Critical Role for TNF in the Induction of Human Antigen-Specific Regulatory T Cells by Tolerogenic Dendritic Cells

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*J Immunol* 2010; 185:1412-1418; Prepublished online 23 June 2010; doi: 10.4049/jimmunol.1000560

http://www.jimmunol.org/content/185/3/1412
Critical Role for TNF in the Induction of Human Antigen-Specific Regulatory T Cells by Tolerogenic Dendritic Cells

Fleur S. Kleijwegt,* Sandra Laban,* Gaby Duinkerken,* Antoinette M. Joosten,* Arnaud Zaldumbide,† Tatjana Nikolic,* and Bart O. Roep*

TNF is a pleiotropic cytokine with differential effects on immune cells and diseases. Anti-TNF therapy was shown to be effective in rheumatoid arthritis but proved inefficient or even detrimental in other autoimmune diseases. We studied the role of TNF in the induction of Ag-specific regulatory T cells (Tregs) by tolerogenic vitamin D3-modulated human dendritic cells (VD3-DCs), which previously were shown to release high amounts of soluble TNF (sTNF) upon maturation with LPS. First, production of TNF by modulated VD3-DCs was analyzed upon maturation with LPS or CD40L with respect to both secreted (cleaved) TNF (sTNF) and expression of the membrane-bound (uncleaved) form of TNF (mTNF). Next, TNF antagonists were tested for their effect on induction of Ag-specific Tregs by modulated DCs and the subsequent functionality of these Tregs. VD3-DCs expressed greater amounts of mTNF than did control DCs (nontreated DCs), independent of the maturation protocol. Inhibition of TNF with anti-TNF Ab (blocking both sTNF and mTNF) during the priming of Tregs with VD3-DCs prevented generation of Tregs and their subsequent suppressive function. These data point to a specific role for mTNF on VD3-DCs in the induction of Ag-specific Tregs.

The online version of this article contains supplemental material. Abbreviations used in this paper: DC, dendritic cell; mTNF, membrane-bound TNF; sTNF, soluble TNF; sTNFRII, soluble TNFR II; T1D, type 1 diabetes; Tcontrol, control T cell; Teff, effector T cell; Treg, regulatory T cell; VD3-DC, vitamin D3-modulated dendritic cell.
DCs) have several immunoregulatory features, such as high programmed death ligand 1 (PD-L1, CD274)/CD86 ratio, an anti-inflammatory cytokine profile, and specific autoreactive T cell killing (22, 23). Most importantly, they induce Ag-specific Tregs. Because VD3-DCs secrete high amounts of TNF upon maturation (23), we investigated the role of TNF in the induction of Ag-specific Tregs by VD3-DCs. We demonstrate in this paper that the qualitative difference caused by modulation with vitamin D3 is not TNF secretion (sTNF) but the presence of TNF on the surface of VD3-DCs (mTNF). Furthermore, we show for the first time that mTNF is critical for the induction of suppressive T cells by VD3-DCs through mTNF–TNFRII interaction, pointing to the important function of mTNF in the induction of immune tolerance.

Materials and Methods

Generation of human VD3-DCs

Human PBMC were isolated by Ficoll gradient from HLA-typed buffy coats, obtained from healthy blood donors. Monocytes were purified from PBMC via positive selection, using CD14-MicroBeads according to the manufacturer’s protocol (Miltenyi Biotec, Auburn, CA). Subsequently, monocytes were seeded in six-well tissue culture plates (Costar, Cambridge, MA) at a density of 2 × 10^6 cells per well and cultured for 6 d in RPMI 1640 medium supplemented with 8% FCS (Greiner, Wemmel, Belgium), human rIL-4 (500 U/ml; Invitrogen), Breda, The Netherlands), and human rGM-CSF (800 U/ml; Invitrogen). On day 3, the culture medium, including the supplements, was refreshed. On day 6, the resulting immature DCs were activated either by the addition of 100 ng/ml LPS (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) or via CD40 triggering by incubating 0.5 × 10^6 DCs with 0.2 × 10^6 CD40L-expressing L cells. Activated mature DCs were harvested for further analysis after 48 h. VD3-DCs (Sigma-Aldrich) were generated by addition of 10^−8 vitamin D3 on day 0 and day 3. For analyzing the effect of TNF on DCs, mature nontreated DCs (NT-DCs) and VD3-DCs were treated for 48 h with 10 μg/ml anti-TNF and 800 U/ml GM-CSF.

