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Direct Stimulation of Human T Cells via TLR5 and TLR7/8: Flagellin and R-848 Up-Regulate Proliferation and IFN-γ Production by Memory CD4$^+$ T Cells[1]

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TLRs are involved in innate cell activation by conserved structures expressed by microorganisms. Human T cells express the mRNA encoding most of TLRs. Therefore, we tested whether some TLR ligands may modulate the function of highly purified human CD4$^+$ T lymphocytes. We report that, in the absence of APCs, flagellin (a TLR5 ligand) and R-848 (a TLR7/8 ligand) synergized with suboptimal concentrations of TCR-dependent (anti-CD3 mAb) or -independent stimuli (anti-CD2 mAbs or IL-2) to up-regulate proliferation and IFN-γ, IL-8, and IL-10 but not IL-4 production by human CD4$^+$ T cells. No effect of poly(I:C) and LPS, ligands for TLR3 and TLR4, respectively, was detected. We also observed that CD4$^+$CD45RO$^+$ memory T cell responses to TLR ligands were more potent than those observed with CD4$^+$CD45RA$^+$ naive T cells. Moreover, among the memory T cells, CCR7$^-$ effector cells were more sensitive to TLR ligands than CCR7$^+$ central memory cells. These data demonstrate for the first time a direct effect of TLR5 and TLR7/8 ligands on human T cells, and highlight an innate arm in T cell functions. They also suggest that some components from invading microorganisms may directly stimulate effector memory T cells located in tissues by up-regulating cytokine and chemokine production. *The Journal of Immunology,* **2005, 175: 1551–1557.**

The innate immune system provides the first line of defense against infection (1–4). Through a limited number of germline-encoded receptors called pattern recognition receptors, innate cells recognize highly conserved structures expressed by large group of microorganisms called pathogen-associated molecular patterns (PAMPs)[3] (5). Among pattern recognition receptors, TLRs are crucial for recognition of pathogen-derived products, and initiate signaling cascades leading to the activation of innate host defenses (6, 7).

TLRs expressed by vertebrates are type 1 transmembrane proteins bearing an intracellular Toll/IL-1R homology domain (8). To date, 10 members of the TLR family that differ in ligand specificities and expression patterns have been described in humans. Among them, TLR2 is crucial for the recognition of a variety of bacterial PAMPs, including lipoproteins and peptidoglycans (9, 10), TLR3 is associated to the recognition of virus dsRNA (11), TLR4 is activated by LPS (12), TLR5 detects bacterial flagellin (13), TLR7 and TLR8 recognize imidazoquinolines (14, 15) and single-stranded viral RNA (16, 17) while TLR9 is required for response to unmethylated CpG DNA (18). The specificities can be extended by the ability of TLRs to heterodimerize and to interact with different adapter and accessory molecules (19). Binding of PAMPs to most of the TLRs initiates a signaling cascade that leads to the nuclear translocation of NF-κB members (20), resulting in the up-regulation of proinflammatory cytokines, reactive oxygen intermediates, and costimulatory molecule expression. TLR expression has been reported in several cell types including myeloid cells, neutrophils, and epithelial cells (6, 7). Although some innate cells (i.e., macrophages and neutrophils) activated via TLRs differentiate into effector cells that kill infectious agents, others, such as dendritic cells, activate T cells and thereby initiate adaptive immune response (21–25).

Previous studies reported that human T lymphocytes express TLR mRNA (26, 27), suggesting that TLR agonists may directly influence T cell effector function. Accordingly, TLR2 has been reported to be functional in T cells (28). In this study, we evaluated the functions of TLR5 and TLR7/8 expressed by highly purified human CD4$^+$ T cells using their ligands flagellin and R-848, respectively. Flagellin, the major component of bacteria flagella, induces cytokine production by epithelial cells, monocytes, and NK cells (13, 29, 30). R-848 is a low m.w. immune response modifier of the imidazoquinoline family, with potent anti-viral and anti-tumor properties (31) elicited through TLR7 and TLR8 (14, 15). In this study, we report that flagellin and R-848 act directly, and in synergy with TCR-dependent and -independent stimuli, in inducing proliferation and cytokine production by human CD4$^+$ T cells. Furthermore, we demonstrate that effector memory T cells, a subpopulation of memory T cells that confer immediate protection in peripheral tissues (32, 33), show enhanced responsiveness to APC-independent TLR activation. These data suggest a new role for TLRs in the activation of adaptive immune cells, and the participation of effector memory T cells in innate immunity.

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[3] Abbreviation used in this paper: PAMP, pathogen-associated molecular pattern; BDCA, blood dendritic cell antigen.

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Materials and Methods

Human T cell purification and cultures

PBMC were isolated from healthy volunteers by standard density gradient centrifugation on Ficoll-Paque (Amersham Pharmacia Biotech). $CD4^+$ T cells were purified by negative selection using MACS technology according to the manufacturer's instructions (Miltenyi Biotec), followed by FACS using PE-Cy7-labeled anti-CD3 mAb and allophycocyanin-labeled anti-CD4 mAb (both from BD Biosciences). FACS sorting was performed using a FACSAria cytofluorometer (BD Biosciences). The absence of contaminating monocytes, B cells, NK cells, and dendritic cells was controlled using anti-CD14, anti-CD19, anti-CD56 (BD Biosciences), anti-blood dendritic cell Ag (BDCA)-1, and anti-BDCA-2 (both from Miltenyi Biotec) mAbs, respectively. In other experiments, peripheral blood $CD4^+$ T cells were separated into $CD45RA^+$ and $CD45RO^+$ cells by FACS sorting using allophycocyanin-labeled anti-CD4 mAb, PE-labeled anti-CD45RA mAb, and FITC-labeled anti-CD45RO mAb (all from BD Biosciences). $CD4^+CD45RO^+$ T cells were separated into $CCR7^+$ and $CCR7^-$ cells by FACS sorting using FITC-labeled anti-CD45RO mAb and PE-labeled anti-CCR7 mAb (R&D Systems). Purity of the cells was >99.9%.

