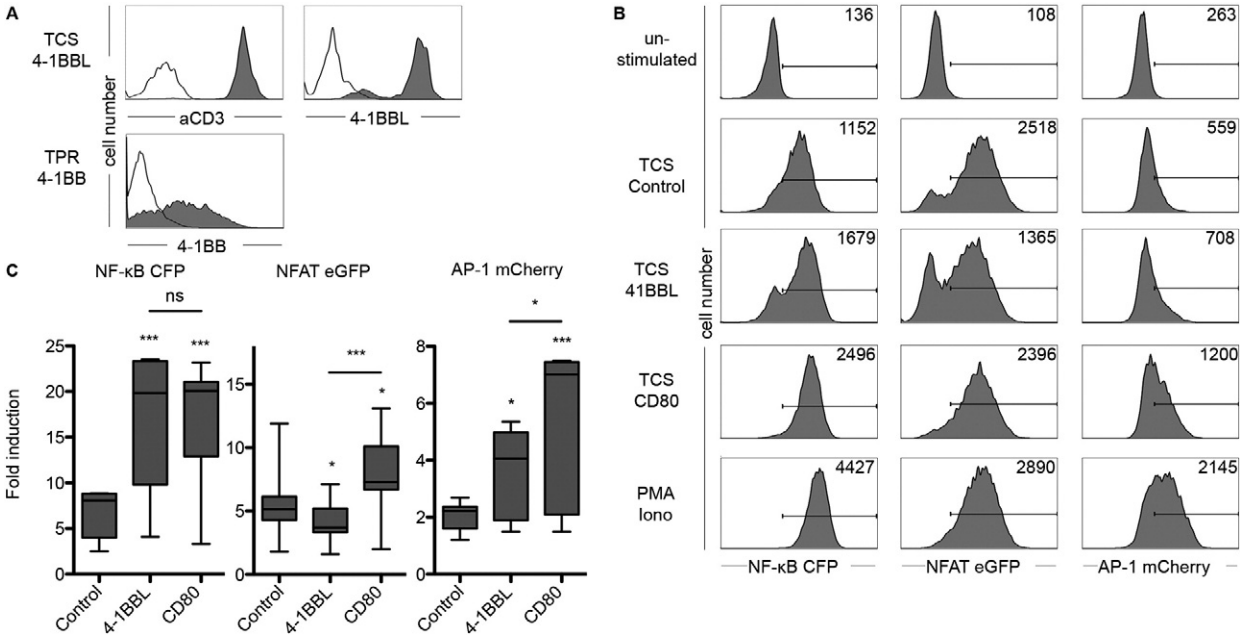

Fig. 6. Effect of signaling via 4-1BB on the transcription factor activity of NF-κB, NFAT and AP-1. A) FACS analysis of the expression of surface molecules on TCS and TPR cells. Open histograms: control cells; filled histograms: TCS or TPR cells expressing the respective molecules. B) Reporter assay to investigate the effect of signaling via 4-1BB on the transcription factor activity of NF-κB, NFAT and AP-1. TPR cells expressing 4-1BB were cocultivated with TCS coexpressing 4-1BBL for 24 h. Activation of NF-κB, NFAT and AP-1 was measured by FACS analysis. Numbers show gMFI. Experiment shown is representative for eleven independently performed experiments. C) Summary of the normalized fold induction of gMFI of the TPR cells compared to unstimulated cells from eleven independent experiments. Statistics by one-way ANOVA, followed by Tukey's multiple comparison post test (*P ≤ 0.05; ***P ≤ 0.001; ns P > 0.05).

