TLR2 Signaling Renders Quiescent Naive and Memory CD4⁺ T Cells More Susceptible to Productive Infection with X4 and R5 HIV-Type 1¹

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It has been recently demonstrated that circulating microbial products are responsible for a systemic immune activation in individuals infected with HIV-type 1. Bacterial products carry structural conserved motifs recognized by TLRs. Some TLR members are expressed in primary human $CD4^+$ T cells but the precise functional role played by these pattern recognition receptors is still imprecise. In this study, we report that engagement of TLR2 in quiescent naive and memory $CD4^+$ T cells leads to the acquisition of an effector-like phenotype. Interestingly, engagement of TLR2 renders both cell subsets more susceptible to productive infection with X4 virions and a higher virus production was seen with R5 viruses. It can be proposed that exposure of resting $CD4^+$ T cells to pathogen-derived products that can engage TLR2 induces the acquisition of an effector-like phenotype in naive and memory $CD4^+$ T lymphocytes, a phenomenon that might result in an acceleration of virus replication, immune dysregulation, and HIV-type 1-mediated disease progression. *The Journal of Immunology*, 2007, 179: 4357–4366.

 ${\bf R}$ eplication of HIV-type 1 (HIV-1)³ relies heavily on the activation state of target cells. Hence, productive HIV-1 infection will ensue in cell subtypes containing the appropriate cellular factors as is the case in effector CD4⁺ T lymphocytes. It is now well-established that these cells are massively depleted at the mucosal interface (i.e., gut and genital tracts) during acute infection (1, 2). Their high permissiveness for HIV-1 infection is linked to expression of both CXCR4 and CCR5, which act as coreceptors for HIV-1, as well as to the presence of cellular factors required for an active virus production. It is known that resting CD4⁺ T cells display a weak permissiveness to a productive HIV-1 infection due to the absence of these cellular factors and, when these cells are now considered as a stable reservoir for the virus (3).

CD4⁺ T lymphocytes constitute a heterogeneous population that can be subdivided into various subsets including naive, shortlived effector, long-lived central memory and effector memory cells. Naive cells have never encountered the Ag, are in a resting state, and express the CD45RA isoform and two markers essential for homing to secondary lymphoid tissues (e.g., lymph nodes,

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spleen, and tonsils), i.e., CD62L and CCR7 (4, 5). These cells circulate in lymphoid tissues through blood and lymph until they encounter their nominal Ag presented by an APC (6, 7). Depending on the duration and strength of the signal received, as well as on the type of cytokines present in the microenvironment, naive cells will differentiate into short-lived effector or long-lived central memory cells, which have distinct roles in immunity. The effector cells migrate to inflamed nonlymphoid tissues to help innate immune cells kill invaders and then they die. In contrast, central memory cells, which express the CD45RO isoform, CD62L and CCR7, recirculate as naive cells among secondary lymphoid tissues through blood and lymph until they re-encounter their nominal Ag. These cells are called "memory cells" because they are able to recognize previous Ags and rapidly orchestrate an efficient immune response. Once activated, they become effector memory cells and will eventually lose CD62L and CCR7, express CD69 and CD25, two markers of cell activation, and acquire chemokine receptors (i.e., CCR5 and CCR3), which are essential for reaching inflamed nonlymphoid tissues (8-11). After accomplishing their effector functions, some effector memory cells will persist in nonlymphoid tissues and provide the long-term immunological memory required for controlling and fighting the invaders at the tissueexternal environment interface.

A rapid and progressive depletion of effector memory CD4⁺ T cells is seen in HIV-1-infected individuals that results in an increased susceptibility to opportunistic and community acquired infections and enhanced microbial translocation to the normally sterile internal environment (12). These features contribute to the establishment of an immune hyperactivation state and T cell turnover in chronically infected individuals, setting up ideal conditions for further viral replication and cellular destruction. When the number of CD4⁺ T cells decreases below 200/mm³, commensal and normally well-controlled microorganisms present in the human flora become pathogenic (13, 14). Indeed, it is known that individuals carrying HIV-1 fail to control and eliminate opportunistic microorganisms and these infections often become invasive and mortal. This particular feature of HIV-1 infection is mostly caused by destruction of effector memory CD4⁺ T cells and the

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³ Abbreviations used in this paper: HIV-1, HIV-type 1; DC, dendritic cell; rh, recombinant human; LTR, long terminal repeat.

inability of the infected host to regenerate the pool of competent effector CD4^+ T lymphocytes.

