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Differential but Direct Abolishment of Human Regulatory T Cell Suppressive Capacity by Various TLR2 Ligands

Hans-Heinrich Oberg, Thi Thuy Hoa Ly, Sandra Ussat, Tim Meyer, Dieter Kabelitz, and Daniela Wesch

CD4⁺CD25^{high} regulatory T cells (Tregs) control cellular immune responses and maintain peripheral tolerance. We investigated whether TLR2 ligands are able to abrogate Treg-induced suppression in humans based on different reports about effects of triacylated lipopeptide Pam3CSK4 in mice. Pretreatment of human Tregs with a mixture of TLR2 ligands Pam3CSK4, FSL-1, and Pam2CSK4 reduced the Treg-mediated suppression of CD4⁺CD25⁻ responder T cells in the majority of the analyzed donors. Differential effects of individual TLR2 ligands are explained by usage of different TLR2 heterodimers in the recognition of Pam3CSK4, FSL-1, and Pam2CSK4. In contrast to the murine system, TLR2 ligand-mediated abrogation of human Treg function was not associated with a downregulation of FoxP3 transcription factor. Furthermore, our results excluded an effect of TLR2 ligands on granzyme A/B release by human Tregs as a potential mechanism to abolish Treg-mediated suppression. Our data suggest that a downregulation of p27Kip1 and restoration of Akt phosphorylation in human Tregs pretreated with TLR2 ligands result in a reversal of suppression on responder T cells. Moreover, our data indicate that a mixture of TLR2 ligands can be used to modulate human Treg activity. The Journal of Immunology, 2010, 184: 4733–4740.

Apart from the effect of exogenous IL-2, it has been suggested that the suppressive capacity of Tregs is abolished by proinflammatory cytokines produced by APC in response to TLR ligands (12–18). TLRs are pattern recognition receptors, which are expressed in APC but also in different subsets of T lymphocytes (19–21). We previously reported that TLR3 provides a costimulatory signal to human γδ T lymphocytes (22), which have been recently confirmed for a subpopulation of human CD8^αβ T cells (23). Selected TLR ligands (TLR2, TLR5, and TLR7/8) also costimulate the anti-CD3 mAb induced proliferation and cytokine production of CD4^αβ T cells (24, 25). Moreover, human Tregs were found to express TLR8 mRNA, and activation by the corresponding ligand ssRNA abrogated the suppression of responder T cells (26), whereas TLR5 ligand flagellin enhanced FoxP3 expression and the suppressive capacity of human Tregs (27). It is possible that there are species-specific differences in the control of Treg activity by TLR ligands. The suppressive activity of human Tregs was not reduced in the presence of triacylated bacterial lipopeptide N-palmitoyl-S-[2,3-bis (palmitoyloxy)-2RS-propyl]-[R]-Cys-[S]-Ser-[S]-Lys (4) trihydrochloride (Pam3CSK4), whereas there are controversial reports of Pam2CSK4 on murine Tregs (28–31). In mice, triacylated lipopeptides, such as Pam3CSK4, are recognized by a TLR1/2 heterodimer (32) or independently of TLR1 (33). Furthermore, Hajjar and colleagues (34) reported on the recognition of triacylated lipopeptides in a TLR1/2-dependent fashion in human cells.

In the current study, we compared the effects of the triacylated lipopeptide Pam3CSK4 and other TLR2 ligands, such as diacylated lipopeptides S-[2,3-bis(palmitoyloxy)-2RS-propyl]-[R]-Cys-[S]-Ser-[S]-Lys (4) trihydrochloride (Pam3CSK4) and S-[2,3-bis(palmitoyloxypropyl)]-Cys-Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Phe (FSL-1, or Pam2CGDPKHP), on Treg-mediated suppression of human CD4⁺ T cell activation. Experiments in TLR-deficient mice indicate that macrophage activating lipopeptide 2, which is structurally similar to FSL-1, is recognized by a TLR2/TLR6 heterodimer, whereas Pam3CSK4 has been reported to be recognized by TLR2 in a TLR6-independent manner (35, 36). Moreover, it has been demonstrated that TLR2 requires TLR6 as a coreceptor for recognition of FSL-1 in human cells (37).

The online version of this article contains supplemental material.

Abbreviations used in this paper: CBA, Cytometric Bead Array; cdk, cyclin-dependent kinase; DDAO-SE, 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one) succinimidyl ester; IF-10, IFN-γ-inducible protein-10; LAU, linear arbitrary unit; MFI, mean fluorescence intensity; TDR, thymidine; Treg, regulatory T cell.

