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Positive T cell co-stimulation by TLR7/8 ligands is dependent on the cellular environment¹

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Abstract

Toll-like receptors (TLRs) are mediators of innate immune responses detecting conserved pathogen-associated molecules. Whereas most TLRs are expressed on the cell surface, TLR3, 7, 8 and 9 are predominantly localized in endosomal compartments. Recent studies reported that TLRs are also expressed by T lymphocytes, resulting in direct co-stimulation of isolated CD4⁺ T cells for example by Pam3CSK4 (TLR2 ligand) or flagellin (TLR5 ligand). We here describe enhanced IFN- γ production and T cell proliferation by anti-CD3 T cell receptor (TCR) or antigenic stimulation of purified human CD4⁺ T cells upon co-culture with TLR7/8 specific single-stranded oligoribonucleotides or small molecule ligands. Surprisingly, TLR7/8 stimulation of CD4⁺ T cells within a whole peripheral mononuclear cell (PBMC) environment did not result in enhanced T cell proliferation, but in a lack of proliferation that was cell-cell contact dependent. Immune cell depletion assays pointed towards a monocyte-mediated effect. Different TLR ligands influenced T cell proliferation differently. The effect of inhibition of T cell proliferation was most prominently seen for TLR7 ligands whereas the effects were minimal for TLR8 and TLR9 ligands indicating that the suppressive phenotype is unique only for certain TLRs. Our results strongly suggest that co-stimulation of T cell proliferation by TLR7/8 agonists is dependent on the specific cellular context.

Keywords

single-stranded oligoribonucleotide (ORN); TLR; T cell activation

Introduction

Toll-like receptors (TLRs) are pathogen recognition receptors that play an essential role in mediating innate immunity. They are able to detect conserved pathogen-associated

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molecular patterns (PAMP) of bacteria, fungi, parasites or viruses (Hopkins et al., 2005). TLRs are type I transmembrane proteins with an extracellular leucine-rich domain and a conserved cytoplasmic domain. TLR1, 2, 4, 5 and 6 are located on the cell surface whereas TLR7, 8 and 9 are located in the endosome (Matsumoto et al., 2002; Heil et al., 2003; Latz et al., 2004; Ahmad-Nejad et al., 2002). TLR3 is detected at both locations dependent on the specific cell type (Matsumoto et al., 2002), (Hewson et al., 2005; Ueta et al., 2005).

To date 10 human TLRs have been identified (Abreu et al., 2004), (Sabroe et al., 2002). TLR1 and TLR6 signal only as a dimer together with TLR2 and recognize bacterial lipopeptides (Wagner 2002), TLR5 is the receptor for flagellin. TLR3 recognizes certain viral double-stranded RNAs, whereas TLR7 and TLR8 recognize single-stranded viral derived RNA. The specific receptor for bacterial DNA sequences with unmethylated CpG motifs is TLR9 (Kawai et al., 2007). TLR receptors are expressed in a variety of different cell types including neutrophils, myeloid cells and epithelial cells (Medzhitov 2001; Takeda et al., 2003). TLR activation is an important step in immune activation, and several different ligands are currently in preclinical or clinical development. For example, the synthetic TLR9 ligand CpG oligodeoxynucleotide CPG 2006 is being tested as a vaccine adjuvant and for the treatment of various types of cancer (Schetter et al., 2004; Krieg 2006; Jurk et al., 2007; Murad et al., 2007; Uhlmann et al., 2003). Single-stranded viral derived RNA, targeting TLR7 and TLR8, can be mimicked by synthetic single-stranded oligoribonucleotides (ORNs) (Heil et al., 2004). Small molecule ligands for TLR7 and TLR8 were as well identified e.g., the imidazoquinoline R-848 and the guanosine analogue loxoribine (Jurk et al., 2002; Lee et al., 2003). One small molecule TLR7 ligand is already approved for the topical treatment of genital warts and certain skin cancers (Beutner et al., 1998; Beutner et al., 1999).

The function of TLRs within immune cells is manifold. Upon binding of the ligand to the TLR, signaling is initiated by the signal transduction domain Toll/IL-1 (TIR) that interacts with adaptor proteins like MyD88, TIRAP/MAL and TRIF (for review see (Akira et al., 2004)). Signaling induces activation of the transcription factor NF- κ B that initiates production of different proinflammatory cytokines, chemokines and upregulation of co-stimulatory molecules (Kawai et al., 2007). Some of the cells of the innate immune system, like macrophages and neutrophils, can differentiate into effector cells upon TLR stimulation (O'Neill 2004). Other cells, like dendritic cells, activate T cells and become effective activators of adaptive immune responses (Akira et al., 2001; Schnare et al., 2001; Iwasaki et al., 2004).

Several studies have reported that some TLRs are expressed by T lymphocytes suggesting also a direct function in stimulating adaptive immune responses (Hornung et al., 2002; Zarembek et al., 2002). A recent publication demonstrated an increase of T cell effector function when these cells were stimulated with the TLR2/6 ligand Pam₃CSK₄, the TLR5 ligand flagellin or the small molecule TLR7/8 ligand R-848 (Caron et al., 2005). Among these T cells, the CD4⁺ effector memory cell population was the main responder to TLR stimulation. Another study described a direct inhibitory influence on CD4 regulatory T cell function upon TLR8 stimulation with poly(dG) ODNs (Peng et al., 2005).

In the present study we show that TLR7/8-activating ORNs are able to enhance T cell proliferation by acting directly on T cells. Positive stimulation of T cells by TLR7/8 agonists could lead to promising novel vaccine adjuvants with broader immune stimulatory effects compared to CpG ODN. However, this positive co-stimulation was abrogated when T cells were in contact with monocytes. We conclude that these monocytes provided inhibitory signals to the TCR-stimulated T cells resulting in a suppression of T cell proliferation. This

inhibitory effect appeared to be mainly induced upon activation of monocyte-expressed TLR8 rather than upon activation of TLR7.