Abs and flow cytometry

Anti-TNF mAb (infliximab, Schering-Plough BV, Utrecht, The Netherlands) was labeled with FITC (Calbiochem, La Jolla, CA) and used to detect the expression of mTNF on cells. Purified rat anti-human CD120b (anti-TNFRII, clone bTNFR-M1), CD4-APC (clone YTS191-1), CD62L-PE (clone SK11), CD134-PE (clone ACT-35), CD25-FITC (clone 2A3), anti-CD80-PE (clone L307.4), anti-CD86-PE (clone IT2.2), and isotype controls IgG2b rat anti-human [clone R35-38], anti-HLA-DR-PE [clone G46-6], anti-CD11c-PE [clone H1-19], anti-CD14-FITC [clone M522], anti-CD40-FITC [clone SC5], anti-CD45R0-PE [clone B-ly6], and IgG1 PE and FITC [clone x40]) were all obtained from BD Pharmingen (San Diego, CA). Anti–CD274-PE (PD-L1; clone MIH1) was obtained from eBioscience (San Diego, CA).

For in vitro culture, NaNO₂ was removed from anti-CD20b and IgG2bκ by gel filtration, using NAP-10 gel filtration columns containing Sephadex G-25 DNA grade media (Amersham Pharmacia Biotech AB, Uppsala, Sweden). End concentration was measured by Bradford assay. Apoptosis was measured by the Annexin V-FITC kit from Miltenyi Biotec (Auburn, CA). Experiments were performed according to the manufacturer’s protocol.

Flow cytometric staining was analyzed on a FACSCalibur (BD Biosciences, San Jose, CA). Analyses were performed with FlowJo 7.5 (Tree Star, Ashland, OR) and WinMDI 2.8.

Induction of Tregs

Naïve CD4+ T cells were incubated 10, 30, or 60 min with TNF (20 ng/ml, kindly provided by DanDrit Biotech, Copenhagen, Denmark), anti-TNFRII (10 μg/ml), or PMA (20 ng/ml)/ionomycin (0.8 μM). Cells were lysed on ice in lysis buffer containing 25 mM HEPES, 25 mM KCl, 25 mM NaF, 5 mM NaPi, 5 mM b-glycerophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM Na3VO4, 1% Nonidet P-40+ protease inhibitors (Roche Diagnostics, Mannheim, Germany). Expression of proteins was determined by SDS-PAGE Western blotting. Phosphorylation level of NF-κB has been determined using an anti-Ser536 phospho NF-κB antibody (Cell Signaling Technology, Beverly, MA). An anti-actin Ab was used as loading control (MP Biomedicals, Santa Ana, CA). Bound Abs were visualized by ECL and quantified by densitometry analysis of scanned films (Image J 1.34S; National Institutes of Health, Bethesda, MD).

Western blotting

Naïve CD4+ cells were obtained from PBMC by negative selection, using the naïve CD4+ selection kit (Dynal; Invitrogen). Subsequently, the naïve CD4+ cells were plated with a mature HLA-mismatched DC (VD3-DCs for Tregs, NT-DCs for control T cells) in a 1:10 ratio for 3 d in IMDM (Lonza, Verviers, Belgium) with 10% human serum. Next, the T cells were rested for 3 d in IMDM with 10% human serum and 10 ng/ml IL-7 and 5 ng/ml IL-15 and subsequently restimulated with mature DCs (VD3-DCs for Tregs, NT-DCs for control T cells) for 4 d. Cells were left to rest for 3 d before use in the suppression assay.