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In this study, we report that TLR2 ligands directly abrogate the suppressive capacity of human Tregs in 75% of the tested donors. We observed that individual TLR2 ligands vary in their efficacy, which correlates with the interindividual heterogeneity in the TLR expression pattern among healthy donors. The abrogation of human Treg function was associated with neither a downregulation of FoxP3 expression nor a proliferative expansion of Tregs. Instead, we found that TLR2 ligands downregulate cdk inhibitor p27kip1 in human Tregs and that an integrated signal of TLR2 ligands and TCR stimulation reconstitutes the defective Akt phosphorylation, thereby abrogating the suppressive capacity of human Tregs.

Materials and Methods

Isolation of T cell populations and cell culture

Bufy coats from healthy adult blood donors were obtained from the Department of Transfusion Medicine in Kiel and Lübeck, Germany. Informed consent was obtained from all donors, and research was approved by the relevant institutional review boards. PBMCs were isolated from the bufy coats by Ficoll-Hypaque (Biochrom, Berlin, Germany). CD4+CD25- responder T cells and Tregs were separated from freshly isolated PBMCs using the magnetic cell separation system (Miltenyi Biotec, Bergisch Gladbach, Germany). Responder T cells were negatively isolated by the following the positive selection of Tregs by CD25 Dynabeads (Invitrogen, Karlsruhe, Germany). To obtain Tregs, magnetic particles were removed from the cells by using DETACH-BEAD (Invitrogen). The purity of the selected responder T cells and Tregs was >97%. The isolated T cells were cultured in serum-free X-VIVO-15 medium (Cambrex Bio Science, Verviers, Belgium) in an APC-free suppression assay as described (38). Briefly, 10^5 or 10^6 purified responder T cells, 2.5 × 10^5 or 5–7.5 × 10^5 purified autologous Tregs, or 2.5 × 10^4 purified autologous Tregs, and 0.5 μg/ml anti-CD3 and anti-CD28 were added and used as TCR stimulus. In addition, experiments, non-Tregs (CD25+CD4+) instead of Tregs were cocultured with responder T cells. To investigate the effect of different TLR2 ligands on various subsets, cells were pretreated with 10^5 responder T cells and 5–7.5 × 10^5 Treg (cultured in 96 round-bottom plates) after 24–72 h and stored at −20°C. Simultaneously, proliferation was measured in these cultures by uptake of [3H]thymidine (TDr).

T cell proliferation assays

Proliferation was measured by uptake of [3H]Thd during the last 16 h of a 3–5 d culture period using a Wallac 1450 Microbeta Trilux counter (PerkinElmer, Rodgau-Jügesheim, Germany). In these assays, 10^5 responder T cells and/or 2.5 × 10^5 Treg were cultured per well in 96 round-bottom plates. Results are expressed as mean cpm ± SD of triplicate cultures. In respect to the responder T cell proliferation, reduced proliferation (i.e., suppression) was calculated as (1 – cpm of responder with Treg/cpm of responder without Treg) × 100.

To discriminate between responder T cells and Tregs, cells were labeled with 5 μM Cell Tracer, either with Far Red 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one) succinimidyl ester (DDAO-SE; Invitrogen) or CFSE (Fluka/Sigma-Aldrich, Tautkirchen, Germany) for 10 min at 37°C. Thereafter, 5 ml cold 10% FCS RPMI 1640 was added and incubated for 5 min on ice. Cells were washed three times with cold 10% FCS RPMI 1640. After centrifugation, Treg cells and 5–7.5 × 10^5 responder T cells were resuspended in X-VIVO-15 (Cambrex Bio Science) and pretreated or not with lipopeptides for 24 h in 96 round-bottom plates. Thereafter, cells were cultured with Activation/Expander Beads (Miltenyi Biotec). These beads were coated with 10 μg/ml each anti-CD3 and anti-CD28 and 0.5 μg/ml anti-CD28 mAb and used as TCR stimulus. In selected experiments, non-Tregs (CD25+CD4+) instead of Tregs were cocultured with responder T cells. To investigate the effect of different TLR2 ligands on various subsets, cells were pretreated with 10^5 responder T cells and 5–7.5 × 10^5 Treg (cultured in 96 round-bottom plates) after 24–72 h and stored at −20°C. Simultaneously, proliferation was measured in these cultures by uptake of [3H]thymidine (TDr).

Cytometric bead array and ELISAs

The BD Cytometric Bead Array (CBA) Human Flex Set was used to measure production of cytokines, such as IL-2, IFN-γ, TNF-α, IL-4, IL-6, the β form of pro-IL-1, and chemokines, such as IL-8, IFN-γ-inducible protein-10 (IP-10), RANTES, MCP-1a, MCP-1b, and MCP-1, in cell culture supernatant samples after 24–72 h. The CBA Human Flex Set was used in combination with a CBA Human Soluble Protein Master Buffer Kit (BD Biosciences) following the instructions of the manufacturer. Samples were analyzed on a BD FACSAri dual-color analyzer (BD Biosciences) using the FCAP Array software (BD Biosciences). Human granzymes A and B were determined by a sensitive sandwich ELISA (BMS2026 and BMS2027) following the procedures outlined by the manufacturer (Bender MedSystems, Vienna, Austria). For assessment of granzymes, cell culture supernatants were collected 24, 48, and 72 h after stimulation.