Material and Methods

Reagents

TLR7/8 agonist Resiquimod (R-848) was commercially synthesized by GLSynthesis, (Worcester, MA). DOTAP was obtained from Roche (Mannheim, Germany). 1-methyl-DL-tryptophane (1-MT DL) was purchased from Sigma (Deisenhofen, Germany). Human IL-2, TNF- α and IFN- γ were obtained from R&D Systems (Wiesbaden-Nordenstadt, Germany). IntronA was obtained from PBL InterferonSource (New Jersey, USA). Pam₃CSK₄ was obtained from EMC microcollections GmbH (Tuebingen, Germany). CpG ODNs 2395, 5457 and 2216 were provided by Coley Pharmaceutical GmbH (Düsseldorf, Germany). ORNs were obtained from BioSpring (Frankfurt, Germany). All ORNs and ODNs were controlled for identity and purity by Coley Pharmaceutical GmbH and had undetectable endotoxin levels (<0.1EU/ml) measured by the Limulus assay (BioWhittaker, Verviers, Belgium). Oligonucleotides were suspended in sterile endotoxin-free Tris-EDTA (ODNs) (Sigma) or in DNase- and RNase-free water (ORNs) (Life Technologies, Eggenstein, Germany), and were stored and handled under aseptic conditions to prevent contamination. All ODNs and ORNs have phosphorothioate (“’”) backbones unless otherwise stated, except ODN 2216 which has a mixed phosphodiester/phosphorothioate backbone and ORN R-1886 which has a complete phosphodiester (“-”) backbone. The sequences are:

ODN 5457 (CpG B-Class): T*C*G*T*C*G*T*T*T*T*T*T*C*G*G*T*C*G*T*T*T*T

ODN 2216 (CpG A-Class): G*G*G-G-G-A-C-G-A-T-C-G-T-C*G*G*G*G*G

ODN 2395 (CpG C-Class):

T*C*G*T*C*G*T*T*T*T*T*C*G*G*C*G*C*G*C*G*C*G

ORN R-0006:

rU*rU*rG*rU*rU*rG*rU*rU*rG*rU*rU*rG*rU*rU*rG*rU*rU*rG*rU*rU

ORN R-0002:

rU*rU*rA*rU*rU*rA*rU*rU*rA*rU*rU*rA*rU*rU*rA*rU*rU*rA*rU*rU

ORN R-1263:

rG*rC*rC*rA*rC*rC*rG*rA*rG*rC*rC*rG*rA*rA*rG*rG*rC*rA*rC*rC

ORN R-1886: rA-rA-rA-rC-rG-rC-rU-rC-rA-rG-rC-rC-rA-rA-rA-rG-rC-rA-rG

Peripheral blood mononuclear cells

PBMC preparations from healthy male and female human donors were obtained from the Blood Bank of the University of Düsseldorf (Germany). PBMCs were purified by centrifugation over Ficoll-Hypaque (Sigma, Deisenhofen, Germany). Purified PBMCs were washed twice with 1x PBS and resuspended in RPMI 1640 culture medium supplemented with 5% (v/v) heat inactivated human AB serum (BioWhittaker, Belgium) or 10% (v/v) heat inactivated FCS, 1.5mM L-glutamine.

Cell isolation and FACS staining

CD4⁺ T cells were isolated from human buffy coats using the RosetteSep™ CD4⁺ T Cell Enrichment Kit, and human CD3⁺ T cells by using the RosetteSep™ Human T Cell Enrichment Cocktail (all from Stemcell Technologies, CellSystems Biotechnologievertrieb GmbH, St. Katharinen, Germany) according to manufacturer's recommendations. Purity was

confirmed by staining with mAb to CD4 (FITC and APC) or CD3 (APC) (all from BD Pharmingen, Heidelberg, Germany) and was typically over 94%. For the isolation of CD3 depleted PBMCs the RosetteSep™ Human CD3⁺ Depletion Kit (Stemcell Technologies) was used according to the manufacturer's instructions. Human monocytes were isolated from PBMCs by CD14 immunomagnetic bead positive selection according to manufacturer's recommendations (Miltenyi Biotech, Bergisch-Gladbach, Germany). Purity was confirmed by staining with mAb to CD14 (BD Pharmingen), and was typically >90%. For the detection of monocytes within PBMCs after ORN/ODN stimulation, PBMCs were stained with a cocktail of CD19-, CD56- and CD3-APC antibodies (all from BD Pharmingen) (CD19-, CD56-, and CD3-negative cells consist mainly of monocytes). A direct stain with anti-CD14 antibody was not possible, since human monocytes lose this CD14 surface expression after activation (unpublished observation).

T cell culture and proliferation assays

Isolated CD4⁺ T cells or PBMCs were stained with CFSE (2.5 μM) (Invitrogen, Karlsruhe, Germany) for 5 minutes at RT and washed three times with PBS/5% FCS. Cells were resuspended in RPMI 1640 culture medium supplemented with 10% (v/v) heat inactivated human AB serum (BioWhittaker) and 1.5mM L-glutamine. Cells were cultured in flat-bottom 96 well-plates (5×10⁵ cells/well) coated with 2μg/ml OKT3 antibody (anti-CD3) (eBioscience, San Diego, USA) for three days. Cells were stained with mAb to CD4 (APC) for CD4⁺ isolated T cells, and with mAb to CD4 (APC), CD8 (PerCP) and CD19 (PE) (all from BD Pharmingen) for cultured PBMCs. Proliferation was measured as a function of dilution of CFSE fluorescence in a FACSCalibur (BD Biosciences).

For allogenic MLRs isolated CD3⁺ T cells from one donor were stained with CFSE (2.5μM) and mixed with APCs (CD3 depleted PBMCs) from an allogenic donor (1×10⁵ T cells with 2×10⁴ APCs). Cells were cultured in conical-bottom 96-well plates for three days. T cell proliferation was measured as dilution of CFSE fluorescence. For SEB stimulation and antigenic T cell stimulation with TT (Calbiochem, Merck Biosciences, Darmstadt, Germany), PBMCs were stained with CFSE (2.5μM), and 5×10⁵ PBMCs/well were stimulated with 2μg/ml SEB (Sigma) for three days, or 2.5μg/ml TT for 6 days in 96-well plates. All cells in the proliferation assays were resuspended in 250μl/well RPMI 1640 medium supplemented with 10% (v/v) heat inactivated human AB serum (BioWhittaker) and 1.5mM L-glutamine. T cells and PBMCs were co-stimulated with the indicated amounts of R-848, Pam₃CSK₄, IL-2, or different ODNs and ORNs. All ORNs were complexed with DOTAP (2μM ORN complexed with 25μg/ml DOTAP). T cells were cultured for the indicated time points in a humidified incubator at 37°C. Supernatants of the cultures were harvested for the measurement of cytokine production.

Cytokine production

Freshly isolated PBMCs were resuspended at a concentration of 3×10⁶/ml to 5×10⁶/ml and added to 96-well round-bottomed plates (200μl/well), which had previously received either nothing or ODNs and ORNs without DOTAP, complexed with DOTAP, or DOTAP alone. Cells were cultured in a humidified incubator at 37°C for the indicated time points. Culture supernatants were collected and, if not used immediately, were frozen at -20°C until required. Amounts of cytokines in the supernatant were assessed using commercially available ELISA Kits (IFN-α or IFN-γ, Diaclone, Besancon, France).