The immune system senses an invasion with microbes via receptors recognizing microbial components known as pathogen-associated molecular patterns (PAMPs) (15). These receptors are identified as pathogens recognition receptors (16). Among these, TLRs are mostly expressed on innate immune cells such as macrophages, dendritic cells (DCs), B lymphocytes, and NK cells (17, 18). TLRs are transmembrane glycoproteins characterized by an extracellular domain bearing various leucine-rich repeat motifs and a cytoplasmic-signaling domain called the Toll/IL-1R homology domain (19). To date, 11 TLRs have been identified in humans (20). These receptors can be classified according to which pathogen-associated molecular patterns they recognize: TLR1, 2, and 6 detect lipids, whereas TLR3, 7, 8, and 9 recognize nucleic acids. TLR4, either associated or not with CD14, identifies various ligands such as LPS, heat shock proteins, and fibronectin, whereas TLR5 binds flagellin (21). These bacterial components are liberated from live bacteria or released upon bacterial lysis (caused by complement, antibiotics, or soluble factors such as lysozyme and β -defensions). Gram-negative bacteria are mostly identified by TLR4 through LPS binding (22, 23) while TLR2 rather recognizes diverse bacterial cell-wall components such as peptidoglycan (24), lipoteichoic acid (25), lipoarabinomannan (26), lipoproteins (27), and porins (28, 29). It has been recently showed that mRNAs encoding for TLR1-5, 7, and 9 are present in CD4⁺ T lymphocytes (30, 31). Further studies have revealed that central memory CD4⁺ T cells express higher levels of TLR2, 4, 5, and 7 than naive ones. Moreover, exposure of these T cell subsets to TLR2, 5, or 7 ligand triggers a signaling cascade resulting in IFN- γ production (30, 31). These data suggest that some TLR ligands can activate resting T cells. Accordingly, we hypothesized that exposure of quiescent naive and memory CD4⁺ T cells to bacterial TLR ligands might modulate susceptibility to HIV-1 infection. We report here that TLR2 triggering increases replication of X4-tropic HIV-1 in naive and memory CD4⁺ T cells and renders these cells susceptible to productive infection with R5-dependent viruses.

Materials and Methods

Abs and reagents

The hybridoma cell line OKT3, which produces the anti-CD35 mAb, and the hybridoma cell line 2.06, which secretes a mAb directed against a monomorphic epitope of the HLA-DR determinant of MHC class II, were obtained from the American Type Culture Collection (ATCC). The anti-ICAM-1 hybridoma R6.5 was provided by S. Benedict (University of Kansas, Lawrence, KS). A purified anti-CD28 Ab (clone 9.3) was provided by J. A. Ledbetter (Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ). PE-conjugated anti-CCR5, anti-CD45RA (clone 5H9), anti-CD45RO (clone UCHL1), and FITC-conjugated anti-CD69 were purchased from BD Pharmingen. The hybridoma cell line 7G7/B6, which produces an anti-CD25 mAb, was provided by J. Bernier (Institut National de la Recherche Scientifique-Institut Armand-Frappier, Laval, Québec, Canada). Hybridomas producing 183-H12-5C and 31-90-25, two Abs recognizing different epitopes of the HIV-1 major viral core protein p24gaa, were supplied by the AIDS Repository Reagent Program (Germantown, MD) and ATCC, respectively. Abs obtained from these cells were purified using mAbTrap protein G affinity columns according to the manufacturer's instructions (Amersham Pharmacia Biotech). Ultra-purified LPS and the TLR2 agonist Pam₃Csk₄ were purchased from InvivoGen.