Cells were cultured in RPMI 1640 medium (Biowhittaker Cambrex) supplemented with 10% FCS (Biowest), 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 10 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from BioWhittaker Cambrex) at 37°C in 5% CO_2. Cells (1×10^6 cells/ml) were cultured for 48 h in 48-well plates in the presence or the absence of 20 or 100 U/ml IL-2 (R&D Systems), 2 μg/ml anti-CD2 paired mAbs (clones T11/1 and T11.2/1; Pelicluster), 2 μg/ml anti-CD3 mAb (clone OKT3, American Type Culture Collection; without or with 5 μg/ml anti-CD28 mAb, clone CD28.2, BD Biosciences), 0.8–20 μg/ml poly(I:C) (Sigma-Aldrich), 0.4–10 μg/ml flagellin (from *Salmonella typhimurium*) or LPS (from 0111:B4 *Escherichia coli* strain), 0.1–5 μg/ml R-848 or Pam_3CSK_4 (all from InvivoGen), 0.5 μg/ml PHA (Murex Biotech) or a combination of these reagents as indicated.

RT-PCR analysis

TLR1 to TLR10 expression was analyzed by RT-PCR on FACS-sorted T cells. Briefly, total cytoplasmic RNA was extracted using TRIzol (Invitrogen Life Technologies), treated with RNase-free DNase (Promega), and reverse transcribed using the Superscript II RNase H-Reverse Transcriptase (Invitrogen Life Technologies) following the manufacturer's recommendations. PCR amplification was performed with an amount of cDNA corresponding to 50 ng of starting total RNA (5 min at 94°C followed by 30 cycles (30 s at 94°C, 30 s at 55°C, and 1 min at 72°C) followed by a final extension of 7 min at 72°C). The primer sequences used for PCR are: hTLR1, sense 5′-CGT AAA ACT GGA AGC TTT GCA AGA-3′, antisense 5′-CCT TGG GCC ATT CCA AAT AAG TCC-3′; hTLR2, sense 5′-GGC CAG CAA ATT ACC TGT GTG-3′, antisense 5′-CCA GGT AGC TCT TGG TGT TCA-3′; hTLR3, sense 5′-ATT GGG TCT GGG AAC ATT TCT CTT C-3′, antisense 5′-GTG AGA TTT AAA CAT TCT TCG C-3′; hTLR4, sense 5′-CTG CAA TGG ATC AAG GAC CA-3′, antisense 5′-TCC CAC TCC AGG TAA GTG TT-3′; hTLR5, sense 5′-CAT TGT ATG CAC TGT CAC TC-3′, antisense 5′-CCA CCA CCA TGA TGA GAG CA-3′; hTLR6, sense 5′-TAG GTC TCA TGA CGA AGG AT-3′, antisense 5′-GGC CAC TGC AAA TAA CTC CG-3′; hTLR7, sense 5′-AGT GTC TAA AGA ACC TGG-3′, antisense 5′-CTT GGC CTT ACA GAA ATG-3′; hTLR8, sense 5′-CAG ATT AGC AGG CGT AAC ACA TCA-3′, antisense 5′-AAT GTC ACA GGT GCA TTC AAA GGG-3′; hTLR9, sense 5′-TTA TGG ACT TCC TGC TGG TGG AGG TGC-3′, antisense 5′-CTG CGT TTT GTC GAA GAC CA-3′; hTLR10, sense 5′-GCT AGT TCT GGG GTT GGC TGT GGC-3′, antisense 5′-CAT TAA TAG CAG CTC GAA GGT TTG CC-3′; GAPDH, sense 5′-GGA GGT GGA GGT CGG AGT C-3′, antisense 5′-GAA GAT GGT GAT GGG ATT TTC-3′. RNA integrity and cDNA synthesis was verified by amplifying GAPDH cDNA. Amplified fragments were size-separated by electrophoresis and visualized by ethidium bromide.

Cytokine quantification

Cells were incubated for 48 h with various stimuli, and the concentrations of IFN-γ, IL-2, IL-4, IL-8, and IL-10 were measured in cell-free culture supernatants by ELISA, according to the manufacturer's recommendations. Specific capture and detection Abs for IFN-γ were from Mabtech (sensitivity of 5 pg/ml) and those for IL-2, IL-8, and IL-10 were from R&D Systems (sensitivity of 50, 15, and 10 pg/ml, respectively). IL-4 was quantified using a commercial kit (R&D Systems; sensitivity of 0.13 pg/ml).

Proliferation assays

Cells (4.10^5/ml) were seeded in triplicate in 96-well flat-bottom plates and stimulated as described above for 72 h. Cells were pulsed during the last 16 h with 0.25 μCi/well [^{3}H]thymidine (Amersham Pharmacia Biotech). Radioactive incorporation was measured by standard liquid scintillation counting, and results expressed in proliferation index (mean of triplicate values) were defined as follows: *A/B*, were *A* and *B* are the cpm values obtained in the presence or absence of TLR ligand, respectively.