stronger costimulator than 4-1BB and we have previously found that TCS expressing CD80 induced higher proliferation in primary human T cells than TCS expressing 4-1BBL (Leitner et al., 2010b; Kober et al., 2008). In line with these results we observed stronger upregulation of reporter gene expression in TPR cells stimulated via CD28 than via 4-1BB. Moreover, we have observed that ligation of the coinhibitory receptors PD-1 and BTLA on the TPR cells resulted in a strong downregulation of NF-κB and NFAT whereas BTLA signals were more potent in reducing AP-1 activity. Thus also molecules not naturally expressed in Jurkat cells can be studied regarding their capacity to induce or attenuate the activity of the transcription factors NF-κB, NFAT or AP-1 when introduced into the TPR cells. Absence of receptors of interest even offers additional possibilities e.g. to introduce receptors with mutated cytoplasmic domains to dissect the intracellular motifs that are critically involved in the generation of activating or inhibitory signals upon receptor engagement. TCS are well suited to provide costimulatory signals to the TPR cells since they display costimulatory ligands in the context of a cell surface and thus allow for a "natural" receptor–ligand interaction. We found that the TCS-TPR system is a sensitive tool to detect the effects of antibodies, which were previously described to block inhibitory pathways (Brown et al., 2003; Peretz et al., 2012; Leitner et al., 2013). Interfering with T cell inhibitory pathways has already been demonstrated to efficiently increase the anti-tumor response in cancer patients (Hamid et al., 2013; Hodi et al., 2010). Consequently, there are currently intense efforts to target so-called checkpoint inhibitors for the treatment of melanoma patients but also for the therapy of other malignancies (Buchbinder and McDermott, 2015; Johnson et al., 2015). In addition to established pathways engaging CTLA-4 or PD-1 such efforts are focusing on emerging pathways like BTLA, TIGIT or 2B4 (CD244) (Pauken and Wherry, 2014; Pardoll, 2012). Our preliminary data indicate that these molecules are functional when introduced in the Jurkat T cell line (Fig. 6 and data not shown). Currently, the biology of most of these novel checkpoint inhibitors is not fully understood. TPR cells in conjunction with TCS can be used as a versatile platform to investigate the impact of such inhibitory pathways on the activation of NF-κB, NFAT or AP-1 in a well-controlled reductionist system. Keeping limitations of studies with transformed T cell lines in mind such studies should be complemented with studies on primary T cells, including T cells that display an exhausted phenotype. Moreover, the TPR cells in conjunction with TCS might be especially valuable for pre-assessing antibodies regarding their ability to efficiently interfere with T cell coinhibitory pathways. They can be used to screen panels of antibodies to coinhibitory ligands or receptors to identify the most promising candidate reagents for profiling in more complex and time consuming assays with primary T cells.

Although the experiments performed in this study focus on the use of the TPR cells to study T cell costimulation, the utility of these cells is obviously not limited to such applications. They could f.i. be used to profile immunosuppressive compounds regarding their effects on NF-κB, NFAT and AP-1 activation in T cell activation or improve chimeric antigen receptor (CAR) design.

T cells armed with chimeric antigen receptors "CARs" that target surface molecules expressed on leukemias or other cancers have great potential to fight malignant cells when adoptively transferred into cancer patients (Turtle et al., 2012; Cheadle et al., 2014). There are continuous efforts to equip CARs with signaling domains that endow them with improved responsiveness and effector function towards cells harboring their target antigens. In this context TPR cells might be an exquisite tool to identify signaling domains that potently activate NF-κB, NFAT and AP-1 and thus might result in improved functionality of the resulting CAR molecules.

In conclusion, our results illustrate the usefulness of fluorescent protein based triple parameter reporter cells to gain a better insight in the transcriptional network that drives cellular activation. In conjunction with TCS, the Jurkat TPR cells allow to study virtually all T cell costimulatory and coinhibitory pathways regarding their impact on NF-κB, NFAT and AP-1 activity. In addition, the TPR cells are an effective tool for the identification and characterization of agents that enhance or suppress T cell activation processes.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jim.2016.01.007.