Detection of cell death

For the measurement of apoptosis and necrosis 5×10⁵ PBMCs/ 96-well were cultured for 24h and 72h with the indicated ligands. Cells were stained with mAb to CD3 (APC) and

apoptosis or necrosis was measured with the AnnexinV/PI detection kit from BD Pharmingen according to the manufacturer's instruction.

Transwell assay

For the transwell stimulation experiment T cells and CD3 depleted PBMCs were isolated as described above and the HTS Transwell-96-System (1 μ M pore size) from Corning (Netherlands) was used. 5×10^5 CD3⁺ T cells, stained with 2.5 μ M CFSE, were added to 96-well flat bottom plates that were precoated with 2 μ g/ml anti-CD3 (OKT3) antibody. 1×10^5 CD3 depleted PBMCs were added to the transwell chamber. As control, the same cells were mixed together and added to a 96-well flat bottom plate without transwell-chamber. Cells were cultured for three days and stimulated with the indicated ligands. For flow cytometric analysis cells were stained with mAb to CD4 (APC) and CD8 (PerCP).

Activation of murine splenocytes

Experiments were performed using C57BL/6 mice, housed at the Institute for Cell biology, Universitätsklinikum Essen (IFZ), Germany, under specific pathogen free conditions. Splenocytes from C57BL/6 mice were stained with CFSE (2.5 μ M) and stimulated for three days on 96-well plates (5×10^5 splenocytes/well). Plates were pre-coated with anti-CD3 antibody (clone: 145-2C11 from BD Pharmingen; 2 μ g/ml). For determination of T cell proliferation, splenocytes were stained with mAb to murine CD4 (PE) and CD8 (PerCP) (BD Pharmingen) and proliferation was measured as the dilution of CFSE in a FACSCalibur.

Isolation of monocytes and real time PCR

5×10^7 PBMCs were stimulated with the different TLR ligands for 24h on 12-well plates. Plates were washed with PBS to remove unbound mononuclear cells. RNA from plate bound monocytes were prepared (RNeasy isolation kit, Qiagen, Hilden, Germany) and IDO level relative to 1000 copies cyclophilin B were determined in the Light Cycler 1.5 system using IDO and cyclophilin B detection kits from Search LC (Heidelberg, Germany).

Statistical analysis

Data shown represent mean $\pm$ SEM of 3-11 different donors. Significance of differences was determined by Student's t-test.

Results

Purified CD4⁺ T cells are co-stimulated with single-stranded TLR7/8 stimulating oligonucleotides

Previous studies have shown that CD4⁺ T cells could be directly co-stimulated by a small molecule TLR7/8 ligand, Resiquimod (R-848), the TLR5 ligand flagellin or the TLR2 ligand Pam₃CSK₄ (Caron et al., 2005). Indeed, T cell express a variety of TLRs with the exception of TLR6, 8 and 10, but including TLR7 (Caron et al., 2005). This co-stimulation was mainly mediated by the memory T cell population (Caron et al., 2005). In order to analyze the influence of different nucleic acid TLR8 and TLR7/8 agonists on T cell activation, we isolated human CD4⁺ T cells and stimulated them with anti-CD3 antibody. Co-stimulation was performed with either IL-2 as positive control or various TLR ligands (Fig. 1): the small molecule TLR7/8 ligand R-848, TLR2/6 ligand Pam₃CSK₄ and with the phosphorothioate ORN R-0006, containing a sequence motif that activates both TLR7 and TLR8 (Forsbach et al., 2007). The anti-CD3 stimulated T cells showed the highest proliferative response upon TLR2 co-stimulation with Pam₃CSK₄ (40% of dividing cells), followed by co-stimulation with IL-2 (34%), R-848 (29%) and the TLR7/8 ligand R-0006

(27%) (Fig. 1A). The observed proliferation increase of 6-13% of the TLR7/8 ligands compared to anti-CD3 stimulation alone was comparable to published data by Caron et al. for R-848 (Caron et al., 2005). The non-stimulatory ORN R-1263 or the transfection vehicle alone did not influence proliferative activity, whereas the CD4⁺ T cell proliferation upon co-incubation with the TLR7/8 ORN was comparable to R-848 (Fig. 1B). Isolated CD8⁺ T cells showed a similar response to the CD4⁺ T cells (data not shown). The TLR8 agonist R-0002 (Forsbach et al., 2007) induced also some T cell proliferation, comparable to anti-CD3 stimulation alone. We did not detect any R-0006-induced proliferation of T cells without anti-CD3 co-stimulation (data not shown).

Determination of IFN- γ production in the supernatants of stimulated T cells revealed that ORN R-0006 induced high amounts of IFN- γ secretion comparable to those levels induced by R-848 and Pam₃CSK₄. IFN- γ expression induced by R-0006 was detected even in the absence of TCR co-stimulation when cultured with IL-2 only. When co-stimulated with IL-2, IFN- γ levels induced by R-0006 were 2-fold increased compared to R-848 and Pam₃CSK₄ (Fig. 1C).

T cell stimulation by single-stranded oligonucleotides is strongly dependent on the cellular environment

In order to analyze T cell responses in a more natural environment, we stimulated human peripheral blood mononuclear cells (PBMCs) with different ORNs and ODNs and determined the response of T cells within the PBMC culture. Surprisingly, we did not detect the same effect on CD4⁺ and CD8⁺ T cells compared to the results observed with purified T cells (Fig. 2A, B). Proliferation of anti-CD3 stimulated T cells was strongly suppressed by R-848 and ORNs R-0006 and R-0002, whereas only a low suppression of proliferation was detected in the presence of the inactive sequence R-1263 at higher concentrations, most likely due to unspecific effects of the transfection reagent. This cytotoxicity is not seen with the transfection reagent alone since DOTAP alone has different attributes as when it is in complex with a ligand (Simberg et al., 2004). Neither DOTAP alone nor three different single-stranded CpG ODN TLR9 agonists (CpG B-Class ODN 5457, A-Class ODN 2216, C-Class ODN 2395) inhibited T cell proliferation (Fig. 2A).

To test if this effect is unique to CD4⁺ T cells we also analyzed proliferation of CD8⁺ T cells. The results were similar to the CD4⁺ T cells: CD8⁺ T cell proliferation was also selectively inhibited by TLR7/8 and TLR8 ligands (Fig. 2B).