Flow cytometry

The following mAbs were used: FITC-labeled anti-CD4, PE- or allophycocyanin-labeled anti-CD25, and control IgG (all from BD Biosciences). PE-conjugated anti-TLR2 mAb clone TL2.1 and PE-conjugated anti-TLR1 mAb clone GDF2.F4 (both from eBioscience, Wien, Austria) were used for surface staining and anti-CD66s mAb clone 66B1153.2 (directed against a cytoplasmic domain) for intracellular staining (Axxora, Liersch, Germany). Unconjugated anti-TR6 was labeled with Alexa Fluor 546 mAb Labeling Kit (Invitrogen) according to the instructions of the manufacturer. For TR6 detection, 1 to 2 × 10^6 cells were washed, fixed, and permeabilized with the Cytofix/Cytoperm Kit (BD Biosciences) and for intracellular FoxP3 expression with eBioscience Fix/Perm Buffer (eBioscience). After blocking, cells were fixed, permeabilized, washed, and stained with either anti-human TR6-Alexa 546 or with anti-human FoxP3-PE (PCH101; eBioscience) Ab for 30 min. After two washing steps, all samples were measured on an FACScalibur flow cytometer (BD Biosciences) using the CellQuest software (CellQuest, Tampa, FL).

For detection of phosphorylation of Akt at serine 742, the modified Phosflow Protocol III (BD Biosciences) was used. Briefly, isolated responder T cells or Tregs were preincubated with medium or TLR2 ligands for 24 h; thereafter, cells were stimulated with TCR or cultured in medium for 0.5, 2, and 5 min in the absence or presence of high concentrations of IL-2 (250 U/ml). Thereafter, cells were fixed with BD Cytofix (BD Biosciences) at 37°C for 10 min, washed, permeabilized with Phosflow Perm Puffer III (BD Biosciences) on ice for 30 min, washed, and stained with PE-labeled anti-phospho-Akt-Ser473 or as a control with anti-phospho-Thr380 (both from BD Biosciences). Flow cytometry was performed on a FACScalibur flow cytometer (BD Biosciences).

p27kip1 Western blot analysis

A total of 2 to 2.5 × 10^5 untreated or TLR2 ligand pretreated Tregs were cultured in medium alone or stimulated with Activation/Expander Beads (Miltenyi Biotec) or Dynabeads CD3/CD28 Expander (Invitrogen) for 24 h. Cells were lysed in Nonidet P-40 lysis buffer (Fluka Chemie, Buchs, Switzerland) with 1% (v/v) detergent in 20 mM Tris-HCl, 150 mM NaCl with protease inhibitors aprotinin, leupeptin, PMSF, sodium pyrophosphate, and sodium fluoride. Samples were separated on 4–12% Nu-PAGE Gel (Invitrogen), and protein was transferred to nitrocellulose membranes (Hybond C-Extra, Amersham Biosciences, Braunschweig, Germany). Blots were blocked with 5% BSA, and p27kip1 was detected by purified mouse anti-p27kip1 Ab (BD Biosciences), followed by HRP-conjugated sheep anti-mouse Ab (Amersham Biosciences). Membranes were stripped and reprobed with anti-β-actin mAb as a control for loading and transfer. Chemiluminescence was detected with LAS-3000 (Raytest, Stubesnach, Germany) and analyzed with AIDA Image analyzer software (Raytest).

Statistical analysis

The paired, two-tailed Student t test was performed.

Results

Lipopeptides abrogate the suppressive activity of human Tregs

We investigated the influence of TLR2 ligands Pam3CSK4, Pam2CSK4, and FSL-1 on the Treg-mediated suppression of human CD4+CD25+ responder T cell proliferation in vitro. Therefore, we isolated highly purified CD4+CD25+FoxP3+ Tregs (Supplemental Fig. 1) and autologous responder cells from different donors. As expected, responder T cell proliferation was inhibited in the presence of highly purified CD4+CD25+FoxP3+ Tregs, but not in the presence of autologous CD25+CD4+ non-Tregs (Supplemental Fig. 2). To examine whether Treg (Fig. 1A, T) or responder T cells (Fig. 1A, R) were affected by TLR2 ligands, each of the two T cell populations was isolated, separately pretreated for 24 h with the...
Further experiments were performed to evaluate in more detail: 1) suppression; 2) abrogation of suppression by the various TLR2 ligands; and 3) statistical significance of these results. Suppression was calculated in relation to parallel cultures set up with responder T cells in the absence of Tregs. The pretreatment of Tregs with PamCysK4, PamCysK4, or FSL-1 induced a significant reversal of the Treg-mediated suppression in 9 out of 14 tested donors (p < 0.05; Fig. 1B, left), whereas in 5 out of 14 donors, pretreatment had no effect (Fig. 1B, right). In contrast, the pretreatment of responder T cells with TLR2 ligands only moderately reduced the suppressive effect in 3 out of 11 tested donors (mainly PamCysK4; Fig. 1C, left). These results indicated that the TLR2 ligand-induced reduction of Treg suppression could be attributed more frequently to a direct effect on Tregs (reduction in 64% of the tested donors with all TLR2 ligands; Fig. 1B) than to a costimulatory effect on responder T cells (reduction in 27% of the donors only with PamCysK4; Fig. 1C).