Results

Human $CD4^+$ T cells express mRNA encoding TLRs

We evaluated whether human $CD4^+$ T cells express mRNA encoding TLRs. Results showed that highly purified $CD4^+$ T cells expressed TLR1–5 (26, 27). TLR7 and TLR9 mRNA were also expressed, although at a lower level. TLR6, 8, and 10 mRNA expression was weak or undetectable (Fig. 1*A*, *upper panel*).

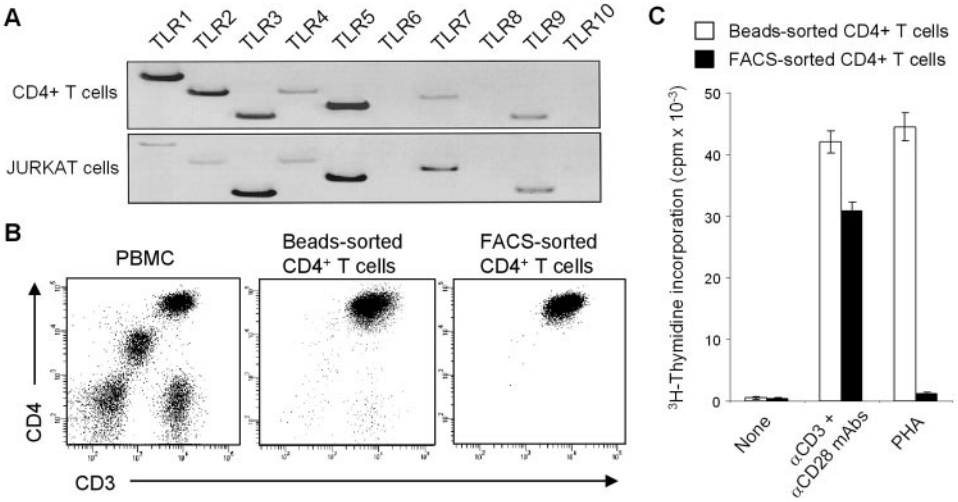

FIGURE 1. RT-PCR analysis of TLR mRNA expression by human $CD4^+$ T cells. *A*, TLR1 to TLR10 mRNA expression was analyzed in highly purified human $CD4^+$ T cells and in Jurkat cells by RT-PCR. *B*, $CD4^+$ T cells were isolated by negative selection using magnetic beads followed by positive selection of $CD3^+CD4^+$ T cells by FACS. At each step, cells were labeled with anti-CD3 and -CD4 mAbs to evaluate the purity of the cell preparation. *C*, FACS-sorted $CD4^+$ T cells failed to proliferate in response to PHA. Beads- (□) and FACS-sorted (■) cells were cultured at 10^6 cells/ml for 3 days in the presence of 0.5 μg/ml PHA or 10 μg/ml anti-CD3 plus 5 μg/ml anti-CD28 mAbs. Proliferation was measured by [^{3}H]thymidine incorporation, and results are expressed in cpm. *A–C*, Results are representative of data obtained with T cells from one of five healthy donors.

Table I. *Phenotype analysis of CD4$^+$ T cell populations*[a]

	PBMC	CD4$^+$ T Cells after Negative Selection	FACS-Sorted CD4$^+$ T Cells
CD3$^+$CD4$^+$	42.5	97.85	99.92
CD8$^+$	14.6	0.54	0.03
CD56$^+$	9.3	0.29	<0.01
CD20$^+$	9.9	0.08	0.02
CD14$^+$	15	0.78	<0.01
BDCA1$^+$	0.5	0.07	<0.01
BDCA2$^+$	0.5	0.14	<0.01

[a] CD4$^+$ T cells were purified by negative selection using immunomagnetic beads followed by positive selection of CD4$^+$ cells by FACS. Purity of the cells was analyzed by FACS at each step, using fluorochrome-labeled mAbs directed against membrane markers of putative contaminating cells, as described in *Materials and Methods*. Results are expressed as a percentage of cell populations and are representative of one of five experiments.

Moreover, the levels of TLR mRNA varied from donor to donor (data not shown).

Analysis of the biological role of TLRs expressed by CD4$^+$ T cells requires excluding contamination with cells expressing high levels of TLRs, such as monocytes. FACS analysis showed that the purity of FACS-sorted CD4$^+$ T cells was >99.9% (Fig. 1*B* and Table I). Importantly, CD14$^+$ monocytes, BDCA1$^+$ myeloid dendritic cells, and BDCA2$^+$ plasmacytoid dendritic cells were undetectable (<0.01%, Table I). The absence of residual accessory function in the CD4$^+$ T cell population was confirmed by the inability of FACS-sorted CD4$^+$ T cells to proliferate in response to PHA, an accessory cell-dependent T cell mitogen (34) (Fig. 1*C*). Purified T cells obtained by negative selection using magnetic beads proliferated in response to PHA (Fig. 1*C*), as residual monocytes were still present in the enriched T cell preparation (Table I). In contrast to PHA, anti-CD3 plus anti-CD28 mAbs induced the proliferation of beads- and FACS-sorted CD4$^+$ T cells (Fig. 1*C*).

Finally, RT-PCR experiments performed on the Jurkat T cell line showed that Jurkat cells express the mRNA encoding most of the TLRs (Fig. 1*A*, *lower panel*).

Together, these data showed that freshly isolated human CD4$^+$ T cells express mRNA encoding TLRs.