TNF antagonists (etanercept, 5 μg/ml, Wyeth Pharmaceuticals BV, Hoofddorp, The Netherlands; and adalimumab, 10 μg/ml, Abbott BV, Hoofddorp, The Netherlands), anti-CD210b (10 μg/ml), and the isotype control IgG2bκ (10 μg/ml) were added only to the coculture of VD3-DCs (for Tregs) and NT-DCs and T cells and not during resting or in the suppression assay. NT-DCs are antagonists in high concentrations (5 μg/ml and 10 μg/ml, respectively) to completely block the high concentration of TNF secreted by guest on April 18, 2017 http://www.jimmunol.org/ Downloaded from

Table I. Expression of costimulatory molecules upon LPS- and CD40L-induced maturation of NT-DCs and VD3-DCs

<table>
<thead>
<tr>
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<th>NT-DCs</th>
<th>VD3-DCs</th>
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<tr>
<td></td>
<td>CD40L</td>
<td>LPS</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>671 ± 218*</td>
<td>584 ± 158</td>
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<tr>
<td>CD80</td>
<td>53 ± 14</td>
<td>55 ± 6</td>
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<td>CD86</td>
<td>120 ± 44</td>
<td>149 ± 24</td>
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*Data represent mean fluorescence intensity ± SD calculated from n = 11 cultures.
by the DCs. Before the start of the coculture, DCs were incubated with TNF antagonists and T cells with CD120b or IgG2a Abs for 15 min.

Suppression assay

Naive CD4+ cells (effector T cells [Teffs]) were obtained from PBMC by negative selection, using the naive CD4+ selection kit; thereafter, we labeled Teff cells with 1 µM CFSE. Tregs or control T cells (donor A) were washed and plated in a round-bottom 96-well plate coated with 1:20,000 anti-CD3 (clone UCHT1, eBioscience) with CFSE-labeled naive CD4+ effector cells (donor A) and an NT-DC (donor B). NT-DCs were from the same donor as the DCs used to induce the Tregs or control T cells. Experiments were performed in triplicate. Cells were plated in a Treg/Teff/DC ratio of 10:10:1. After 4 d, cells were recovered and stained for CD4, and CFSE dilution of CD4 responder cells was analyzed on a FACS-Calibur. Immediately prior to the sample acquisition, 10,000 Flow-Count Fluospheres (Beckman Coulter, Woerden, The Netherlands) were added. For each sample, equal numbers of microbeads (5000) were acquired to facilitate comparison between samples.

Cytokine assays

Supernatant from DCs was taken after maturation or after 48 h incubation with anti-TNF or no treatment. Cytokine analysis was done with a Luminex 17-plex kit by Bio-Rad (Hercules, CA) according to the manufacturer’s protocol.

Calculation of proliferation index and statistical analysis

The divisions of responder cells were calculated by a proliferation index, as shown in the formula below, where \( n \) is the maximal division of the assay:

\[
PI(d) = \frac{\sum_{d=0}^{d=n} \text{Events}(d)}{\sum_{d=0}^{d=n} \text{Events}(d) \cdot 2^d}
\]

T cells primed by NT-DC stimulating responder T cells are depicted as the 100% proliferation value, and a proliferation index of 1.0 (no division) forms the 0% proliferation value. The statistical analyses were performed with a Student \( t \) test.

Results

CD40L induces similar production of TNF by VD3-modulated and control DCs

We previously demonstrated that VD3-DCs produce significantly more TNF than do NT-DCs when stimulated with LPS (23). To investigate whether this effect depends on TLR signaling, we stimulated VD3-DCs and NT-DCs to mature with either LPS or CD40L and compared TNF secretion in the supernatant upon 48 h of culture. In line with previous data, greater concentrations of TNF were detected in the supernatant of LPS-stimulated VD3-DCs than in NT-DCs (Fig. 1). When stimulated to mature with CD40L, VD3-DCs and NT-DCs released comparable amounts of TNF, showing that TNF secretion depends on the maturation protocol. Tolerogenic VD3-DCs released equal amounts of TNF in both CD40L- and LPS-stimulated cultures.
Maturation with LPS or CD40L induces tolerogenic phenotype characteristics identical to those of VD3-DCs with low HLA-DR and costimulatory receptor (CD80 and CD86) expression (Table I). Phenotypes of control NT-DCs were also similar in LPS- and CD40L-stimulated cultures.

**VD3-DCs express more mTNF than do NT-DCs**

Cells produce TNF as mTNF, which is subsequently cleaved off and released from the membrane as sTNF (10). Because TNF measured in the supernatant represented only the soluble form of the molecule, we also analyzed mTNF on the surface of VD3-DCs and NT-DCs (Fig. 2). VD3-DC cultures contained significantly more cells with increased expression levels of mTNF on the surface than did NT-DCs ($p < 0.05$). Similar high expression of mTNF was detected when VD3-DCs were matured with either LPS or CD40L (Fig. 2).