Therefore, we focused in the next series of experiments on the pretreatment of Tregs with TLR2 ligands. We scrutinized the difference between donors in whom Tregs were affected by TLR2 ligands and those in whom Tregs were not affected. We observed that the donor-dependent variability in the effects of TLR2 ligands on abrogation of Treg-mediated suppression was possibly due to donor-dependent differences in the TLR expression pattern. As shown in Fig. 2 for 17 individual donors, Tregs always expressed TLR2 but neither TLR1 nor TLR6 in the nonabrogative group (Fig. 2Aa). Tregs expressing TLR2 and TLR6 (Fig. 2Ab) reacted to the preincubation with PamCysK4 and FSL-1 with an abrogation of the Treg-mediated suppression (Fig. 2Bb), whereas TLR1/TLR2 expressing Tregs responded to pretreatment with PamCysK4 (Fig. 2Bc). However, we also observed partial abrogation after pretreatment with PamCysK4 but not with FSL-1 of Tregs, which did not express TLR6, possibly suggesting a signaling via TLR2 independent of TLR6 for PamCysK4 (Fig. 2Ab, ▲; Fig. 2Ac, ☆/×). Similar to responder T cells, the pretreatment of Tregs with the TLR2 ligands alone did not result in an upregulation of TLR expression, ruling out that pretreatment of the cells with TLR2 ligands induced an activation of these cells (Supplemental Fig. 3, med). The addition of Activation/Expander Beads (Miltenyi Biotech) coated with anti-CD3, anti-CD28, and anti-CD2 mAbs, which are used in the suppression assay after the pretreatment of Tregs as a TCR stimulus (TCR), induced an upregulation of TLRs, also of the weakly expressed TLRs. Initially not expressed TLRs were upregulated after combined stimulation via TCR and TLR2 ligands (Supplemental Fig. 3, TLR1). Moreover, we recognized that Tregs, which responded strongly to one TLR2 ligand, reacted only moderately to the two other TLR2 ligands (Supplemental Fig. 4), again in line with the respective TLR expression on these cells.

**TLR2 ligand-pretreated Tregs did not suppress cytokine and chemokine production**

The different effects of the individual TLR2 ligands convinced us to use a mixture of TLR2 ligands in subsequent experiments. Besides proliferation, the cytokine and chemokine production of responder T cells was also impaired when cocultured with untreated Tregs. Pretreatment of Tregs with TLR2 ligands reverted the suppressive capacity, thereby restoring IL-2, IFN-γ, IP-10 (CXCL-10), and RANTES (CCL5) production. IL-8 (CXC-8) was not suppressed in the presence of Tregs, but was enhanced in the presence of TLR2 ligand-pretreated Tregs. The data were obtained from four independent experiments measured after 48 h (data not shown) and 72 h (Fig. 3). In contrast, production of TNF-α, MIP-1α (CCL3), and MIP-1β (CCL4) was not restored after coculture of responder T cells with TLR2 ligand-pretreated Tregs (Fig. 3). No significant levels of Th2 cytokines, such as IL-4, IL-10, and IL-6, were measured different TLR2 ligands, washed, and analyzed in the suppression assay (38). We observed that the Treg-mediated suppression of responder T cell proliferation (R/T) was completely abrogated after pretreatment of Tregs with a mixture of PamCysK4, FSL-1, and PamCysK4 in the majority (four of five) of experiments (representative experiment shown in Fig. 1A, R/T+TLR2L). In contrast, pretreatment of responder T cells with TLR2 ligands (Fig. 1A, T/R+TLR2L) did not result in a reduction of suppression in the majority of the experiments, although the pretreatment had a costimulatory effect on responder T cells in the absence of Tregs (Fig. 1A, R+TLR2L). These results clearly show that human responder T cell proliferation can be modulated by a direct effect of TLR2 ligands on Tregs in the absence of APCs. Moreover, we observed that the tested lipopeptides did not exert any effect on their own in the absence of anti-CD3/anti-CD28/anti-CD2 stimulation (data not shown).
in these culture supernatants. Moreover, untreated or TLR2 ligand-pretreated Tregs did not produce any of the mentioned cytokines and/or chemokines in the absence or presence of anti-CD3/anti-CD28/anti-CD2 mAb activation (data not shown). Next, we analyzed whether a sustained FoxP3 expression in human Tregs correlated with the inability of Tregs to produce cytokines or chemokines after TLR2 ligand pretreatment. As expected, human Tregs expressed FoxP3 after TCR triggering (Supplemental Fig. 5) instead of Activation/Expansion Beads (Miltenyi Biotec). Production of the indicated cytokines and chemokines was determined in cell-culture supernatants by BD CBA Human Flex Sets. Mean ± SD of four different experiments after 72 h of culture are shown. Significances are represented as *p < 0.05 and **p < 0.01. (†) Presented a t test value of 0.069.