Ligands for TLR2, 5, 7, and 8 up-regulate IFN-γ production by human CD4$^+$ T cells

Therefore, we analyzed the effect of Pam_3CSK_4, poly(I:C), LPS, flagellin, and R-848, ligands for TLR2, TLR3, TLR4, TLR5, and TLR7/8, respectively, on IFN-γ production by T cells.

Pam_3CSK_4, flagellin, and R-848 induced a limited production of IFN-γ (<2 ng/ml) but synergized with suboptimal concentrations of anti-CD3 mAb, anti-CD2 mAbs, or IL-2 in inducing IFN-γ production by T cells from six healthy subjects (Fig. 2*A*). According to the subject tested, variations in the levels of IFN-γ produced in response to one of the TLR ligands and in the intensity of T cell response to each of the ligands were observed (Fig. 2*A*). As an example, in the presence of the anti-CD2 mAbs, increase in IFN-γ production varied from 2.6- to 24.5-fold in response to Pam_3CSK_4, from 1.9- to 18.5-fold in response to flagellin, and from 3.1- to 36.7-fold in response to R-848 for the six subjects tested. Accordingly, Fig. 2 shows representative data obtained with two of six subjects. The effects of Pam_3CSK_4, flagellin, and R-848 on IFN-γ production were dose dependent and maximal at concentrations of 1, 5, and 1 μg/ml, respectively (Fig. 2*B*). These concentrations were used throughout the study. TLR ligands-enhanced IFN-γ production by activated T cells was time dependent (Fig. 2*C*). In the presence of immobilized anti-CD3 mAb, the effect of Pam_3CSK_4, flagellin, or R-848 was significant after 16 h of stimulation and

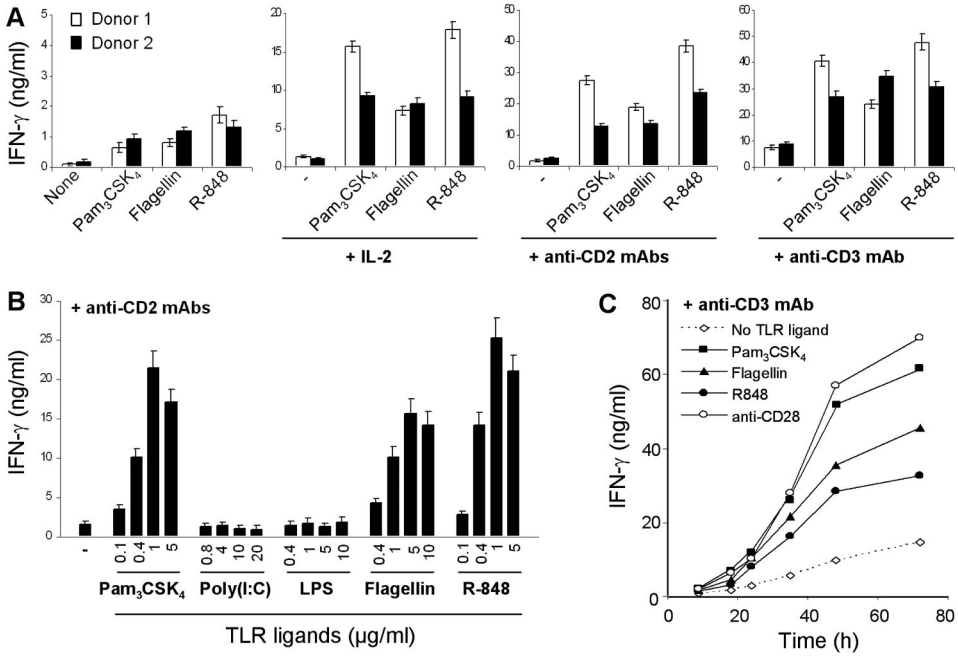

FIGURE 2. Pam_3CSK_4, flagellin, and R-848 up-regulate IFN-γ production by human CD4$^+$ T cells. *A*, Highly purified CD4$^+$ T cells were either unstimulated or stimulated with 100 U/ml IL-2, 2 μg/ml anti-CD2 mAbs or anti-CD3 mAb, in the absence or presence of 1 μg/ml Pam_3CSK_4 (TLR2 ligand), 5 μg/ml flagellin (TLR5), or 1 μg/ml R-848 (TLR7/8). IFN-γ was quantified by ELISA in the 48-h cell-free supernatants. *B*, CD4$^+$ T cells were stimulated with anti-CD2 mAbs in the absence or presence of increasing doses of Pam_3CSK_4, poly(I:C) (TLR3), LPS (TLR4), flagellin, or R-848. IFN-γ was quantified after 48 h. *C*, CD4$^+$ T cells were stimulated with anti-CD3 mAb in the absence or presence of TLR ligands or anti-CD28 mAb. IFN-γ was quantified by ELISA at different time-points after stimulation. *A–C*, Results are expressed in nanograms per milliliter as mean ± SD of three experiments and are representative of the data obtained with T cells purified from the peripheral blood of two (□ and ■) of six healthy donors (*A*) or from one of three donors (*B* and *C*).

maximal at day 3. Although the levels of IFN-γ induced by an anti-CD28 mAb (used as a control of the maximum IFN-γ secretion capacity of T cells) were higher, kinetics of IFN-γ production in the presence of TLR ligands were similar to those obtained in the presence of the anti-CD28 mAb (Fig. 2*C*).