We next analyzed if proliferation of other cell types, like B cells is also affected by co-incubation with TLR7/8 agonists. PBMCs were stimulated with the B-Class TLR9 agonist CpG ODN 5457 or the TLR7/8 agonist ORN R-0006 and induction of B cell proliferation was measured (Fig. 2C). As described before (Hartmann et al., 2000; Cong et al., 2003), CpG ODN 5457 induced strong B cell proliferation whereas ORN R-0006 did not induce proliferation under these experimental conditions. In addition, B cell proliferation induced by CpG ODN 5457 was not inhibited by R-0006, suggesting that the suppressive effect of TLR7/8 ligands is specific to T cells within PBMCs.

Inhibition of T cell proliferation is not mediated by TLR7/8 induced cytokines

Single-stranded ORNs with certain sequence motifs induce a variety of cytokines from human PBMCs (Forsbach et al., 2008; Hornung et al., 2005). To determine whether the induction of IFN- γ and TNF- α secretion by the ORNs within the PBMC culture mediates the suppression of T cell proliferation, we added physiologically relevant amounts (5-40 ng/ml) of these cytokines to the cell cultures, corresponding to the cytokine concentrations detected upon PBMC stimulation with R-0006 and R-0002 (Forsbach et al., 2008) and data not

shown). Addition of IFN- γ and TNF- α in this physiologic range had no influence on anti-CD3 induced T cell proliferation (data not shown).

IFN- α is also very strongly induced by R-0006 complexed to DOTAP (Forsbach et al., 2008). Furthermore, complexation to DOTAP also yielded a much stronger cytokine response of CPG ODN of any type (B-Class and C-Class). IFN- α production from human PBMC by R-0006 and CPG C ODN is comparable when both substances are complexed to DOTAP (Fig. 3 A).

IFN- α was shown to be able to inhibit T cell function. For example CD4⁺ T cells, stimulated with an anti-CD3 antibody, could be blocked in the G₀/G₁ phase of the cell cycle upon addition of IFN- α (Romerio et al., 2002). To test if the suppressive effect of R-0006 is caused by the high amounts of type I interferons produced by R-0006 complexed to DOTAP, we also delivered CpG B-Class ODN 5457 and CpG C-Class ODN 2395 with DOTAP and assayed for proliferation inhibition. Although the DOTAP formulated CpG C-Class ODN did induce the same amount of IFN- α as R-0006 (Fig. 3A) it did not lead to the same extent of proliferation inhibition (Fig. 3B). However, DOTAP formulated ODNs showed a much stronger inhibition of T cell proliferation than unformulated ODNs (Fig. 3B). This effect could be mediated by the elevated IFN- α levels in the culture induced by formulated ODNs compared to unformulated ODNs.

To further analyse the possible effect of IFN- α , we treated PBMCs with IFN- α alone, and in combination with the non-stimulatory ORN R-1263 which should simulate the culture environment induced by a stimulatory ORN, and compared it to R-0006 alone (Fig. 3C). We detected some inhibition of T cell proliferation when IFN- α alone or in combination with R-1263 at doses up to 20ng/ml was added. However, although ORN R-0006 alone does induce comparable levels of IFN- α upon PBMC culture (Fig. 3A) compared to DOTAP formulated CPG C, the inhibition conferred by R-0006 was much stronger. Therefore, the inhibition of T cell proliferation appears not to be exclusively mediated by IFN- α , although contribution of IFN- α stimulated by this ORN cannot be completely excluded.

Negative T cell co-stimulation of TLR7/8 ligands is not restricted to anti-CD3 antibody stimulation and is not IDO-mediated

Next we wanted to investigate whether the inhibition of T cell proliferation is unique to anti-CD3 stimulation of the TCR or if antigenic restimulation of memory T cells also leads to the same phenotype. Therefore, we cultured PBMCs with the recall antigen tetanus toxoid (TT) to stimulate CD4⁺ memory T cells. The results were similar to the anti-CD3 stimulated naive T cells in PBMC cultures. Compared to anti-CD3 induced T-cell proliferation, R-0006 mediated inhibition of TT induced T cell proliferation is more powerful especially at lower concentrations of R-0006. One reason for this could be the kind of T cell stimuli: anti-CD3 stimulation activates the whole T cell population whereas TT is specific for a smaller memory subpopulation of T cells. Additionally, the anti-CD3 antibody could provide a stronger activation signal to T cells since it binds with higher affinity to the TCR than MHC presented TT (Krogsgaard et al., 2005)

Especially the TLR7/8 agonist R-0006, but also the TLR8 agonist R-0002 induced a strong inhibition of TT-induced CD4⁺ T cell proliferation, whereas the non-stimulatory control ORN R-1263 did not (Fig. 4A). When PBMCs were stimulated with the superantigen staphylococcal enterotoxin B (SEB), we saw similar inhibition of CD4⁺ and CD8⁺ T cell proliferation upon co-incubation with R-0006 (Fig. 4B).

In additional experiments, we stimulated T cells in a mixed lymphocyte reaction (MLR). Similar to the results described above, TLR7/8 co-stimulation by R-0006 led to a strong

inhibition of T cell proliferation, but the TLR9 agonist CpG C-Class ODN 2395 did not (Fig. 4C).

It has been shown that upregulation of indoleamine 2,3-dioxygenase (IDO) in murine splenic CD19⁺ dendritic cells inhibits T cell proliferation (Mellor et al., 2005). IDO is induced by high doses of CpG ODNs *in vivo* in mice (Wingender et al., 2005a; Mellor et al., 2005), and IFN- γ induces IDO in monocyte-derived dendritic cells, inhibiting human T cell proliferation *in vitro* (Munn et al., 2002). Blocking of IDO by 1-methyl tryptophan (1-MT) in these studies led to a reversal of the suppressive phenotype. PBMCs produce IFN- γ when incubated with ORN R-0006 or CpG ODNs, and this IFN- γ should be able to induce IDO expression within the PBMC cell population (Sioud 2005; Kadoya et al., 1992; Hassanain et al., 1993; Chon et al., 1995). Indeed, we observed increased IDO levels in isolated monocytes after stimulation with R-0006, CpG C-Class ODN 2395 or IFN- γ (Fig. 4D). To test whether this R-0006-induced IDO up-regulation may be responsible for the suppressive phenotype, we treated the cells with two different concentrations of 1-MT to inhibit IDO function (Fig. 4C). Inhibition of IDO with 1-MT had, if at all, a slight effect on the T cell proliferation in the MLR, suggesting that the TLR7/8 ligand induced inhibition is not IDO mediated. Although IFN- γ is able to induce much higher amounts of IDO than R-0006, it did not inhibit T cell proliferation (data not shown), further making an involvement of IDO unlikely.