**FIGURE 2.** Efficacy of TLR2 pretreatment correlates with TLR1, -2, and -6 expression on Tregs. A, Tregs from 17 representative donors were stained with anti-TLR1, -2, or -6 mAbs or the appropriate isotype control. The expression of TLR1, -2, and -6 is depicted as mean fluorescence intensity (MFI) after subtraction of the MFI of isotype control. Donors were grouped into TLR2+, TLR1−, and TLR6− (a); TLR2+, TLR1+, and TLR6+ (b); and TLR2−, TLR1dim, and TLR6− (c). B, Tregs were pretreated for 24 h without (black bars) or with Pam3CSK4 (light gray bars), FSL-1 (white bars), or Pam2CSK4 (dark gray bars) and thereafter cocultured with CD4+CD25+ responder T cells and stimulated with anti-CD3/anti-CD28/anti-CD2 mAb-coated T cell Activation/Expansion Beads (Miltenyi Biotec). [3H]ThdR uptake was analyzed after 5 d, and suppression was calculated as (1−cpm of responder with Treg/cpm of responder without Treg) × 100. Median ± SD of the 17 experiments is shown.

**FIGURE 3.** Mixture of TLR2 ligands partially abrogates Treg-mediated suppression of cytokine and chemokine production. A total of 1 × 10^6 CD4+CD25− responder T cells (R, black bars), coculture of responder T cells and 7.5 × 10^5 of untreated (R/T, light gray bars) or TLR2 ligand mixture-pretreated Tregs (R/T, dark gray bars) were stimulated with T Cell Activation/Expansion Beads (Miltenyi Biotec). Production of the indicated cytokines and chemokines was determined in cell-culture supernatants by BD CBA Human Flex Sets. Mean ± SD of four different experiments after 72 h of culture are shown. Significances are represented as *p < 0.05 and **p < 0.01. (†) Presented a t test value of 0.069.

**TLR2 ligand pretreatment did not enhance granzyme production in Tregs**

In further experiments, we examined the effect of TLR2 ligands on the abrogation of the suppressive capacity of Tregs in more detail. Although the exact molecular mechanism of Treg-mediated suppression is not completely clear, enhanced levels of intracellular granzyme A in activated human Tregs and granzyme B in murine Tregs have been reported to be responsible for suppression of responder T cells by inducing cell death (5–7). Therefore, we examined whether the pretreatment of human Tregs with TLR2 ligands influenced the production of granzymes. Human Tregs produce little if any granzyme A 72 h after TCR stimulation (in the absence of exogenous IL-2) and, quite unexpectedly, a much lower amount than responder T cells (Fig. 5A). Responder T cells started to produce granzyme A 24 h after TCR triggering, which was neither suppressed in the presence of untreated Tregs nor influenced significantly by TLR2 ligand-pretreated Tregs at any time point (Fig. 5A). We obtained comparable results when we stimulated responder T cells with anti-CD3/anti-CD28 mAb for TCR triggering (Supplemental Fig. 5) instead of Activation/Expansion Beads-coated (Miltenyi Biotec) anti-CD3, anti-CD28, and anti-CD2 mAb (Fig. 5A). Moreover, responder T cells produced much more granzyme B than granzyme A after TCR stimulation (Fig. 5B, Supplemental Fig. 5).

Similar to granzyme A, human Tregs did not or only slightly produced granzyme B after TCR stimulation (Fig. 5B), even in the presence of exogenous IL-2 (data not shown). Strikingly, the observed high granzyme B production of responder T cells 72 h after stimulation was significantly suppressed after coculturing these cells with untreated Tregs (Fig. 5B, R/T). Surprisingly, Treg-mediated suppression of granzyme B production was partially abolished by TLR2 ligand-pretreated Tregs (Fig. 5B). Thus, our data rule out that an enhanced production of granzyme A or B by human Tregs is responsible for the suppression of responder T cells. Moreover, the granzyme A/B production of Tregs is not influenced by pretreatment of Tregs with TLR2 ligands. Additionally, we demonstrate that granzyme B production by responder T cells can be used as an additional read-out assay to investigate suppression by Tregs.