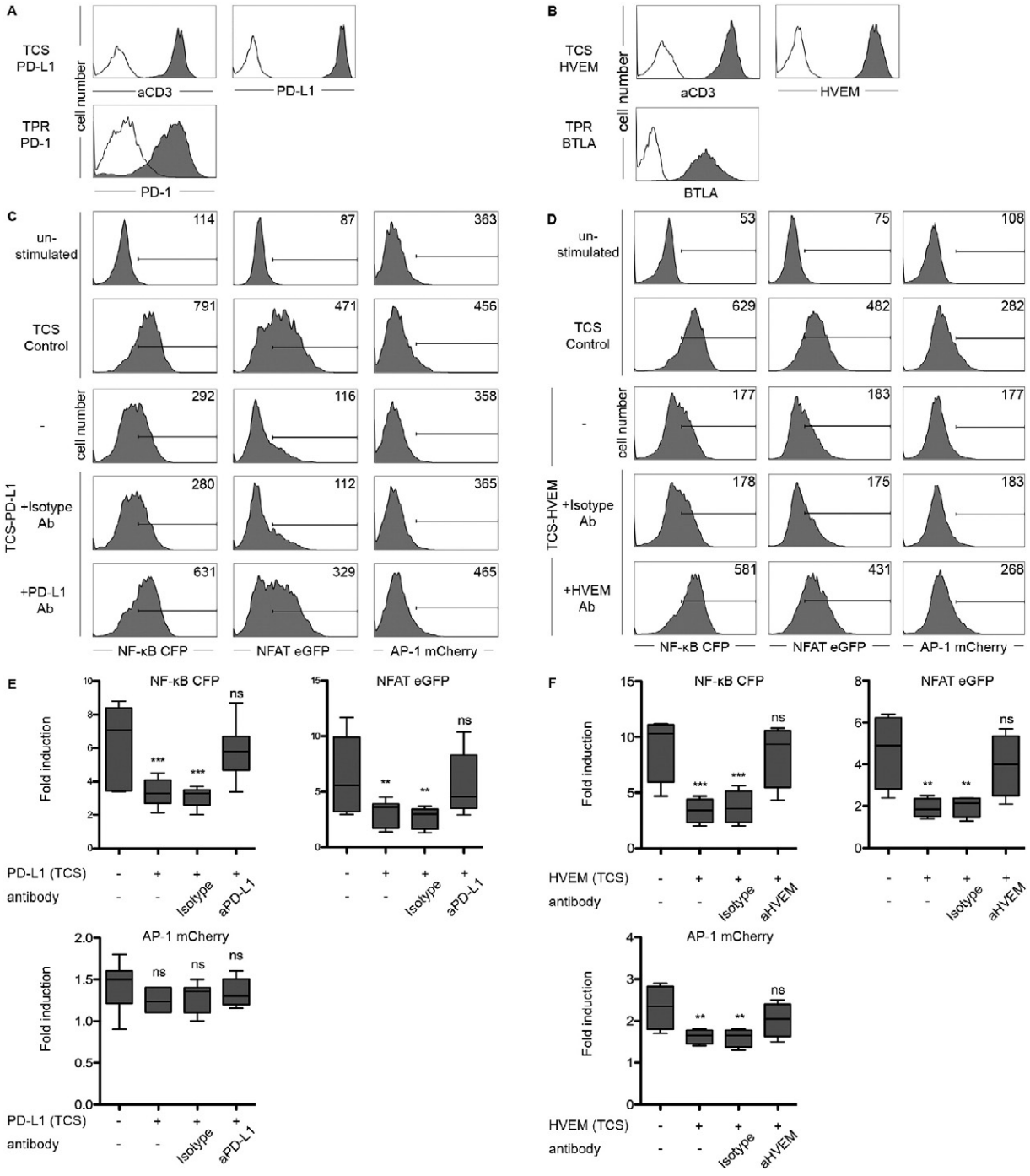

Fig. 7. Effect of signaling via PD-1 and BTLA on the transcription factor activity of NF-κB, NFAT and AP-1. A, B) FACS analysis of the expression of surface molecules on TCS and TPR cells. Open histograms: control cells; filled histograms: TCS or TPR cells expressing the respective molecules. C) Reporter assay to investigate the effect of signaling via PD-1 on the transcription factor activity of NF-κB, NFAT and AP-1. TPR cells expressing PD-1 were cocultivated with TCS coexpressing PD-L1. Anti-PD-L1 and isotype control antibody were used at a final concentration of 5 μg/ml. Activation of NF-κB, NFAT and AP-1 was analyzed by FACS. Numbers show gMFI. Experiment shown is representative for seven independently performed experiments. D) Reporter assay to investigate the effect of signaling via BTLA on the transcription factor activity of NF-κB, NFAT and AP-1. TPR cells expressing BTLA were cocultivated with TCS expressing HVEM. Anti-HVEM and isotype control antibody were used at a final concentration of 5 μg/ml. Activation of NF-κB, NFAT and AP-1 was analyzed by FACS. Numbers show gMFI. Experiment shown is representative for four independently performed experiments. E) Summary of the normalized fold induction of gMFI of the TPR cells compared to unstimulated cells from seven independent experiments. NF-κB, NFAT and AP-1. Statistics by one-way ANOVA, followed by Tukey's multiple comparison post test (*$P \le 0.05$; **$P \le 0.01$; ***$P \le 0.001$; ns $P > 0.05$). F) Summary of the normalized fold induction of gMFI of the TPR cells compared to unstimulated cells from four independent experiments of NF-κB, NFAT and AP-1. Statistics by one-way ANOVA, followed by Tukey's multiple comparison post test (**$P \le 0.01$; ***$P \le 0.001$; ns $P > 0.05$).