Recently, viral single-stranded RNA has been identified as the natural ligand for TLR-7 and TLR-8 (16, 17). Although in a lesser extent than R-848, we observed that PolyU ssRNA also up-regulated IFN-γ production by $CD4^+$ T cells (data not shown). Finally, at any concentrations tested, and even in the presence of the additional stimuli, anti-CD3 mAb, anti-CD2 mAbs, or IL-2, poly(I:C), and LPS did not affect IFN-γ production (Fig. 2*B*). Together, these data reinforce results from others showing that TLR2 is functional in human T cells (28) and demonstrate, for the first time, a functional role for TLR5 and TLR7/8 ligands in stimulating human T cells.

TLR ligands up-regulate T cell proliferation and IL-2 and IL-10 production, but differentially affect IL-8 production

We next analyzed more precisely the nature of the T cell response to TLR 2, 5, and 7/8 ligands. Used alone, Pam_3CSK_4, flagellin, and R-848 had a limited effect on T cell proliferation (Fig. 3) and failed to modulate IL-2 and IL-10 production (data not shown). However, they synergized with anti-CD3 mAb, anti-CD2 mAbs, or IL-2 in up-regulating T cell proliferation (Fig. 3) and IL-2 and IL-10 production (Fig. 4*A*). In parallel, IL-4 production was observed in response to anti-CD3 plus anti-CD28 mAbs (used as a positive control), but remained undetectable after treatment with TLR ligands alone or in combination with anti-CD3 mAb, anti-CD2 mAbs, or IL-2 (data not shown).

Pam_3CSK_4, flagellin, and R-848 induced IL-8 production by T cells and synergized with IL-2 in up-regulating IL-8 production (Fig. 4*B*). Surprisingly, in the presence of anti-CD3 or anti-CD2 mAbs, flagellin, and Pam_3CSK_4 up-regulated IL-8 production while, in contrast, R-848 decreased it (Fig. 4*B*). This decrease was dose dependent (Fig. 4*C*) and observed at the transcriptional level (data not shown). Finally, no effect of poly(I:C) and LPS was observed either on T cell proliferation or on IL-8 and IL-10 production (data not shown).

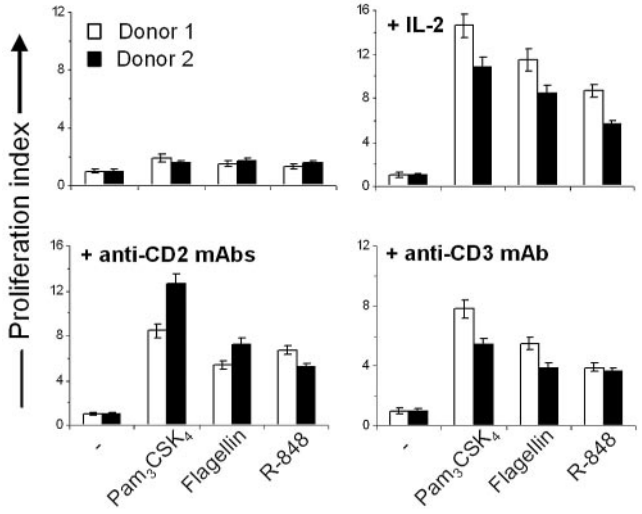

FIGURE 3. Pam_3CSK_4, flagellin, and R-848 up-regulate human $CD4^+$ T cell proliferation. Highly purified $CD4^+$ T cells were either unstimulated or stimulated with 20 U/ml IL-2, 2 μg/ml anti-CD2 mAbs or anti-CD3 mAb, in the absence or presence of 1 μg/ml Pam_3CSK_4, 5 μg/ml flagellin, or 1 μg/ml R-848. Proliferation was measured after 72 h. Results are expressed in proliferation index as mean ± SD, $n = 3$, and are representative of data obtained with T cells from two (□ and ■) of six healthy donors.