**TLR2 ligand-induced downregulation of p27Kip1 and reconstitution of Akt phosphorylation in Tregs reverse their suppressive capacity**

Another possible explanation for the abrogation of Treg-mediated suppression as measured by [3H]ThdR uptake in the coculture assays
could be an enhanced proliferative capacity of Tregs receiving a strong activation signal via TCR/TLR2 stimulation. Tregs express higher levels of the cell-cycle inhibitor protein p27Kip1 in comparison with responder T cells (Fig. 6A, 6B). We examined in four independent experiments the expression of p27Kip1 and possible effects of TLR2 ligand preincubation. Pretreatment with TLR2 ligands alone did not exert any effect on the p27Kip1 expression of responder T cells, but TCR stimulation decreased expression of p27Kip1, and responder T cells are able to proliferate in the absence of Tregs (Fig. 6A, data not shown). In contrast, preincubation of Tregs with TLR2 ligands induced a reduction of p27Kip1, but, quite unexpectedly, to the same extent as TCR stimulation (Fig. 6B). These findings suggested that Tregs might start to proliferate after TCR stimulation and possibly more effectively after a combined signal of TLR2 ligands and TCR. Therefore, it was necessary to discriminate between responder T cells and Tregs among proliferating cells in the cocultures. We did this by labeling both populations with cell trace dyes, responder T cells with Far Red DDAO-SE, and Treg with green fluorescence CFSE. As expected, responder T cells proliferated upon TCR stimulation in the absence of Tregs (Fig. 7Aa). The proliferation of responder T cells was inhibited in the presence of untreated Tregs (Fig. 7Ab), but not after addition of Tregs pretreated with a mixture of TLR2 ligands (Pam2CSK4, Pam3CSK4, and FSL-1; Fig. 7Ac). More importantly, these experiments revealed that TLR2 ligand-pretreated Tregs did not proliferate (Fig. 7Af) or produce cytokines, such as IFN-γ and IL-2, after TCR stimulation (data not shown). Therefore, TLR2 ligand-mediated abrogation of suppression is not due to the proliferation of Tregs in the coculture assays.

To examine whether additional regulators of cell proliferation are influenced by TLR2 ligand-pretreated Tregs, a Proteome...
Profilor Array for human Phospho-MAPKs (R&D Systems, Wiesbaden, Germany) was performed. The Phospho-MAPK Array revealed a defect in the phosphorylation of Akt in Tregs in comparison with responder T cells (data not shown). These data were validated by the Phosflow method (Fig. 7B). Moreover, with the Phosflow method, it was possible to confirm the defect in the phosphorylation of Akt at serine 473 and not at threonine 308 as already described by others (9). Moreover, we observed that pretreatment of Tregs with TLR2 ligands and TCR stimulation partially overcame the defect in phosphorylation of Akt (Fig. 7B, right panel), although the extent of phosphorylated Akt was much lower in Tregs compared with responder T cells under the same conditions (Fig. 7B). These findings support the observation that pretreatment of human Tregs with TLR2 ligands combined with TCR stimulation results in a loss of their suppressive capacity, but not in a proliferation of Tregs. In further experiments, we asked whether the failure of Treg proliferation might be due to the absence of sufficient IL-2 in the coculture assay for both T cell subsets. We observed that the addition of high concentrations of IL-2 (250 U/ml) resulted in an enhanced phosphorylation of Akt in TCR/TLR2L-treated Tregs (Fig. 7B, right panel) and proliferation of Tregs (Fig. 7C).

Discussion

Our results have identified a previously unknown capacity of TLR2 ligands, such as diacylated Pam2CSK4, FSL-1, and triacylated Pam3CSK4, to reduce Treg-mediated suppression of proliferation and cytokine/chemokine production of CD4+CD25+ human responder T cells. Importantly, only the pretreatment of Tregs but not of responder T cells abrogated the suppressive effect after TCR triggering, which correlated with TRL1, TLR2, and TLR6 expression on human Tregs. Together with the recently described similar effect of poly-G oligonucleotides and natural TLR8 ligand ssRNA (26), it is becoming increasingly clear that the suppressive capacity of Tregs is counterregulated by TLR ligands not only indirectly via APCs or dendritic cells but also directly.

However, the effects of lipopeptides on Tregs displayed a donor-dependent variability, which might be due to the usage of different TLR2 heterodimers in the recognition of Pam2CSK4, FSL-1, and Pam3CSK4, respectively. Peng and colleagues (26) described an unresponsiveness of human Tregs to TLR2 ligand Pam3CSK4, which we confirmed in only 75% of our tested donors. We suggest that the absence of TLR1, which forms a heterodimer with TLR2 and is necessary for recognition of triacylated lipopeptides (32, 39), explains the observed unresponsiveness of human Tregs to Pam3CSK4 more obviously than a species-specific difference between murine and human Tregs. Moreover, the abrogation of Treg-mediated suppression after pretreatment of TLR2+/TLR6 Tregs with Pam3CSK4 support a TLR6-independent signaling of TLR2 for Pam3CSK4, which is described so far only for murine splenic B lymphocytes or bone marrow-derived monocytes from wild-type and TLR-deficient mice (34, 36), but not for human T lymphocytes.