Potential conflict of interest

SJ, JL and PS have entered into negotiations for marketing and distribution of reporter cell lines.

Acknowledgments

This work was supported by the Austrian Science Fund (FWF, project 21964-B20). We appreciate the excellent technical assistance of Claus

Wenhardt and Petra Cejka. We wish to acknowledge Herbert Strobl, Philip Schatzlmeier and Hannes Stockinger for providing constructs and cell lines.

References

Abraham, R.T., Weiss, A., 2004. Jurkat T cells and development of the T-cell receptor signalling paradigm. Nat. Rev. Immunol. 4 (4), 301–308.

Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R.J., Rahmsdorf, H.J., et al., 1987. Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. Cell 49 (6), 729–739.

Ballou, L.M., Lin, R.Z., 2008. Rapamycin and mTOR kinase inhibitors. J. Chem. Biol. 1 (1–4), 27–36.

Boss, V., Talpade, D.J., Murphy, T.J., 1996. Induction of NFAT-mediated transcription by Gq-coupled receptors in lymphoid and non-lymphoid cells. J. Biol. Chem. 271 (18), 10429–10432.

Brown, J.A., Dorfman, D.M., Ma, F.R., Sullivan, E.L., Munoz, O., Wood, C.R., et al., 2003. Blockade of programmed death-1 ligands on dendritic cells enhances T cell activation and cytokine production. J. Immunol. 170 (3), 1257–1266.

Buchbinder, E.I., McDermott, D.F., 2015. Cytotoxic T-lymphocyte antigen-4 blockade in melanoma. Clin. Ther. 37 (4), 755–763.

Cheadle, E.J., Gornall, H., Baldan, V., Hanson, V., Hawkins, R.E., Gilham, D.E., 2014. CAR T cells: driving the road from the laboratory to the clinic. Immunol. Rev. 257 (1), 91–106.

Chen, L., Flies, D.B., 2013. Molecular mechanisms of T cell co-stimulation and co-inhibition. Nat. Rev. Immunol. 13 (4), 227–242.

Crabtree, G.R., Clipstone, N.A., 1994. Signal transmission between the plasma membrane and nucleus of T lymphocytes. Annu. Rev. Biochem. 63, 1045–1083.

DeBenedette, M.A., Shahinian, A., Mak, T.W., Watts, T.H., 1997. Costimulation of CD28- T lymphocytes by 4-1BB ligand. J. Immunol. 158 (2), 551–559.

Derdak, S.V., Kueng, H.J., Leb, V.M., Neunkirchner, A., Schmetterer, K.G., Bielek, E., et al., 2006. Direct stimulation of T lymphocytes by immunosomes: virus-like particles decorated with T cell receptor/CD3 ligands plus costimulatory molecules. Proc. Natl. Acad. Sci. U. S. A. 103 (35), 13144–13149.

Flanagan, W.M., Corthesy, B., Bram, R.J., Crabtree, G.R., 1991. Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. Nature 352 (6338), 803–807.

Frantz, B., Nordby, E.C., Bren, G., Steffan, N., Paya, C.V., Kincaid, R.L., et al., 1994. Calcineurin acts in synergy with Pma to inactivate I-kappa-B/Mad3, an inhibitor of Nf-kappa-B. EMBO J. 13 (4), 861–870.

Hamid, O., Robert, C., Daud, A., Hodi, F.S., Hwu, W.J., Kefford, R., et al., 2013. Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. N. Engl. J. Med. 369 (2), 134–144.

Hess, A.D., Bright, E.C., 1991. The effect of the CD28 activation pathway on the immunosuppressive action of cyclosporine. Transplantation 51 (6), 1232–1240.

Hodi, F.S., O'Day, S.J., McDermott, D.F., Weber, R.W., Sosman, J.A., Haanen, J.B., et al., 2010. Improved survival with ipilimumab in patients with metastatic melanoma. N. Engl. J. Med. 363 (8), 711–723.

Jain, J., McCaffrey, P.G., Valge-Archer, V.E., Rao, A., 1992. Nuclear factor of activated T cells contains Fos and Jun. Nature 356 (6372), 801–804.

Johnson, D.B., Peng, C., Sosman, J.A., 2015. Nivolumab in melanoma: latest evidence and clinical potential. Ther. Adv. Med. Oncol. 7 (2), 97–106.