Cells

293T cells were provided by W. C. Greene (The J. Gladstone Institutes, San Francisco, CA) and TZM-bl indicator cells were obtained from the AIDS Repository Reagent Program. These cell lines were cultured in DMEM supplemented with 10% FBS. To obtain the studied primary CD4⁺ T cell subsets, PBMCs from healthy donors were isolated by Ficoll-Hypaque gradient centrifugation. Thereafter, CD4⁺ T cells were purified from freshly isolated PBMCs by immunomagnetic negative selection as indicated by the manufacturer (StemCell Technologies). Bulk CD4⁺ T cells were separated into pure naive (CD45RA-expressing) and memory (CD45RO-expressing) subpopulation by negative selection as described by the manufacturer (Miltenyi Biotec). The purity of isolated CD4⁺ T cell subsets was determined by cytofluorometry and was always >98%. Lymphocytes were cultured in RPMI 1640 medium supplemented with 10% FBS with or without recombinant human (rh) IL-2 (30 U/ml) as indicated.

Plasmids and production of viral stocks

pNL4-3 (32) and pNL4-3Balenv (33) are full-length infectious molecular clones of HIV-1. In pNL4-3Balenv, the env gene of the X4 (T)-tropic NL4-3 strain has been replaced with that of the R5 (macrophage)-tropic Bal strain. Viruses were produced by the calcium phosphate coprecipitation method in 293T cells as described previously (34). Virus preparations were normalized for virion content using an in-house enzymatic assay specific for the major viral p24^{gag} protein. In this test, 183-H12-5C and 31-90-25 Abs are used in combination to quantify p24^{gag} levels (35).

FACS analysis

To monitor cell surface expression of CCR5, CD25, CD69, ICAM-1, and HLA-DR on naive and memory CD4⁺ T lymphocytes, samples from two different healthy donors were incubated with anti-CCR5-PE, anti-CD25, anti-CD69-FITC, anti-ICAM-1, anti-HLA-DR or an appropriate isotype-matched irrelevant control Ab for 30 min at 4°C. Cells were next washed with PBS and then incubated when appropriate with an R-PE-conjugated goat anti-mouse IgG for 30 min at 4°C. After two washes with PBS, cells were fixed in 2% paraformaldehyde and analyzed by FACS (Epics ELITE ESP; Coulter Electronics).

Stimulation and virus infection assays

Primary naive and memory CD4⁺ T cells (1×10^5) were seeded in 96-well flat-bottom tissue-culture plates and either left untreated or treated with the following reagents. Briefly, purified CD4⁺ T lymphocyte subsets were subjected to a treatment with cross-linked OKT3 (i.e., OKT3 at 1 µg/ml and goat anti-mouse at 5 μ g/ml) or Pam₃Csk₄ (5 μ g/ml), an agonist of TLR2. Cells stimulated with the mitogenic agent PHA-L used in combination with rhIL-2 were used as a positive control. After 24 or 72 h of stimulation, photographs of cell cultures were taken to estimate cell aggregates. For virus infections studies, the purified cell subsets were either left untreated or treated with the tested stimuli for 24 or 72 h and washed before infection with NL4-3 or NL4-3Balenv (10 ng of p24^{gag} per 1×10^5) for 24 h. Next, cells were washed again to eliminate uninternalized viruses. Supernatants were harvested at 3 days postinfection. Virus replication was estimated by incubating cell-free supernatants with TZM-bl, an indicator cell line derived from HeLa cells that carries a stably integrated luciferase reporter gene placed under the control of the HIV-1 regulatory element (i.e., long terminal repeat (LTR)) (36). This experimental strategy allows for rapidly assessing the amount of infectious virus particles contained in the supernatant from infected cells, in contrast to the measurement of the extracellular p24gag content which does not discriminate between infectious and noninfectious viruses.