**Blocking of TNF during induction of Tregs by VD3-DCs abrogates the suppressive function of Tregs**

Modulated VD3-DCs are potent inducers of Ag-specific Tregs that suppress proliferation of responder T cells in vitro (23). To investigate the importance of sTNF versus mTNF for the tolerogenic capacity of VD3-DCs, we cocultured VD3-DCs with HLA-mismatched naive CD4$^+$ T cells in the presence of TNF antagonists. Currently available antagonists cannot selectively block mTNF, because etanercept (sTNF receptor) binds predominantly to sTNF, whereas adalimumab (human anti-TNF Ab) binds to both mTNF and sTNF (13). We assessed the contribution of mTNF to Treg induction indirectly by comparing the effect of blocking sTNF alone (using etanercept) with the effect of blocking both sTNF and mTNF (using adalimumab).

Addition of different TNF antagonists to the coculture had no effect on the expression of CD25, CD62L, and OX40 in T cells stimulated with VD3-DCs or NT-DCs (Fig. 3). Next, we tested the suppressive capacity of T cells induced in the presence of TNF antagonists. Naive T cells induced by NT-DCs with or without TNF antagonists were used as a control. Notably, TNF antagonists were not used in the suppression assay, but solely during coculture of VD3-DCs with T cells. Teffs in the suppression assay were naive CD4$^+$ T cells from the same donor as Tregs and control T cells (Tcontrols). Proliferation of Teffs was strongly suppressed when cocultured with Tregs derived with VD3-DCs alone or Tregs generated in the presence of etanercept, as suggested by the lower division rates of CFSE-labeled cells (Fig. 3). In marked contrast, proliferation of Teffs was not suppressed by Tregs induced in the presence of anti-TNF. Proliferation of Teffs with Tcontrols derived from cocultures with NT-DCs was similar and unaffected by the presence of TNF antagonists during the induction. We further quantified the suppressive efficiency of Tregs, confirming that Tregs generated by VD3-DCs alone or with etanercept had similar suppression potential, whereas addition of anti-TNF Ab during induction of VD3-DCs prevents generation of suppressive T cells by VD3-DCs.

**Anti-TNF does not change phenotype or viability of VD3-DCs**

We investigated whether anti-TNF prevents the induction of Tregs by changing the phenotype or survival of DCs. Incubation of NT-DCs and VD3-DCs with anti-TNF for 48 h did not change expression of PDL-1 or costimulatory molecules CD80 and CD86 on DCs (Fig. 4) or other DC markers (Supplemental Fig. 1). An anti-inflammatory cytokine profile of the VD3-DCs (low IL-12 and high IL-10) was maintained upon anti-TNF treatment. Likewise, production of IL-1b and IL-6 did not change because VD3-DCs cultured with or without anti-TNF produced 1.2 ± 0.1 ng/ml and 1.2 ± 0.4 ng/ml of IL-1b and 9.6 ± 4.4 ng/ml and 11.1 ± 7.8 ng/ml of IL-6, respectively.

To analyze whether anti-TNF causes death of VD3-DCs via reverse signaling through mTNF and thus prevents induction of Tregs, we analyzed AnnexinV/PI profiles upon anti-TNF treatment (Fig. 5). Anti-TNF did not affect viability of DCs. VD3-DC cultures contain more early apoptotic (AnnexinV$^+$PI$^-$) cells and similar number of late apoptotic cells (AnnexinV$^+$PI$^+$) when compared with NT-DCs. Equal numbers of viable VD3-DCs were present in both treated and nontreated cultures.

Taken together, anti-TNF does not affect the phenotype, cytokine profile, or viability of VD3-DCs. Therefore, differential effects of anti-TNF on the outcome of the VD3-DC/T cell coculture cannot be attributed to the change induced on the DCs, but conceivably result from blocking of the interaction of mTNF with its receptor.