Jorgl, A., Platzer, B., Taschner, S., Heinz, L.X., Hocher, B., Reisner, P.M., et al., 2007. Human Langerhans-cell activation triggered in vitro by conditionally expressed MKK6 is counterregulated by the downstream effector RelB. Blood 109 (1), 185–193.

June, C.H., Ledbetter, J.A., Gillespie, M.M., Lindsten, T., Thompson, C.B., 1987. T-cell proliferation involving the CD28 pathway is associated with cyclosporine-resistant interleukin 2 gene expression. Mol. Cell. Biol. 7 (12), 4472–4481.

Khalaf, H., Jass, J., Olsson, P.E., 2010. Differential cytokine regulation by NF-kappaB and AP-1 in Jurkat T-cells. BMC Immunol. 11, 26.

Kober, J., Leitner, J., Klauser, C., Woitek, R., Majdic, O., Stockl, J., et al., 2008. The capacity of the TNF family members 4-1BBL, OX40L, CD70, GITRL, CD30L and LIGHT to costimulate human T cells. Eur. J. Immunol. 38 (10), 2678–2688.

Leitner, J., Klauser, C., Pickl, W.F., Stockl, J., Majdic, O., Bardet, A.F., et al., 2009. B7-H3 is a potent inhibitor of human T-cell activation: no evidence for B7-H3 and TREML2 interaction. Eur. J. Immunol. 39 (7), 1754–1764.

Leitner, J., Grabmeier-Pfistershammer, K., Steinberger, P., 2010a. Receptors and ligands implicated in human T cell costimulatory processes. Immunol. Lett. 128 (2), 89–97.

Leitner, J., Kuschei, W., Grabmeier-Pfistershammer, K., Woitek, R., Kriehuber, E., Majdic, O., et al., 2010b. T cell stimulator cells, an efficient and versatile cellular system to assess the role of costimulatory ligands in the activation of human T cells. J. Immunol. Methods 362 (1–2), 131–141.

Leitner, J., Drobits, K., Pickl, W.F., Majdic, O., Zlabinger, G., Steinberger, P., 2011. The effects of Cyclosporine A and azathioprine on human T cells activated by different costimulatory signals. Immunol. Lett. 140 (1–2), 74–80.

Leitner, J., Rieger, A., Pickl, W.F., Zlabinger, G., Grabmeier-Pfistershammer, K., Steinberger, P., 2013. TIM-3 does not act as a receptor for galectin-9. PLoS Pathog. 9 (3), e1003253.

Leitner, J., Herndler-Brandstetter, D., Zlabinger, G.J., Grubeck-Loebenstein, B., Steinberger, P., 2015. CD58/CD2 is the primary costimulatory pathway in human CD28-CD8+ T cells. J. Immunol.

Li, Q., Verma, I.M., 2002. NF-kappaB regulation in the immune system. Nat. Rev. Immunol. 2 (10), 725–734.

Lieberson, R., Mowen, K.A., McBride, K.D., Leautaud, V., Zhang, X., Suh, W.K., et al., 2001. Tumor necrosis factor receptor-associated factor (TRAF)2 represses the T helper cell type 2 response through interaction with NFAT-interacting protein (NIP45). J. Exp. Med. 194 (1), 89–98.

Macian, F., 2005. NFAT proteins: key regulators of T-cell development and function. Nat. Rev. Immunol. 5 (6), 472–484.

Magness, S.T., Bataller, R., Yang, L., Brenner, D.A., 2004. A dual reporter gene transgenic mouse demonstrates heterogeneity in hepatic fibrogenic cell populations. Hepatology 40 (5), 1151–1159.

Matsuda, T., Cepko, C.L., 2004. Electroporation and RNA interference in the rodent retina in vivo and in vitro. Proc. Natl. Acad. Sci. U. S. A. 101 (1), 16–22.

Noguchi, T., Ikeda, M., Ohmiya, Y., Nakajima, Y., 2008. Simultaneous monitoring of independent gene expression patterns in two types of cocultured fibroblasts with different color-emitting luciferases. BMC Biotechnol. 8, 40.

Northrop, J.P., Ullman, K.S., Crabtree, G.R., 1993. Characterization of the nuclear and cytoplasmic components of the lymphoid-specific nuclear factor of activated T cells (NF-AT) complex. J. Biol. Chem. 268 (4), 2917–2923.