Nuclear extracts and EMSA

Primary naive and memory CD4⁺ T cells (5 \times 10⁶) were either left untreated (mock) or treated for 24 h at 37°C with Pam₃Csk₄ (5 μ g/ml) in a final volume of 2.5 ml of RPMI 1640. As a positive control, we used CD4⁺ T cells (5 \times 10⁶) that were activated for 3 days with PHA-L (1 μ g/ml) in the presence of rhIL-2 (30 U/ml) and stimulated for 4 h with OKT3 (1 μ g/ml) and anti-CD28 (1 μ g/ml) in a final volume of 2.5 ml of RPMI 1640. Cells were then washed twice with ice-cold PBS, and nuclear extracts were prepared according to a previously reported protocol (37). EMSAs were performed by incubating 10 μ g of nuclear proteins with 20 μ l of 1× binding buffer (10 mM HEPES (pH 7.9), 4% glycerol, 1% Ficoll, 25 mM KCl, 1 mM DTT, 0.5 mM EDTA, 25 mM NaCl) containing 2 µg of poly(dI-dC), 10 µg of nuclease-free BSA fraction V, and 0.8 ng of a $[\gamma^{-32}P]$ dsDNA oligonucleotide for 20 min at room temperature. The consensus NF-kB-binding site (5'-ATGTGAGGGGACTTTCCCAGGC-3') and the consensus binding site for Oct-2A (5'-GGAGTATCCAGCTCCG TAGCATGCAAATCCTCTGG-3') (used as control for nonspecific competition) were used as probes and/or in competition assays. The DNAprotein complexes were resolved from free-labeled DNA by electrophoresis in native 4% (w/v) polyacrylamide gels in 0.5× Tris borate/ EDTA buffer. The gels were subsequently dried and exposed to Kodak x-ray film. Cold competition assays were conducted by adding a 100-fold molar excess of an unlabeled dsDNA oligonucleotide simultaneously with the labeled probe.

FIGURE 1. TLR2 triggering induces homotypic aggregation in both naive and memory $CD4^+$ T cells. Naive (A) and memory (B) $CD4^+$ T cells (1 × 10⁵) were either left untreated (mock) or treated with OKT3 (1 µg/ml), Pam₃Csk₄ (5 µg/ml), and PHA-L (1 µg/ml)/rhIL-2 (30 U/ml) (used as a positive control) for 24 and 72 h. Images are presented at ×20 magnification and each is representative of three independent experiments.



Bio-Plex cytokine assay

A commercial Bio-Plex cytokine test that can detect and quantify 10 different cytokines (i.e., IL-1*β*, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-15, TNF- α , and IFN- γ) through the use of the Luminex 100 apparatus was purchased from Bio-Rad. The Luminex technology is a bead array cytometric analyzer designed to study numerous analytes simultaneously by using spectrally distinct beads in a single well of a microtiter plate, using very small sample volumes (i.e., as little as 25 µl). Briefly, purified naive and memory $\tilde{CD4}^+$ T cells were either left untreated (i.e., mock) or treated for 24 h at 37°C with the following stimuli: the TLR2 ligand Pam₃Csk₄ (5 μ g/ml), the TLR4 ligand LPS (0.1 μ g/ml), or cross-linked OKT3 (1 μ g/ ml). Quantification was achieved by measuring concentrations of the studied cytokines in cell-free supernatants according to the manufacturer's instructions. The lowest detection limits for the tested cytokines are: 4.38 pg/ml for IL-1*β*, 1.6 pg/ml for IL-2, 0.33 pg/ml for IL-4, 2.27 pg/ml for IL-6, 4.82 pg/ml for IL-7, 2.12 pg/ml for IL-8, 3.35 pg/ml for IL-10, 2.08 pg/ml for IL-15, 2.25 pg/ml for TNF- α , and 3.72 pg/ml for IFN- γ .

Statistical analysis

Statistical analyses were conducted according to the methods outlined in Zar (38) and Sokal and Rohlf (39). Briefly, homoscedasticity was determined using the variance ratio test and the means were compared using a single-factor ANOVA followed by appropriate post-hoc multiple comparisons (Tukey's or Dunnett's). Values of p of <0.05 (*) were deemed statistically significant, whereas p values <0.01 (**) were considered highly significant. Computations were conducted using GraphPad PRISM version 3.03 statistical software.