**VD3-DCs require interaction of mTNF with TNFRII to induce Tregs**

Previous studies have demonstrated that the interaction between TNF and TNFRII on natural Tregs is important for their suppressive function (14, 15). Furthermore, mTNF binds preferentially to TNFRII (11–13). This finding prompted us to test whether the interaction between mTNF and TNFRII plays a role in the induction of VD3-DCs. We cocultured VD3-DCs with naive CD4$^+$ T cells in the presence of anti-TNFRII Ab (anti-CD120b) or isotype control, using the same protocol as for TNF antagonists. Anti-TNFRII Abs may have either stimulating or blocking effect when bound to TNFRII. Stimulation of TNFRII induces phosphorylation of NF-$\kappa$B (15). Using Western blot analysis, we observed that incubation of naive CD4$^+$ T cells with anti-TNFRII Ab did not induce phosphorylation of NF-$\kappa$B (Supplemental Fig. 2), confirming the blocking function of this Ab.

Subsequently, the suppressive function of Tregs induced in the presence of anti-TNFRII Ab was analyzed (Fig. 6). Induction of T cells with suppressive activity by VD3-DCs was prevented equally by either anti-TNFRII or anti-TNF Ab. This observation indicated that the interaction between mTNF and TNFRII is essential for the induction of T cells with suppressive function by VD3-DCs.
Discussion

The role of TNF in the immune response is versatile. Blockade of TNF by antagonists has been to suppress the inflammatory response (2). However, the role of TNF in regulation has been inconsistent, whereas most studies focused on the role of TNF in the Treg–Teff interaction (14, 16, 24). We previously reported that VD3-DCs secrete high amounts of TNF (23), raising the question whether TNF contributes to the tolerogenic function of VD3-DCs. Our data demonstrate that the expression of mTNF, rather than sTNF, distinguishes VD3-DCs from control NT-DCs. Furthermore, mTNF is essential for the induction of Ag-specific suppressive T cells by VD3-DCs through mTNF–TNFRII interaction. This finding points to an important function of mTNF in the induction of immune tolerance.

Reports on the role of TNF in immune suppression are inconsistent. TNF was previously shown to downmodulate Treg function (15, 16). Either TNF or a TNFRII agonist during the suppression assay prevented the suppression of proliferation of target cells. In contrast, in mice, TNF promoted the expansion and function of Tregs (14). Our approach differs from previous studies in the sense that we blocked sTNF and mTNF during the coculture with VD3-DCs and therefore assessed the function of TNF in the capacity of tolerogenic DCs to induce Tregs. Our data underline the importance of mTNF as an additional immunoregulatory helper in the induction of Tregs by VD3-DCs.

Addition of different TNF antagonists during induction of Tregs had profoundly different consequences for their functional efficiency in immune suppression. Whereas sTNFRII (etanercept) did not decrease the suppressive function of induced Tregs, but rather enhanced it, anti-TNF (adalimumab) totally abrogated the induction of Tregs. Two TNF antagonists that were used in this study have different affinities for mTNF or sTNF (11–13). Etanercept primarily blocks sTNF, whereas adalimumab blocks both sTNF and mTNF. In our view, this difference in affinity may endorse opposite effects of TNF antagonists on the Treg induction. In the clinical setting, different effects of etanercept and adalimumab are observed in inflammatory bowel disease; anti-TNF (adalimumab) can be an effective therapy, whereas sTNFRII (etanercept) is not (25, 26).

The interaction of different forms of TNF with TNF receptors is not equally strong. mTNF preferentially binds to TNFRII (12), whereas dissociation rates of sTNF from TNFRII is ∼20–30 times faster than from TNFRI (27). Therefore, the increased expression of mTNF on VD3-DCs conceivably signals via TNFRII on T cells. Indeed, in our study, blockade of TNFRII during Treg induction abrogated their suppressive function, similar to the effect of anti-TNF. High expression of TNFRII on Tregs supports the conceivable importance of this receptor in immune regulation (28, 29).

Addition of exogenous TNF to Treg/Teff coculture in a nonspecific suppression assay was shown to modulate Treg function via TNFRII (14–16). The importance of the superior mTNF–TNFRII interaction of different forms of TNF with TNF receptors is not equally strong. mTNF preferentially binds to TNFRII (12), whereas dissociation rates of sTNF from TNFRII is ∼20–30 times faster than from TNFRI (27). Therefore, the increased expression of mTNF on VD3-DCs conceivably signals via TNFRII on T cells. Indeed, in our study, blockade of TNFRII during Treg induction abrogated their suppressive function, similar to the effect of anti-TNF. High expression of TNFRII on Tregs supports the conceivable importance of this receptor in immune regulation (28, 29).