Single-stranded ORNs lead to a block of T cell proliferation rather than induction of apoptosis or necrosis

The lack of dividing T cells in the cultures treated with TLR7/8 ligands could have been due to a block of cell cycle or the induction of apoptosis or necrosis. To further investigate the potential mode of action, we stimulated PBMCs with TLR7/8 ligands and anti-CD3 antibody for 24h and stained them with AnnexinV and propidium iodide (PI), allowing discrimination between live, early apoptotic and late apoptotic (or necrotic) cells. We first analyzed the percentage of live cells (AnnexinV⁻, PI⁻) within the whole PBMC population (Fig. 5A), and the fraction of live cells within the CD3⁺ T cell population (Fig. 5B). Analysis of all three populations (AnnexinV⁻, PI⁻; AnnexinV⁺, PI⁻ or AnnexinV⁺, PI⁺) demonstrated no difference between ORN R-0006 and the control ORN R-1263 at 24h. All tested oligos showed a slight reduction in live cells within the culture (Fig. 5 A/B). We also measured apoptosis after 72h, the timepoint used for the analysis of T cell proliferation, but the results were similar to the 24h measurement (data not shown). These data strongly suggest that inhibition of T cell proliferation by TLR7/8 ligands is not due to apoptosis or necrosis.

Inhibition of T cell proliferation is cell contact-dependent

To further characterize the mechanism of the inhibitory effect of TLR7/8 ligands in PBMC cultures, we determined whether the inhibition of T cell proliferation was mediated by soluble factors like cytokines or by cell to cell contact by use of a transwell system, shown schematically in Fig. 6A. When the T cells had no contact to antigen presenting cells, we detected no inhibition of anti-CD3 induced T cell proliferation (Fig. 6B), although anti-CD3 stimulation together with IL-2 showed a strong T cell proliferation, demonstrating that the T cells are functional within this system. Anti-CD3 stimulation alone exhibited 10-15% proliferation comparable to what was detected for purified T cells (Fig. 1A, B). Interestingly, in contrast to the purified T cell cultures R-0006 showed no increase of T cell proliferation in the transwell system (Fig. 6B). This could have been due to IFN- α that is produced by R-0006 in the T cell depleted population, interfering with T cell proliferation to a certain extend as shown in Fig. 3A.

The same T cells and T cell-depleted PBMCs were also directly mixed and incubated on an anti-CD3 precoated control plate. Here, we detected a clear inhibition of T cell proliferation with the stimulatory ORNs similar to the untouched PBMC cultures, indicating that purification of the T cells did not influence the inhibitory effects of the T cell proliferation within the whole cell population. CpG C-Class ODN or the control ORN R-1263 did not induce any inhibition (Fig. 6C) as shown previously (Fig. 2 and 3).

T cell inhibition is mediated by monocytes and stimulation of TLR8 leads to a stronger suppressive phenotype than TLR7 stimulation

The transwell experiments strongly suggested that the mechanism of inhibition is cell contact dependent. In order to determine the cell type responsible for the effect, we depleted different cell populations from the entire PBMC population before stimulating the cells with anti-CD3 antibody. Depletion of NK cells did not result in any change of the phenotype (data not shown). However, when depleting CD14⁺ monocytes we could not detect any R-0006 specific inhibition of T cell proliferation (Fig. 7A). T cells within the complete PBMC population showed a strong proliferative inhibition in the presence of R-0006 (Fig. 7A, very left panel), whereas CD4⁺ and also CD8⁺ T cells proliferated after depletion of monocytes from these cultures (Fig. 7A right panel) in the range of purified T cells stimulated by anti-CD3 (Fig. 1B). The overall T cell proliferation is lower in the monocyte-depleted culture (Fig. 7A) compared to the proliferation level of T cells in the complete PBMC environment (Fig. 6C) since monocytes are strong APCs for T cells. However, even with the lower proliferation levels the difference between the presence and absence of monocytes and their effect on T cell proliferation is obvious. Like in the transwell experiment, T cells within monocyte depleted PBMCs show no proliferation increase after treatment with R-0006 which again may be explained by interfering IFN- α induction.

Next we investigated if changes in expression of any surface molecule could be involved in mediating a positive or negative effect on T cell stimulation. Within R-0006-stimulated PBMCs we analyzed a set of surface markers that are known to act as co-receptors for T cells and can alter T cell stimulation (CD86, CD80, CD252, CD95, 41BBL on monocytes, and CD28, ICOS, CTLA4, CD134, CD27, CD95, 41BB, CD247 on T cells) but we did not observe any specific change in the expression of these markers by flow cytometry (data not shown).

PD-L1 is a negative regulator of T cell activation, and its engagement of the receptor PD-1 can result in cell cycle arrest (Carter et al., 2002). To analyze PD-L1 expression on monocytes, we stimulated PBMCs with anti-CD3 antibody together with ORN R-0006 or the control ORN R-1263, and analyzed expression of PD-L1. Stimulation of PBMCs with 0.1 μ M R-0006 resulted in a significant increase of PD-L1 expression on monocytes, which was not observed with the nonstimulatory ORN R-1263 (Fig. 7B). However, at higher R-0006 concentrations (1 μ M) PD-L1 expression returned to background levels. This may suggest that the increase of PD-L1 on monocytes cannot be the main mechanism for inhibition of T cell proliferation, since stimulation of T cells with 1 μ M R-0006 still resulted in a strong block of proliferation, although the kinetics for PD-L1 expression versus T cell proliferation inhibition may be different.

Monocytes were reported to express only TLR8 (Bekeredjian-Ding et al., 2006). We next analyzed the effect of stimulation with a TLR7, TLR8 or TLR7/8 agonist on monocytes. Consequently, TLR8-specific stimulation should lead to strongest inhibition of T cell proliferation. All ORNs used so far were not specific for TLR7 alone, they were stimulating either TLR8 (R-0002) or both receptors (R-0006) simultaneously (Forsbach et al., 2007). Recently a RNA sequence motif was discovered that specifically activates the TLR7 receptor (Jurk et al., submitted). When we stimulated PBMCs with an ORN containing this

TLR7-specific motif (R-1886), we indeed detected no difference in T cell inhibition compared to the non-stimulatory control ORN R-1263, indicating that inhibition of T cell function most probably is not mediated by TLR7 stimulation (Fig. 7C).