Our observation that Tregs of several donors reacted only to one TLR2 ligand and Tregs from other donors to two or three TLR2 ligands confirmed our initial idea to use a mixture of TLR2 ligands in subsequent experiments. The coculture of responder T cells with untreated Tregs inhibited cytokine/chemokine production. However, pretreatment of Tregs with a TLR2 ligand mixture restored the production of Th1 cytokines (IL-2, IFN-γ) and chemokines (e.g., RANTES, IP-10) in responder T cells to a comparable level with responder T cells alone. Interestingly, IL-8, which is involved in the recruitment of inflammatory cells, was not suppressed by untreated Tregs. However, IL-8 was further enhanced in the presence of TLR2 ligand-pretreated Tregs, suggesting that recruitment of inflammatory cells is continuously ensured and is further increased after the entry of microorganisms. The inability of Tregs to produce IL-2, IFN-γ, RANTES, and IP-10 is caused by the constitutive expression of FoxP3, which can bind to the forkhead-binding sites located within promoter regions of cytokine genes and thus suppress cytokine production and probably chemokine production (40). In accordance with data in the mouse model (30), we observed that the treatment of human Tregs with TLR2 ligands did not downregulate FoxP3 protein expression. In contrast to the murine counterpart (28–30), TLR2 treatment and TCR activation of human Tregs induced a further upregulation of...
intracellular FoxP3, explaining the sustained suppression of cytokine production in human Tregs after activation. Moreover, these data support our assumption that responder T cells cocultured with TLR2 ligand-pretreated Tregs are the key producers of the measured cytokines and chemokines, which are essential for proliferation and antimicrobial effector function of responder T cells.

In a further attempt to identify the effect by which TLR2 ligands abolish suppressive capacity of Tregs, we examined the release of granzyme A and B. The intracellular accumulation of granzymes is discussed to contribute to Treg-mediated suppression by killing responder T cells (5–7). Freshly isolated human Tregs do not express intracellular granzyme B and only little granzyme A (5, 41, 42). However, Gondek and colleagues (6) described an intracellular upregulation of granzyme B protein expression 72 h after anti-CD3 stimulation of murine Tregs in the presence of IL-2 and a reduced ability of Tregs from granzyme B−/− mice to suppress proliferation of responder T cells. In humans, anti-CD3/anti-CD46 treatment has been used to generate adaptive Tregs from naive CD4+CD25− T cells after 4 d of culture, which express granzyme B and low amounts of granzyme A (5, 41). Instead of intracellular expression of granzymes, we examined the release of granzyme A and B in our experiments. We observed that freshly isolated Tregs released only low amounts of granzyme A and B, which are not increased 24–72 h after activation. Furthermore, our observation that responder T cells alone produced much higher amounts of granzyme A and B than Tregs argues against release of granzymes as a mechanism of Treg-mediated suppression.

During the attempts to identify the effect by which TLR2 ligands abrogate Treg suppression, we observed that granzyme B is strongly upregulated in CD4+CD25+ responder T cells after activation. Nonapoptotic and extracellular functions (e.g., cleavage of viral and host proteins and surface receptors or remodeling of extracellular matrix by granzyme B produced by CD4+ T cells) are described already by others (43–46). Interestingly, in our study, granzyme B release of CD4+CD25− responder T cells was suppressed in the presence of untreated Tregs and partially restored with TLR2 ligand-pretreated Tregs. Thus, granzyme B production by CD4+CD25− responder T cells seems to be an useful additional read-out assay to examine Treg suppression.