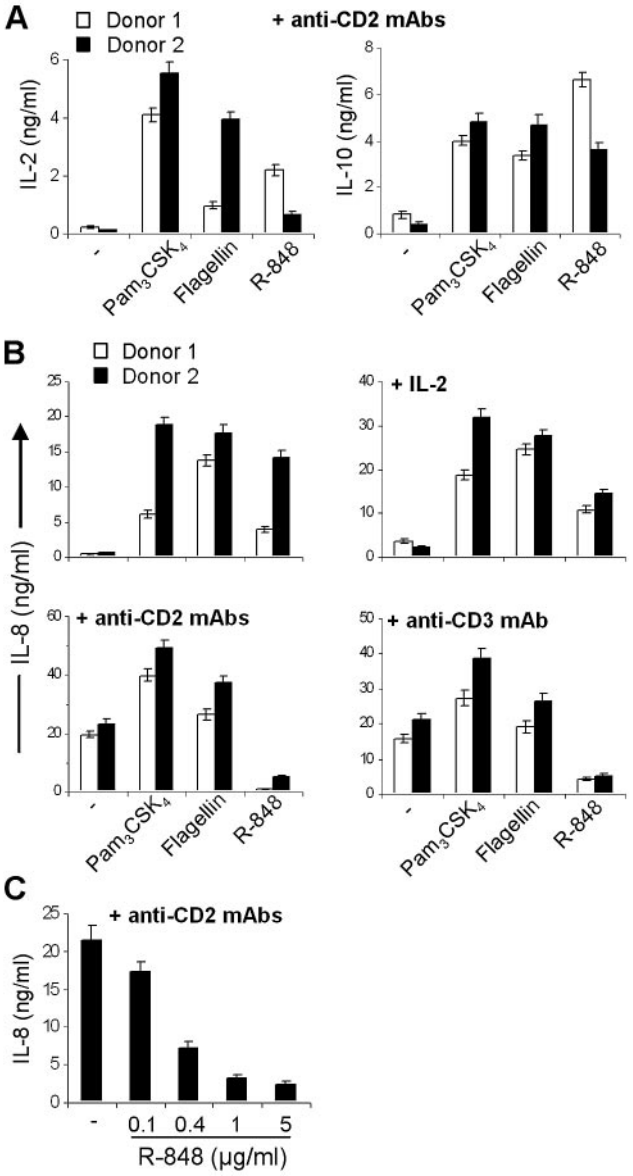

FIGURE 4. Pam_3CSK_4, flagellin, and R-848 affect cytokine production by $CD4^+$ T cells. *A* and *B*, Highly purified $CD4^+$ T cells were either unstimulated or stimulated with 100 U/ml IL-2, 2 μg/ml anti-CD2 mAbs or anti-CD3 mAb, as indicated in the figures, in the absence or presence of 1 μg/ml Pam_3CSK_4, 5 μg/ml flagellin, and 1 μg/ml R-848. IL-2 (*A*, *left panel*), IL-10 (*A*, *right panel*), and IL-8 (*B*) were quantified in the 48-h cell-free supernatants. Results are expressed as mean ± SD, $n = 3$, and are representative of the data obtained with T cells from two (□ and ■) of six healthy donors. *C*, $CD4^+$ T cells were stimulated with anti-CD2 mAbs in the absence or presence of increasing doses of R-848, and IL-8 was quantified after 48 h. Results are expressed in nanograms per milliliter as mean ± SD, $n = 3$, and are representative of the data obtained with T cells from one of three healthy donors.

Effector memory $CCR7^-CD45RO^+$ T cells are highly sensitive to TLR ligands

We then compared naive vs memory T cell sensitivity to Pam_3CSK_4, flagellin, and R-848. Highly purified $CD4^+$ T cells were separated into $CD45RA^+$ (naive) and $CD45RO^+$ (memory) T cells and incubated with TLR ligands in the absence or presence

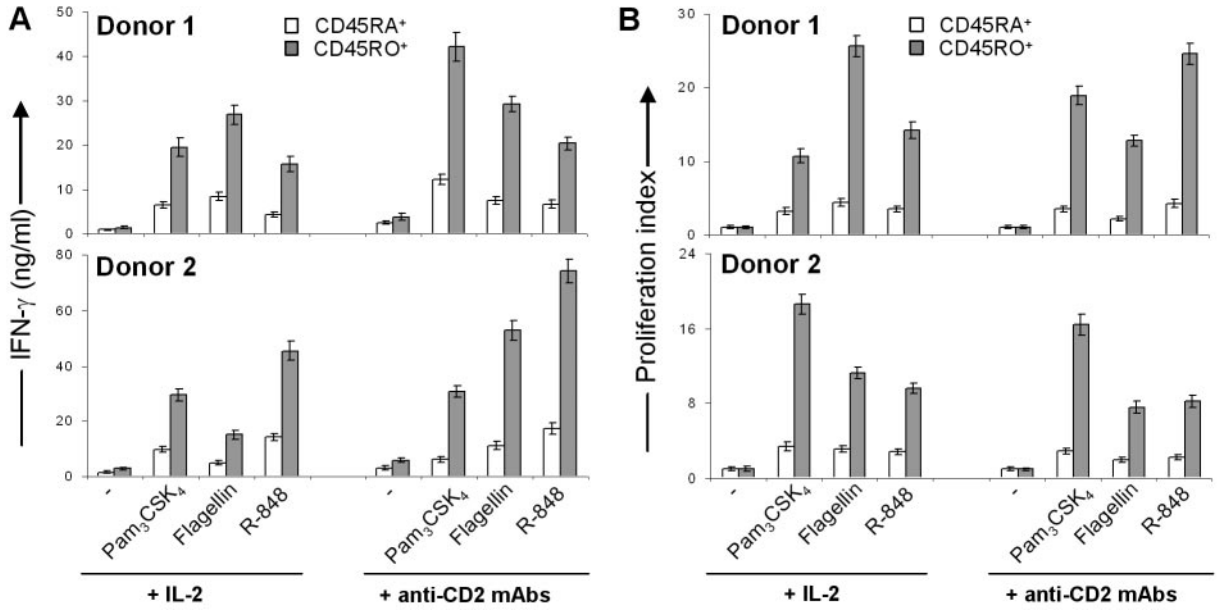

FIGURE 5. Memory T cells are more sensitive to Pam_3CSK_4, flagellin, and R-848 than naive T cells. Naive ($CD45RA^+$) (□) and memory ($CD45RO^+$) T cells (▨) were purified from $CD4^+$ T cells by FACS sorting and either unstimulated or stimulated with IL-2 (100 U/ml for cytokine production or 20 U/ml for proliferation assay) or anti-CD2 mAbs (2 μg/ml) in the absence or presence of 1 μg/ml Pam_3CSK_4, 5 μg/ml flagellin, or 1 μg/ml R-848. IFN-γ was quantified in the 48-h cell-free supernatants (*A*), and proliferation was determined after 72 h (*B*). Results are expressed as mean ± SD, $n = 3$, and are representative of the data obtained with the T cells of two of six healthy donors.

of anti-CD2 mAbs, IL-2 (Fig. 5), or anti-CD3 mAb (data not shown). Whatever the costimulus used, TLR ligands up-regulated IFN-γ production (Fig. 5*A*) and T cell proliferation (Fig. 5*B*) more efficiently in memory than in naive T cells. Representative results obtained with two of six subjects are presented. These data showed that memory $CD4^+$ T cells are more sensitive to TLR ligands than naive T cells.