In contrast to humans, mice express a functionally impaired TLR8 (Heil et al., 2004; Gorden et al., 2006; Hemmi et al., 2002). To support the hypothesis that TLR8 mediated activation of monocytes is responsible for the inhibitory effect on T cells, we used a T cell proliferation assay set up with murine splenocytes. R-0006 is also a very strong cytokine inducer in the murine system (Forsbach et al., 2007). Murine splenocytes stimulated with anti-CD3 antibody in the presence of stimulatory ORNs showed no inhibition of T cell proliferation within the splenocyte population (Fig. 7D), consistent with our hypothesis that the inhibitory effect is mediated through TLR8.

Discussion

Recent studies suggested that different TLRs are expressed on different T cell subsets (Hornung et al., 2002; Zarembek et al., 2002; Dolganiuc et al., 2006; Caron et al., 2005). The expression of these TLRs on T cells argues for the potential of the respective TLR ligands to directly influence T cell function. In this study we demonstrate that stimulation of human purified T cells by TLR7/8 ORNs enhances T cell proliferation. It has been shown that highly purified CD4⁺ T cells can respond to R-848, flagellin and Pam₃CSK₄ and that CD4⁺ effector memory T cells are the main population responding to TLR7, TLR8, TLR2, TLR5 and TLR6 stimulation (Caron et al., 2005). We confirm previous studies reporting enhanced proliferation of isolated CD4⁺ T cells, and additionally demonstrate that single-stranded ORNs with a TLR7/8-specific sequence motif are able to result in increased T cell proliferation and cytokine production upon co-stimulation of the TCR. However, for T cells in the environment of PBMCs (with contact to APCs) this pattern of co-stimulation is completely changed.

For adequate T cell activation two signals are required. The first signal is normally provided by an antigen, bound to a MHC molecule stimulating the TCR, and the second signal is mediated by different co-receptors (Frauwirth et al., 2002). TLR7/8, TLR2/6 and TLR5 can provide such a second signal of co-stimulation to the T cells. It was shown that TLR signaling modulates T cell responses induced by TCR stimulation rather than inducing direct cellular responses (Kabelitz 2006).

Most TLR receptors, except TLR6, 8 and TLR10, were detected on peripheral T cells, although in varying expression levels from study to study (Kabelitz 2006). For example, TLR8 was not detected on conventional T cells, but on CD4⁺CD25⁺ regulatory T cells (Peng et al., 2005). However, most of such studies, with some exceptions analyzed only expression levels of certain TLRs without any functional characterization (Caron et al., 2005; Peng et al., 2005). Here we examined different functional effects of TLR activation on T cells that could open up new directions in the development of T cell modulating drugs. Indeed, we found that the TLR7/8-specific ORN R-0006 was able to induce proliferation of purified conventional T cells. Our results suggest that positive co-stimulation through single-stranded ORNs appears to be mediated by a direct TLR7 stimulation of the T cells. This would be in accordance with the observation that TLR7 is expressed on purified CD4⁺ T cells and TLR8 not (Caron et al., 2005).

Surprisingly, T cells behaved completely different when stimulated by TLR7/8 ORN agonists within a whole PBMC culture. The stimulatory TLR7/8 ORN agonist R-0006 led to a strong inhibition of anti-CD3 induced T cell proliferation. The TLR7/8 ligand R-848 and

the TLR8-specific ORN R-0002 as well showed a similar inhibition, suggesting that the inhibition may be mediated by TLR7 and/or TLR8.

A previous study showed that IFN- γ has an anti-proliferative effect on T cells (Gajewski et al., 1988). However, although T cells produce IFN- γ in the presence of the TLR7/8 agonists, exogenously added TNF- α or IFN- γ had no influence on T cell activation. In addition, cell proliferation in general was not inhibited since the ORNs did not inhibit CpG ODN TLR9-mediated B cell proliferation, ruling out a potential general anti-proliferative effect on all cells, or toxicity induced in the cultures resulting in cell death.

IFN- α has been described to block S-phase entry of stimulated T cells (Erickson et al., 1999). We also detected inhibition of T cell proliferation by exogenously added IFN- α , however, the effect was much stronger with the TLR7/8-specific ORN. Additional results arguing against IFN- α as the responsible mediator of the suppressive effect were acquired using a strong IFN- α producer acting through TLR9. The CpG C-Class ODN (Honda et al., 2005; Vollmer et al., 2004; Hartmann et al., 2003) did not similarly suppress T cell proliferation upon co-stimulation with the TCR. However, when formulated with DOTAP, the CpG C-Class ODN produced similar amounts of IFN- α compared to R-0006, but the inhibition of T cell proliferation was much lower than induced by R-0006. These data indicate that IFN- α expression is not sufficient to explain the inhibition of T cell proliferation induced by ORN R-0006. It is likely that the mechanism for suppression by R-0006 may also involve to some extent IFN- α mediated effects. The presence of IFN- α might as well explain the effect seen in the transwell and depletion experiments where we could not detect any increase in T cell proliferation despite depletion of monocytes.

The inhibition of proliferation was not restricted to naive T cells that are dependent on co-signals for activation. We observed a similar inhibition with antigen- (TT) activated memory T cells that require no co-stimulation (Kearney et al., 1994; Pihlgren et al., 1996; Curtsinger et al., 1998). In addition, TCR superantigen stimulation by SEB was strongly inhibited upon TLR7/8 ligand co-incubation. Superantigens are able to stimulate T cells that express a particular class of β -domains of the T cell receptor, in association with MHC-Class-II bearing cells (Niedergang et al., 1995), and do not require peptide-specific MHC recognition (Seth et al., 1994). This suggests that inhibition of T cell proliferation through TLR7/8 stimulation is not due to a lack or suppression of a positive T cell activation co-signal. In addition, the TLR7/8-activating ORN exhibited a strong inhibition of allogeneic MLRs, whereas making it unlikely that a defective antigen recognition is involved. Taken together, suppression of T cell proliferation mediated by TLR7/8-activating ORNs is independent from the stimuli required to induce T cell proliferation. Inhibition of T cell activation is also not due to cell death occurring in T cells or other cell populations. It appears more likely that incubation with TLR7/8 ligands inhibits cell cycle progression of T cells since R-0006 co-stimulated T cells did not undergo apoptosis but failed to divide.