In further experiments, we examined other putative explanations for the abolishment of suppressive capacity of Tregs by TLR2 ligands including: 1) a reduction of high levels of cytosolic cAMP potentially produced by human Tregs similar to murine Treg (8), which induce inhibition of proliferation and IL-2 production upon gap junction-mediated transfer into responder T cells; 2) a down-regulation of cdk inhibitor p27Kip1 in human Tregs; and/or 3) a restoration of the defect Akt phosphorylation in human Tregs possibly followed by a strong activation signal, which might result in an enhanced proliferative capacity of Tregs. We observed that: 1) pretreatment of Tregs with TLR2 ligands reduced cytosolic cAMP levels in Tregs in only one out of four tested donors, suggesting that this is not the major target of TLR2 ligand effects (not shown); 2) pretreatment with TLR2 ligands and TCR activation drastically reduced the expression of the cdk inhibitor p27Kip1 in human Tregs to a level comparable to that of responder T cells, suggesting that Tregs can potentially enter cell cycle; and 3) an integrated signal of TLR2 ligand pretreatment and TCR stimulation restored phosphorylation of Akt at serine 473 and reduced the suppressive capacity of Tregs. However, Tregs did not proliferate even in coculture with responder T cells, which are able to produce IL-2 in the presence of TLR2 ligand-pretreated Tregs. We suppose that IL-2 is consumed mainly by responder T cells and not by TLR2 ligand-pretreated Tregs in coculture of the two T cell subsets, because the addition of high concentrations of IL-2 resulted in the proliferation of both subsets. These data are in contrast to the murine counterpart in which TLR2 ligand treatment of Tregs is linked to a concomitant proliferative activity of these cells in the absence of IL-2 (28). Others, however, have reported that the addition of exogenous IL-2 is necessary for proliferation of murine Tregs upon TCR/TLR2 ligand stimulation in vitro (29, 30). In contrast to the experiments with murine Tregs by Chen et al. (30), we observed that pretreatment of human Tregs with TLR2 ligands resulted in an abrogation of their suppressive activity on cytokine production by responder T cells. The reason for this discrepancy might be explained by the usage of different TLR2 ligands. Chen et al. (30) used Pam3CSK4 in their experiments, whereas we applied a mixture of TLR2 ligands in most experiments based on the reduced expression of TLR1 on human Tregs and thus the expected non-reactivity of most donors against Pam3CSK4. There are no data available about the expression of TLR1 on murine Tregs analyzed in vitro or in vivo in the different mouse models (28–30). Our results indicate that expression of TLR1 is required for recognition of Pam3CSK4 by human T cells, whereas Pam3CSK4 has been reported to be recognized in a TLR1/2-dependent fashion or independently of TLR1 in mice (32, 33).

In conclusion, our data clearly demonstrate that human TLR2 ligand-pretreated Tregs lose their suppressive capacity and are silenced based on restoration of Akt phosphorylation at serine 473 and a downregulation of cdk inhibitor p27Kip1 in these cells. The silencing of TLR2 ligand-pretreated Tregs restored cytokine/chemokine production and proliferation exclusively in responder T cells after TCR stimulation, which is appropriate if responder T cells must be rapidly activated against undesired microorganisms.

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Disclosures

The authors have no financial conflicts of interest.

References


**SUPPLEMENTAL FIGURE 1:** Purity of magnetically isolated Treg cells. Treg cells were stained with anti-human FoxP3-PE and appropriate isotype control by intracellular staining and anti-CD25 by surface staining as indicated. Results with 5 donors are shown.

**SUPPLEMENTAL FIGURE 2:** Treg inhibit proliferation of responder T cells. 1x10⁴ CD⁴⁺CD25⁻ responder T cells (R) and 2.5x10³ Treg (T) or 2.5x10³ non-Treg (NT) were cocultured or cultured alone, and stimulated with T Cell Activation/Expansion Beads. Proliferation was measured by ³H-TdR incorporation after 5 days. One representative experiment out of four is shown.

**SUPPLEMENTAL FIGURE 3:** TCR stimulation but not TLR pre-treatment increases TLR expression. Untreated or TLR2 ligand pre-treated responder T cells (R, left panel) or Treg (T, right panel) were stained with anti-TLR-1, -2 or -6 mAb or the appropriate isotype controls as indicated before (med) and after TCR stimulation. One representative experiment out of four is shown. The numbers indicate the median fluorescence intensity.

**SUPPLEMENTAL FIGURE 4:** Treg display different reactivity patterns to TLR2 ligand preincubation. Treg were pre-treated for 24 hours without (black bars) or with 1µg/ml Pam₂CSK₄ (light grey bars), FSL-1 (white bars) or Pam₃CSK₄ (dark grey bars). For suppression assay, co-culture of 1x10⁴ CD⁴⁺CD25⁻ responder T cells and 2.5x10³ Treg were stimulated with T Cell Activation/Expansion Beads. ³H-TdR-uptake was analyzed after 5 days and suppression was calculated as [(1-cpm of responder with Treg/cpm of responder without Treg) x100]. Ten different experiments with pre-treated Treg are shown ordered by their reaction to Pam₂CSK₄ (bars on the left hand side, seven donors), FSL-1 (bars in the middle, four donors) or Pam₃CSK₄ (bars on the right hand side, five donors). Significance is indicated as * (p < 0.05).
**SUPPLEMENTAL FIGURE 5:** Responder T cells produce more granzyme B than granzyme A. 1x10^5 CD4^+CD25^- responder T cells were cultured in medium or stimulated with T Cell Activation/Expansion Beads. Granzyme A and B production were determined by ELISA, and production after stimulation was calculated as fold increase of the medium control. Four different experiments for granzyme A and six different experiments for granzyme B after 72 hours of culture are shown.