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interaction was not assessed in these studies, precluding comparison of these data with our findings. We propose that mTNF–TNFRII binding is essential during the induction of Tregs, rather than in their subsequent activity, and bear most relevance to Ag-specific suppression, because cells have to be in close contact for Ag recognition.

Our data do not discern conclusively whether the block of mTNF–TNFRII interaction during coculture of VD3-DCs and T cells completely prevents the induction of Tregs, or prevents Tregs from acquiring their cell-contact-mediated suppressed function. Anti-TNF treatment did not change the regulatory phenotype and viability of VD3-DCs. Furthermore, our preliminary data show that Tregs induced in the presence of anti-TNF retain their IL-10 capacity, implying that mTNF–TNFRII blockade interferes in the induction of Tregs equipped with complete regulatory arsenal, whereas it may allow differentiating of T cells with partial regulatory potential.

Current experimental tools do not allow straightforward translation of our in vitro findings into the in vivo setting. Mechanisms that mouse and human DCs use to induce Tregs conceivably differ owing to the qualitative differences between the mouse and human immune systems (30). Our preliminary analyses indicate that unlike human VD3-DCs, mouse VD3-DCs do not express increased mTNF (data not shown). Nonetheless, experiments with anti-TNF treatment in mouse models have been performed (9, 31), and show that anti-TNF can indeed prevent induction of Tregs. Although this finding would corroborate our claim that TNF is critical in the induction of T cell regulation, the mechanisms through which this may occur could be diverse. Indeed, systemic treatment of mice with anti-TNF has additional consequences, which include modulation of survival of autoreactive T cells, quantitative changes of CD4+CD25+ T cells, or block of the migration of autoreactive T cells toward the inflammatory signals (9, 31, 32). These findings preclude investigating the contribution of mTNF alone in the interaction between VD3-DCs and T cells in vivo. Future advances in experimental models or availability of TNF antagonists that selectively block mTNF might allow preclinical in vivo studies in the future.

The ability to induce tissue-specific Tregs and tolerance is indispensable to battle autoimmune diseases. We show that mTNF produced by human VD3-DCs is important for their tolerogenic function. It is, therefore, conceivable that anti-TNF therapy may act adversely in different patients or disease pathways. Indeed, a recent study shows that anti-TNF treatment promotes skewing toward a Th1/Th17 cytokine profile in an experimental model of rheumatoid arthritis (33), whereas sTNF has also been suggested to promote specific killing of autoreactive T cells in human diabetestes, pointing to beneficial effects in the immunopathogenesis of rheumatoid arthritis (33), whereas sTNF has also been suggested to promote specific killing of autoreactive T cells in human diabetes (33), whereas sTNF has also been suggested to promote specific killing of autoreactive T cells in human diabetes (33), whereas sTNF has also been suggested to promote specific killing of autoreactive T cells in human diabetes.

Acknowledgments
We thank Jun Wang of the Rheumatology Department of Leiden University Medical Center, Leiden, The Netherlands, for the kind gifts of TNF antagonists and mTNF-Ab.

Disclosures
The authors have no financial conflicts of interest.

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References


Supplementary Legend

**Figure S1. Effect of anti-TNF on phenotype of VD3-DC.**
Mature DC were cultured with anti-TNF therapy for 48 hours. The DC markers CD14, CD1a, CD11c, HLA-DR and CD40 were measured with (black thick line) and without (filled histogram) anti-TNF therapy. Dashed line is isotype control. Histograms are representatives of three independent experiments.
Figure S2. No agonistic effect of anti-TNFRII, clone hTNFR-M1.

CD4⁺ T-cells were isolated from PBMC and treated with medium, anti-TNFRII (clone hTNFR-M1), TNF or PMA/iono for 0, 10, 30 and 60 min. There was investigated for agonistic effects of anti-TNFRII on NFκB signaling by phosphorylation of P65 (Pser 536). Actin control is shown in the lower column.