Additional experiments led us to the surprising observation that suppression of T cell proliferation in the presence of a TLR7/8 agonist is cell contact dependent. Moreover, the monocyte population appears to be responsible for the proliferation inhibition. Our experiments exclude several possible mechanisms through which monocytes may transmit a negative signal to T cells. First, IDO is described to have an essential negative impact on T cell activation, and is induced by type I and II interferons (Hassanain et al., 1993; Kadoya et al., 1992; Chon et al., 1995), or by TLR ligands (Munn et al., 1998). Intravenous administration of a TLR9 agonist under certain conditions can induce IDO upregulation and results in a suppression of T cell responses (Wingender et al., 2005b; Mellor et al., 2005). 1-MT is a tryptophane analogue that acts as a potent IDO inhibitor, and is shown to reverse suppression of T cell proliferation in the presence of IDO⁺ dendritic cells (Hwu et al., 2000).

IDO as well is induced in monocytes by different TLR7/8 ligands (Furset et al., 2007), which were able to suppress autologous T cell activation. This effect could be restored by treating the cells with 1-MT (Furset et al., 2007). Similar to TLR9 CpG ODN agonists, the TLR7/8 ORN ligand induced high amounts of IFN- γ , and upregulated IDO expression in monocytes. However, T cell proliferation was not restored by treatment with the IDO inhibitor 1-MT. Second, exogenously added IFN- γ or TNF- α or a CpG C-Class ODN, inducing very high amounts of IDO, did not inhibit T cell proliferation. Overall, our data strongly suggest that the mechanism of inhibition is not due to IDO activation.

We also tested if positive or negative co-stimulatory molecules on monocytes may be involved in the inhibition of T cell proliferation which may be differentially affected upon treatment with TLR7/8 agonists. One such surface molecule with a negative regulatory function for T cells is PD-L1 which is a B7 family member and has recently been shown to down-regulate T cell activation through the programmed death-1 (PD-1) receptor (Freeman et al., 2000; Latchman 1997; Carter et al., 2002). It was also reported that in HIV-1 infected individuals upregulation of PD-L1 is one mechanism of T cell inhibition. The same study also showed that HIV-1-derived TLR7/8 ligands are able to increase MyD88-dependent PD-L1 expression on monocytes, dendritic cells and myeloid dendritic cells (Meier et al., 2008). We confirmed this finding, and observed PD-L1 upregulation on monocytes upon treatment with TLR7/8 ORN agonists. However, upregulation of PD-L1 was only induced at lower but not higher ORN concentrations at which T cell proliferation inhibition also occurs. Therefore, a lack of upregulated PD-L1 at ORN concentrations inducing strongest inhibition may argue against an involvement of this cell surface molecule in the suppressive effect on T cell proliferation.

Inhibition of T cell proliferation appears to be mediated by TLR8 stimulation of monocytes as TLR8 but not TLR7 is expressed on monocytes (Smits et al., 2008). Therefore, although direct TLR7 stimulation of T cells leads to activation of T cell proliferation, this positive proliferation signal is overcome by the inhibitory signal mediated by TLR8-stimulated monocytes. In this scenario, isolated T cells expressing TLR7 would be directly co-stimulated, whereas monocytes expressing TLR8 would deliver a negative signal to the activated T cells, resulting in a block of T cell proliferation. Indeed, we did not detect any T cell inhibition induced by an ORN exclusively stimulating TLR7 signaling. ORN R-0006 is a strong TLR7 and TLR8 stimulator (Forsbach et al., 2008), whereas ORN R-0002 only stimulates TLR8, although to a lesser extent (Forsbach et al., 2007). This would also explain why the inhibitory effect of ORN R-0006 was more pronounced than that of ORN R-0002.

An additional indication for TLR8 being the main mediator of T cell inhibition was identified in our murine T cell stimulation experiments. Stimulation of murine splenocytes with R-0006 did not lead to inhibition of T cell proliferation, although R-0006 is a potent cytokine inducer in mice *in vitro* and *in vivo* (Forsbach et al., 2007). Mice do have an impaired TLR8 (Hemmi et al., 2002; Gordon et al., 2006; Heil et al., 2004), which would explain why R-0006 was not inhibitory for T cells. The percentage of monocytes within the murine splenocyte population is comparable, although being somewhat lower to that in human PBMCs (Lagasse et al., 1996; Lopez-Gonzalez et al., 2007). However, even with a lower number of monocytes in the murine splenocyte cultures, one would have expected to observe a negative effect on T cell proliferation in the presence of a functional TLR8 in the monocyte population.

In summary, we demonstrate that T cell activation is directly increased by single-stranded ORNs containing a TLR7/8 stimulating RNA sequence motif. However, T cells within a PBMC environment are in cellular contact with monocytes which appear to suppress T cell activation upon TLR8 activation. Since R-848 gave promising results including enhanced

antigen-specific T cell activation in a monkey study by stimulating both TLR7 and TLR8 (Wille-Reece et al., 2006), the question has to be resolved if our observed effect is an *in vitro* phenotype that is not seen *in vivo*, or if an ORN exclusively stimulating TLR7 would be better suited for certain vaccination strategies. This monkey study demonstrated that the highest Th1 responses were elicited by immunization with HIV Gag protein together with either a CpG ODN or the TLR7/8 agonist R-848, especially when R-848 was conjugated to the HIV Gag protein. It was superior to the immunization with a TLR8-only agonist that led to significant lower Th1 responses. It is also possible that the negative effect of monocytes on T cells can be overcome in an *in vivo* setting.

Finally, our results emphasize the importance to control for the cellular environment, and again point out the differences between the murine and human innate immune system.

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3 Abbreviations used in this Paper

ORN	oligoribonucleotide
ODN	oligodeoxyribonucleotide
CPB	cyclophilin B
CFSE	carboxyfluorescein succinimidyl ester
DOTAP	1,3-dioleoyloxy-3-(trimethylammonium)propane
PAMP	pathogen-associated molecule pattern
poly I:C	polyriboinosinic:polyribocytidylic acid
PBMC	peripheral blood mononuclear cells
TT	tetanus toxoid

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FIGURE 1. Isolated CD4⁺ T cells are co-stimulated by TLR7/8 specific single-stranded ORNs
 A, Magnetic bead purified CD4⁺ T cells were stained with CFSE (2.5μM) and stimulated with plate-bound anti-CD3 antibody (2μg/ml) for three days together with Pam₃CSK₄ (4μg/ml), R-848 (1μg/ml), or ORNs (1μM) formulated with 12.5 μg/ml DOTAP. The proliferation of T cells was measured as the dilution of CFSE. One representative out of six experiments is shown. B, Isolated T cells were stimulated like in (A) and proliferation was calculated as the dilution of CFSE. Concentration of ORNs (R-0006, R-0002, R-1263) was 0.125μM, prepared with 1.55μg/ml DOTAP. Data shown represent mean ± SEM of five donors. C, Supernatants of the T cell cultures were harvested and IFN-γ production was measured in an ELISA. For stimulation without anti-CD3 antibody, 100 U/ml IL-2 were used. Data shown represent mean ± SEM of six donors. Significance of differences was determined by Student's t-test.