Results

TLR2 signaling triggers cluster formation in naive and memory $CD4^+$ T cells

It is now well-established that treatment of macrophages and DCs with some TLR ligands results in cell activation (40–42). As for naive and memory CD4⁺ T cells, it is known that TLR2 stimulation triggers IFN- γ and IL-8 secretion (30), but the possible phenotypic changes occurring in such cells following TLR2 engagement remains largely unknown. To define whether TLR2 stimulation has an effect on the phenotype of naive and memory CD4⁺ T cells, the TLR2 ligand Pam₃Csk₄ was used to treat the studied CD4⁺ T cell subsets. In brief, quiescent naive and memory CD4⁺ T cells were purified from PBMCs of different healthy donors and were exposed to the TLR2 agonist Pam₃Csk₄ for either 24

or 72 h. These stimulation time lengths were selected based on the idea that activation is a relatively fast event and several activation markers and phenotypic changes are observed within these time frames. Data depicted in Fig. 1 indicate that treatment of both naive (Fig. 1A) and memory $CD4^+$ T cells (Fig. 1B) with Pam_3Csk_4 induces the formation of small cellular aggregates after 24 h and clusters were larger and more numerous after 72 h of treatment. The TLR2-mediated cell clusters are much smaller and less compact than the ones obtained with PHA-L/rhIL-2, a combination of reagents that was used as a positive control. Engagement of the TCR/CD3 complex with the OKT3 mAb was also very efficient at inducing cluster formation. Interestingly, no synergistic effect was observed when OKT3 was used in combination with the TLR2 agonist Pam₃Csk₄ (data not shown). Taken together, these results suggest that TLR2 stimulation influences the activation status of both naive and memory CD4⁺ T cells.

TLR2 engagement increases expression of activation markers on naive and memory $CD4^+$ T cells

It has been demonstrated that some cell surface molecules such as CD69, CD25, ICAM-1 and HLA-DR are up-regulated following T cell activation and differentiation (43-46). Given that TLR2 engagement leads to homotypic aggregation of quiescent naive and memory CD4⁺ T lymphocytes, we next assessed whether treatment with the TLR2 agonist Pam₃Csk₄ can increase expression of some specific cell surface markers. To this end, we performed immunostaining of CD69 and CD25, which are considered as two early activation state markers, as well as of ICAM-1 and HLA-DR after exposure to the tested stimuli. As expected, treatment of purified CD4⁺ T cell subsets either with OKT3 or the PHA-L/rhIL-2 combination resulted in an increase in CD25, CD69, ICAM-1, and HLA-DR expression (Table I). It is of interest to note that a more significant induction of most of the studied cell surface markers was seen with the TLR2 agonist Pam₃Csk₄ compared with treatment with OKT3. For example, CD69 was up-regulated by ~4and 14-fold in naive and memory CD4⁺ T cells, respectively. Comparable results were obtained when studying expression of the

Table I. Expression of CD69, CD25, ICAM-1, and HLA-DR in both naive and memory $CD4^+$ T cells after treatment with the TLR2 ligand

		Na	ive (%) ^a			Mei	mory (%) ^a	
Conditions	CD69	CD25	ICAM-1	HLA-DR	CD69	CD25	ICAM-1	HLA-DR
Isotype Mock OKT3 Pam ₃ Csk ₄ Pam ₃ Csk ₄ +OKT3 PHA I	3.9 5.2 9.0 15.8 18.9	2.0 1.6 5.5 6.0 9.4	2.0 3.5 5.0 5.8 9.2	1.0 3.6 4.4 4.8 6.6	1.0 2.6 4.4 14.6 17.8	1.0 0.9 2.2 9.2 8.7	1.0 1.0 3.4 8.5 9.8	1.0 5.7 8.9 13.2 17.2
PHA-L +rhIL-2	22.7	10.8	20.2	8.7	27.0	12.0	43.4	18.5

^a %, Percentage of cells expressing the studied cell surface marker was defined by flow cytometry.