Pardoll, D.M., 2012. The blockade of immune checkpoints in cancer immunotherapy. Nat. Rev. Cancer 12 (4), 252–264.

Pauken, K.E., Wherry, E.J., 2014. TIGIT and CD226: tipping the balance between costimulatory and coinhibitory molecules to augment the cancer immunotherapy toolkit. Cancer Cell 26 (6), 785–787.

Peretz, Y., He, Z., Shi, Y., Yassine-Diab, B., Goulet, J.P., Bordi, R., et al., 2012. CD160 and PD-1 co-expression on HIV-specific CD8 T cells defines a subset with advanced dysfunction. PLoS Pathog. 8 (8), e1002840.

Pfistershammer, K., Majdic, O., Stockl, J., Zlabinger, G., Kirchberger, S., Steinberger, P., et al., 2004. CD63 as an activation-linked T cell costimulatory element. J. Immunol. 173 (10), 6000–6008.

Pfistershammer, K., Klauser, C., Pickl, W.F., Stockl, J., Leitner, J., Zlabinger, G., et al., 2006. No evidence for dualism in function and receptors: PD-L2/B7-DC is an inhibitory regulator of human T cell activation. Eur. J. Immunol. 36 (5), 1104–1113.

Platzer, B., Jorgl, A., Taschner, S., Hocher, B., Strobl, H., 2004. RelB regulates human dendritic cell subset development by promoting monocyte intermediates. Blood 104 (12), 3655–3663.

Popow, I., Leitner, J., Grabmeier-Pfistershammer, K., Majdic, O., Zlabinger, G.J., Kundi, M., et al., 2013. A comprehensive and quantitative analysis of the major specificities in rabbit antithymocyte globulin preparations. Am. J. Transplant. 13 (12), 3103–3113.

Ratzinger, F., Haslacher, H., Poeppl, W., Hoermann, G., Kovarik, J.J., Jutz, S., et al., 2014. Azithromycin suppresses CD4(+) T-cell activation by direct modulation of mTOR activity. Sci. Rep. 4, 7438.

Ruff, V.A., Leach, K.L., 1995. Direct demonstration of NFATp dephosphorylation and nuclear localization in activated HT-2 cells using a specific NFATp polyclonal antibody. J. Biol. Chem. 270 (38), 22602–22607.

Saoulli, K., Lee, S.Y., Cannons, J.L., Yeh, W.C., Santana, A., Goldstein, M.D., et al., 1998. CD28-independent, TRAF2-dependent costimulation of resting T cells by 4-1BB ligand. J. Exp. Med. 187 (11), 1849–1862.

Schichl, Y.M., Resch, U., Hofer-Warbinek, R., de Martin, R., 2009. Tristetraprolin impairs NF-kappaB/p65 nuclear translocation. J. Biol. Chem. 284 (43), 29571–29581.

Shaulian, E., Karin, M., 2002. AP-1 as a regulator of cell life and death. Nat. Cell Biol. 4 (5), E131–E136.

Steinberger, P., Majdic, O., Derdak, S.V., Pfistershammer, K., Kirchberger, S., Klauser, C., et al., 2004. Molecular characterization of human 4Ig-B7-H3, a member of the B7 family with four Ig-like domains. J. Immunol. 172 (4), 2352–2359.

Turtle, C.J., Hudecek, M., Jensen, M.C., Riddell, S.R., 2012. Engineered T cells for anti-cancer therapy. Curr. Opin. Immunol. 24 (5), 633–639.

Vodjdani, G., Coulombel, C., Doly, J., 1988. Structure and characterization of a murine chromosomal fragment containing the interferon beta gene. J. Mol. Biol. 204 (2), 221–231.

Waibler, Z., Sender, L.Y., Merten, C., Hartig, R., Kliche, S., Gunzer, M., et al., 2008. Signaling signatures and functional properties of anti-human CD28 superagonistic antibodies. PLoS One 3 (3), e1708.

Watts, T.H., 2005. TNF/TNFR family members in costimulation of T cell responses. Annu. Rev. Immunol. 23, 23–68.

Wiederschain, D., Wee, S., Chen, L., Loo, A., Yang, G., Huang, A., et al., 2009. Single-vector inducible lentiviral RNAi system for oncology target validation. Cell Cycle 8 (3), 498–504.

Wu, J., Katzav, S., Weiss, A., 1995. A functional T-cell receptor signaling pathway is required for p95vav activity. Mol. Cell. Biol. 15 (8), 4337–4346.