Two populations of $CD4^+CD45RO^+$ memory T cells, which differ in tissue localization and functions, can be distinguished according to CCR7 expression: the $CCR7^+$ central and $CCR7^-$ effector T cells (32). Therefore, we compared the sensitivity of these two populations to TLR2, TLR5, and TLR7/8 ligands. These populations were purified from $CD4^+CD45RO^+$ T cells by FACS sorting and were stimulated with anti-CD2 or anti-CD3 mAbs in the absence or presence of Pam_3CSK_4, flagellin or R-848 (Fig. 6). Results showed that, whatever the stimuli used, effector memory T cells produced higher levels of IFN-γ in response to TLRs than central memory T cells (Fig. 6). As an example, in response to anti-CD2 mAbs, increase in IFN-γ production in $CCR7^-$ compared with $CCR7^+$ cells varied from 1.9- to 3.8-fold in response to Pam_3CSK_4, from 1.7- to 2.9-fold in response to flagellin, and from 1.9- to 3.5-fold in response to R-848, for the five subjects tested. As a positive control, effector memory $CD4^+$ T cells stimulated by anti-CD3 plus anti-CD28 mAbs produced higher levels of IFN-γ than central memory T cells (32).

Together, these data show that effector memory T cells exhibit an enhanced response to TLR activation.

Discussion

In this study, we demonstrate that flagellin and R-848, ligands for TLR5 and TLR7/8, respectively, stimulate human peripheral blood $CD4^+$ T cells with a prominent effect on $CD45RO^+CCR7^-$ effector memory T lymphocytes.

Previous studies reported that human peripheral blood T cells express TLR mRNA (26, 27). This study aimed to evaluate whether TLRs may have a functional role in T lymphocytes. As most of the hemopoietic cells express TLRs and respond to TLR ligands (7), the use of highly purified T cells was a prerequisite. $CD4^+$ T cells were purified from peripheral blood by a two-step purification protocol combining immunomagnetic and FACS sorting. Following this protocol, no contaminating B cells, NK cells (that produce IFN-γ and proliferate in response to TLR2 and TLR5 ligands; Ref. 30), and APCs (such as monocytes and myeloid or plasmacytoid dendritic cells) that are highly sensitive to TLR-mediated activation, were phenotypically and functionally detected in our purified T cells. This data was reinforced by the observation that poly(I:C), a TLR3 ligand that induces IL-8 production by monocytes (G. Caron and P. Jeannin, unpublished data), did not induce IL-8 production by purified $CD4^+$ T cells.

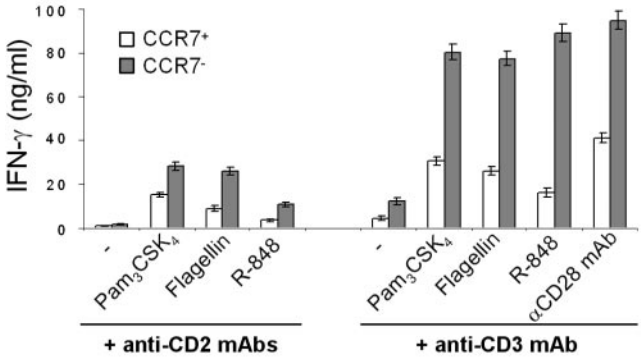

FIGURE 6. Effector memory T cells exhibit an enhanced response to TLR activation. Effector ($CCR7^-$) (▨) and central ($CCR7^+$) memory T cells (□) were purified from $CD4^+CD45RO^+$ T cells by FACS sorting and either unstimulated or stimulated with anti-CD2 or anti-CD3 mAbs in the absence or presence of TLR ligands. IFN-γ was quantified in the 48-h cell-free supernatants. As a positive control, cells were stimulated with anti-CD3 plus anti-CD28 mAbs. Results are expressed in nanograms per milliliter as mean ± SD, $n = 3$, and are representative of the data obtained with the T cells of one of five healthy donors.

In agreement with previous studies (26, 27), we observed that human $CD4^+$ T cells express mRNA encoding most of the members of the TLR family, except TLR10 (26). Ligands for TLR2 (Pam_3CSK_4), TLR5 (flagellin), and TLR7/8 (R-848) up-regulate proliferation and IL-2, IL-8, IL-10, and IFN-γ production by human $CD4^+$ T cells, in the absence of APCs. These data are, to our knowledge, the first demonstration that human T cells are sensitive to TLR5 and TLR7/8 ligands. We also reinforce recent data showing that bacterial lipopeptide (Pam_3CSK_4) costimulates human $CD4^+$ T cell proliferation and IFN-γ production (28).