 α subunit of the IL-2R (i.e., CD25), which is augmented by ~3and 9.2-fold in naive and memory CD4⁺ T lymphocytes, respectively. Surface expression of ICAM-1 was up-regulated by ~2.9and 8.5-fold compared with an increase of ~4.8- and 13.2-fold for HLA-DR in naive and memory CD4⁺ T cells, respectively. The percentage of cells expressing the studied activation markers was further enhanced when the TLR2 agonist was used in combination with OKT3. All activation markers were more rapidly induced on the surface of memory than naive CD4⁺ T cells by the TLR2 ligand because an increase is already seen after 24 h of treatment (data not shown). These data confirm that TLR2 engagement changes the activation status of quiescent naive and memory CD4⁺ T cells.

TLR2 engagement leads to NF- κ B induction in naive and memory CD4⁺ T cells

Activation of CD4⁺ T lymphocytes results in nuclear translocation of certain transcription factors like NF- κ B, NFAT, and AP-1, which regulate the expression of a broad range of genes involved in inflammation and immune response (47–49). Considering that ICAM-1 and HLA-DR are regulated by NF- κ B (47, 50) and that TLR2 stimulation enhances expression of these cell surface markers in the studied cell subsets, we hypothesized that NF- κ B might be induced following TLR2 engagement. Therefore, purified naive and memory CD4⁺ T cells were treated with the TLR2 agonist Pam₃Csk₄ for 24 h and nuclear proteins were extracted and analyzed by EMSA. As illustrated in Fig. 2*A*, NF-κB was induced upon treatment of naive and memory CD4⁺ T with Pam₃Csk₄. For example, fold increases over unstimulated cells are 1.7 and 3 for naive and memory CD4⁺ T cells, respectively (Fig. 2*B*). Competition assays performed with cold specific or unrelated oligonucleotides confirmed the specificity of the retarded NF-κB-specific complexes. Our results thus suggest that stimulation of TLR2 triggers a signaling cascade resulting in nuclear translocation of NF-κB in both naive and memory CD4⁺ T cells.

Replication of X4 virions is increased in quiescent naive and memory $CD4^+$ T cells treated with the TLR2 agonist

It is well-known that HIV-1 replicates predominantly in effector CD4⁺ T cells (51). It is thought to be associated with the presence of cellular factors such as NF- κ B, NFAT, and AP-1, which are crucial for viral replication (47–49). Some of these transcription factors are also present in memory CD4⁺ T cells explaining why such cells are also permissive to HIV-1 replication, but to a lesser extent than effector CD4⁺ T lymphocytes. In contrast, these intracellular constituents are not present in the nucleus of naive CD4⁺ T cells, which might be the cause of their very low natural susceptibility to productive HIV-1 infection. Based on our previous data, we tested whether treatment of quiescent naive and memory CD4⁺ T cells with the studied TLR2 ligand can modulate HIV-1

FIGURE 2. NF- κ B is induced upon treatment of naive and memory CD4⁺ T cells with the TLR2 agonist. A, Naive and memory CD4⁺ T cells (10 \times 10⁶) were either left untreated (mock) or treated with Pam_3Csk_4 (5 µg/ml) for 24 h. The positive control consisted of cells treated first with PHA-L (1 μ g/ml)/rhIL-2 (30 U/ml) and next stimulated with OKT3 (1 µg/ml)/CD28 (1 µg/ml). The nuclear extracts were incubated with a NF-kB-labeled probe, and the complexes were resolved on a native 4% polyacrylamide gel. Competitions were performed with a 100-fold molar excess of either specific (i.e., NF-κB) or nonspecific (Oct-2A) oligonucleotides. The data shown are representative of two independent experiments. B, Fold increase over unstimulated cells for naive and memory CD4⁺ T cells have been determined by estimating the signal band intensities by laser densitometry scanning.