FIGURE 2. ORNs inhibit anti-CD3 induced T cell proliferation within PBMCs

A, PBMCs were isolated from different donors, stained with CFSE (2.5 μM) and stimulated with anti-CD3 antibody (2 μg/ml) and the indicated ligands on 96-well plates for three days. ORNs were formulated with DOTAP (4 μM ORN with 50 μg/ml DOTAP). ODNs were used in the absence of DOTAP. For analysis PBMCs were stained with mAb to CD4, CD8 (B) and CD19 (C), and the proliferation of the different cell types was measured as dilution of CFSE fluorescence. Data shown represent mean ± SEM of eleven (A, B) and 3 donors (C).

FIGURE 3. Strong inhibitory effect of ORNs is not mediated by TLR7/8 induced cytokines
 A, PBMCs were stimulated with the indicated amounts of ORNs and DOTAP. ODNs were used either unformulated or formulated with DOTAP (4 μM ODN or ORN with 50 μg/ml DOTAP). Supernatants of stimulated PBMCs were analyzed for IFN- α production. IFN- α amounts were measured after 24 hours. B, PBMCs were stimulated for three days with ORNs, unformulated CpG ODNs or DOTAP formulated CpG ODNs. The proliferation of CD4⁺ T cells within PBMCs was measured as the dilution of CFSE by FACS. C, PBMCs were stimulated with the indicated amounts of ORNs and DOTAP (1 μM ORN with 12.5 μg/ml DOTAP), or either with 0.5 μM ORN R-1263 together with different concentrations of IFN- α , or with R-0006. The proliferation of CD4⁺ T cells within PBMCs was measured as the dilution of CFSE. All data shown represent mean $\pm$ SEM of three donors.

FIGURE 4. TLR7/8 ligand R-0006 leads to inhibition of T cell proliferation independent of the specific T cell stimulus

A, PBMCs were stimulated with 2.5µg/ml TT for six days together with the indicated TLR agonists. ORN R-0006 and R-1263 were formulated with DOTAP (1µM ORN with 12.5µg/ml DOTAP). The recall antigen stimulation of CD4⁺ T cells within PBMCs was measured as the dilution of CFSE. Data shown are mean ± SEM of six donors. B, PBMCs were stimulated with 2µg/ml SEB for three days and proliferation of CD4⁺ and CD8⁺ T cells was measured. Data shown represent mean ± SEM of six donors. C, T cells from one donor and CD3 depleted APCs from another donor were isolated and mixed in a 5:1 ratio (T cells:APCs in a MLR) for three days. For inhibition of IDO function, two different concentrations of 1-MT (100µM and 500µM) were added to the cultures. Proliferation of T cells in allogenic MLR was measured as the dilution of CFSE. One experiment out of three is shown. D, PBMCs were stimulated with the indicated ligands (1.3µM CpG C-Class ODN 2395 or ORN R-0006 with DOTAP 16.6µg/ml) or 20ng/ml IFN-γ for 24h. Plates were washed and RNA from plate bound monocytes was prepared. IDO mRNA levels relative to 1000 copies cyclophilin B are depicted. Data shown are mean ± SEM of three donors

FIGURE 5. Lack of T cell apoptosis or necrosis upon TLR7/8 stimulation

A, PBMCs were stimulated with the different ORNs (4μM with 50μg/ml DOTAP) for 24h and stained with AnnexinV and PI together with anti-CD3 Ab. Percentage of the AnnexinV⁺, PI⁻ population within the whole PBMCs or within the pre-gated CD3⁺ T cell population (B) is indicated. Data shown represent mean ± SEM of three donors.

FIGURE 6. Inhibition of T cell proliferation is cell contact dependent

A, Schematic overview of the transwell assay system. Flat bottom 96-well plates were coated with anti-CD3 Ab (2 μ g/ml) and T cells were cultured on these plates. In the transwell chamber T cell depleted PBMCs were stimulated. T cells and T cell depleted PBMCs from one donor were isolated and mixed either directly together (5×10^5 T cells with 1×10^5 T cell depleted PBMCs) for the control plate (C), or were stimulated in different chambers in the transwell system (B). Before mixing, T cells were stained with 2.5 μ M CFSE. Cells were stimulated for three days with the indicated ligands. ORNs were formulated with DOTAP (4 μ M with 50 μ g/ml DOTAP). As control T cells were stimulated with anti-CD3 Ab together with 100 U/ml IL-2. Data shown represent mean $\pm$ SEM of 4 donors.

FIGURE 7. TLR7/8 stimulated monocytes mediate inhibition of T cell proliferation

A, PBMCs were depleted of CD14⁺ monocytes by magnetic bead separation. Depleted PBMCs or non-depleted PBMCs, as control, were stained with CFSE (2.5 μ M) and stimulated for three days with anti-CD3 antibody (2 μ g/ml) and the indicated TLR ligands. ORNs were formulated with DOTAP (2 μ M ORN with 25 μ g/ml DOTAP). Proliferation of CD4⁺ and CD8⁺ T cells was measured after three days of culture. Data shown are mean $\pm$ SEM of six donors. B, PBMCs were stimulated for 24h with the indicated ligands with 0.1 μ M ORNs formulated with 1.25 μ g/ml DOTAP or 1 μ M ORN formulated with 12.5 μ g/ml DOTAP. PBMCs were stained with a cocktail of anti-CD19, anti-CD56, anti-CD3 antibodies together with anti-PD-L1 antibody. Monocytes (as CD56⁺CD3⁺CD19⁺ population) were analyzed for PD-L1 expression after 24h. Data shown represent mean $\pm$ SEM of six donors (two independent experiments). Significance of differences was determined by Student's t-test. C, PBMCs were stained with CFSE (2.5 μ M) and stimulated for three days with anti-CD3 antibody (2 μ g/ml) and the indicated TLR ligands. ORNs were formulated with DOTAP (2 μ M ORN with 25 μ g/ml DOTAP). Proliferation of CD4⁺ and CD8⁺ T cells was measured after three days of culture. Data shown are mean $\pm$ SEM of three donors. D, Splenocytes from C57BL/6 wt mice were isolated and stained with CFSE (2.5 μ M), and stimulated with the indicated TLR ligands. ORNs were formulated with DOTAP (4 μ M ORN with 50 μ g/ml DOTAP). Data shown represent mean $\pm$ SEM of four wt C57BL/6 mice.