Flagellin and R-848 induce the production of cytokines by $CD4^+$ T cells. Flagellin is a bacterial component that is highly immunogenic in the absence of adjuvant (35), a property related to its ability to stimulate dendritic cells (36). In the absence of adjuvant, flagellin favors in vivo the development of a Th2-polarized response (37). Flagellin did not induce IL-4 or CCL17 (thymus and activation-regulated chemokine) and CCL22 (macrophage-derived chemokine) production (data not shown), two chemokines produced by activated T cells that favor the recruitment of Th2 cells (38, 39). These results suggest that the ability of flagellin to favor the induction of Th2 responses in vivo may involve cell types other than T cells, such as dendritic cells. In agreement with this hypothesis, and as previously reported by others, we observed that, in contrast to IFN-γ used as a positive control (40), flagellin did not affect LPS-induced IL-12 p70 production by human monocyte-derived dendritic cells (P. Jeannin, unpublished data). Recent studies reported that viral ssRNA activate innate cells via TLR7 (16, 17). Resiquimod (R-848) belongs to the imidazoquinoline family and exhibits potent antitumor and antiviral activities (31). Imidazoquinolines induce the production of IL-12, IFN-α, and IFN-γ by murine and human mononuclear cells and, in contrast to flagellin, favor the development of Th1-polarized responses (41). R-848 is a synthetic agonist for TLR7 and TLR8 and is easily usable in in vitro assays. In the presence of APCs, R-848 shifts human allergen-specific $CD4^+$ Th2 lymphocytes into IFN-γ-producing cells (42). Our study shows a direct effect of R-848 on IFN-γ production by human T cells with no induction of IL-4 or Th2-attracting chemokines (data not shown). These data show that ssRNA, released during virus-infected cell lysis, can induce the production of IFN-γ that, in turn, exhibits direct antiviral properties (43).

An activation of T lymphocytes by PAMPs should occur upon epithelial cell barrier disruption. As a consequence, PAMP-activated T cells may produce cytokines involved in the recruitment and activation of inflammatory cells to initiate appropriate immune responses. IL-8 is involved in the recruitment of inflammatory cells and IFN-γ plays a critical role in the activation of epithelial cells. At the opposite, the immunomodulatory cytokine IL-10 controls the outcome of the immune response and maintains tissue homeostasis. Surprisingly, we observed that R-848, in contrast to flagellin and Pam_3CSK_4, decreased in a dose-dependent manner anti-CD2 mAbs-induced IL-8 production. This effect appears restricted to IL-8 production as R-848 potentiates the anti-CD2 mAbs-induced IL-2, IL-10, and IFN-γ synthesis. This result suggests that signaling pathways associated to TLR2, TLR5, and TLR7/8 may differ (44), and additional experiments are required to explain this observation.

Flagellin and Pam_3CSK_4 induced proliferation of human $CD4^+$ T lymphocytes. The in vivo relevance of this result remains unclear. Recently, Tulic et al. (45) reported that ex vivo LPS application in nasal mucosa induced the proliferation of T lymphocytes. Whether the induction of proliferation was direct or not remains unclear. Nevertheless, this study shows that proliferation of T lymphocytes localized within tissues can be initiated by TLR agonists.

TLR ligands have a limited T cell stimulatory activity when used alone (except on IL-8 production) but synergized with TCR-dependent and -independent stimuli. This result is in accordance with data showing that myeloid cells are more sensitive to PAMP-mediated activation than T cells and suggests 1) that T cells require a strong PAMP-mediated activation signal to be efficiently stimulated, and 2) that an activation of T cells by PAMPs may occur during a massive entry of microorganisms.

Although human T cells express TLR3 and TLR4 mRNA, we failed in detecting an effect of their ligands (poly (I:C) and LPS, respectively) on proliferation and cytokine production by T cells. Recent studies reported that murine regulatory $CD4^+CD25^+$ T cells respond to LPS (46), and that dsRNA directly enhances activated $CD4^+$ T cell survival (47). However, in accordance with our data, Komai-Koma et al. (28) also failed to detect an effect of LPS or poly(I:C) on human $CD4^+$ T cells. The absence of effect of these ligands may be related to 1) the absence of accessory molecules on T cells, such as CD14 that belongs to the LPS-receptor complex, 2) the intracellular localization of the TLR proteins that can be modified upon activation, as previously reported for TLR5 (48) or, 3) the absence of TLR proteins. Additional experiments are required to investigate these hypotheses.

We observed that memory $CD4^+$ T cells are more sensitive to TLR-mediated activation than naive $CD4^+$ T cells. This result could be attributed to the fact that memory T cells, which have already encountered the Ag, require less stringent stimulatory signals than naive T cells to be activated (49). The observation that effector memory $CD4^+$ T cells are also more sensitive than central memory $CD4^+$ T cells reinforces this hypothesis (32, 33). Immune memory is displayed by central and effector memory T cells that exhibit distinct phenotypic and biologic characteristics (33). Central memory T cells, which mediate reactive memory, express CCR7, home to the T cell areas of secondary lymphoid organs and have little effector function. They readily proliferate and differentiate into effector cells in response to an antigenic stimulation. Effector memory T cells support protective memory. They lack CCR7 expression and then migrate to inflamed peripheral tissues where they display immediate effector function. Our results also underline that T cells that have already encountered Ags are more sensitive to TCR-independent TLR-mediated stimulation. Based on these observations, it is tempting to speculate that the pool of Ag-primed T cells, which enter peripheral tissues to mediate inflammatory reaction, could be efficiently stimulated by PAMPs and contribute to contain invasive pathogens through the production of cytokines such as IL-8 and IFN-γ that favor phagocyte recruitment and activation, respectively. Finally, the observation that both memory T cell subsets respond to TLR ligands suggest that stimulation with PAMPs may contribute to the maintenance of a local polyclonal repertoire of memory T cells.

Although it has been recently suggested that TLR2 could be unique in its ability to costimulate T cell functions, in this study, we demonstrate that TLR5 and TLR7/8 ligands act synergistically with TCR-dependent and TCR-independent stimuli to up-regulate cytokine production and proliferation of human T cells with an optimal effect on memory T cells. This mechanism may participate in local memory T cell homeostasis and contribute, together with costimulatory signals such as IL-2 or CD58, to stimulation of effector T cells recruited at the site of microorganism entry.

Disclosures

The authors have no financial conflict of interest.

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