FIGURE 3. TLR2 engagement promotes replication of X4 virus in naive and memory CD4⁺ T lymphocytes. Purified naive (A) and memory (B) CD4⁺ T cells (1 \times 10⁵) were either left untreated (mock) or treated with OKT3 (1 µg/ml), Pam₃Csk₄ (5 µg/ml), LPS (0.1 μ g/ml) (used as a negative control), Pam₃Csk₄ (5 µg/ml)/OKT3 (1 µg/ml), and LPS (0.1 μ g/ml)/OKT3 (1 μ g/ml) in the absence (left panels) or presence (right panels) of rhIL-2 (30 U/ml) for 24 and 72 h. The positive controls consisted of cells treated with PHA-L and rhIL-2 (upper and lower left panels). Next, cells were incubated with the X4tropic strain NL4-3 (10 ng of p24 per 1×10^5 cells) for 24 h. Cell-free supernatants were harvested at 3 days postinfection and production of infectious viruses was estimated through the use of the TZM-bl indicator cell line. Two days later, TZM-bl cells were lysed to monitor luciferase activity. Results are expressed as fold increase of luciferase activity of cells treated with the listed stimuli over mock-treated cells. The data shown represent the mean \pm SDs of quintuplicate samples and are representative of eight separate experiments.

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replication. In some samples, the TLR2 agonist Pam₃Csk₄ was also used in combination with OKT3 and all the tested stimuli were used in the absence or presence of rhIL-2 to define whether this cytokine can modulate the response of the studied CD4⁺ T cell subsets to stimulation through TLR2. In this series of investigations, the TLR4 agonist LPS was used as a negative control because CD4⁺ T cells do not express the CD14-signaling coreceptor. Following an incubation period of 24 or 72 h with the tested stimuli, cells were infected with the prototypic X4-using NL4-3 strain of HIV-1 and virus production was estimated through the use of the TZM-bl cell line. It is important to highlight that TZM-bl cells express neither TLR2 nor CD14 (the latter cell surface constituent would allow signaling through TLR4) (data not shown). Results from Fig. 3A indicate that treatment of naive CD4⁺ T cells with the TLR2 agonist Pam₃Csk₄ before exposure to NL4-3 is sufficient to enhance virus production (i.e., 1.7-fold increase after 24 h of treatment). A more significant increase in virus production was seen when such cells were stimulated with the TLR2 ligand and OKT3 (i.e., 2-fold increase). Moreover, the TLR2-mediated HIV-1 production was markedly enhanced in the presence of rhIL-2 (i.e., a 27-fold increase in presence of rhIL-2). Similarly, the TLR2- and TCR/CD3-mediated virus replication was also augmented upon addition of rhIL-2 (i.e., a 19-fold augmentation in presence of rhIL-2). A similar TLR2-mediated increase in HIV-1 production was seen when experiments were conducted in memory CD4⁺ T cells (Fig. 3B). Again, the noticed augmentation in virus production was more significant in presence of rhIL-2. In an attempt to demonstrate that the observed TLR2-mediated augmentation in luciferase activity seen in TZM-bl is not due to soluble factors produced by the stimulated CD4⁺ T cells, purified naive and memory CD4⁺ T cells from two different healthy donors were either left untreated or treated for 24 h at 37°C with the following stimuli: cross-linked OKT3, the TLR2 ligand Pam3Csk4, or the TLR4 ligand LPS. Next, cell-free supernatants were harvested and added to TZM-bl indicator cells. In some samples, TZM-bl cells were incubated with complete culture medium only (i.e., CTRL; used to

estimate the basal level of luciferase activity). None of the tested cell-free supernatants was able to drive HIV-1 LTR-driven luciferase in TZM-bl indicator cells (data not shown), therefore indicating that the TLR2-dependent increase in HIV-1 LTR-driven reporter gene activity is really associated with an increase in HIV-1 production. Altogether, we provide evidence here that TLR2 engagement enhances replication of X4-tropic virus in both quiescent naive and memory CD4⁺ T cells.

Engagement of TLR2 increases CCR5 expression and renders cells more permissive to R5 virions

Upon cell activation, naive and memory CD4⁺ T cells acquire an effector phenotype. The effector phenotype is characterized by expression of some specific cell surface molecules, including chemokine receptors like CCR5 and CCR3, the role of which is to allow migration of effector cells to inflamed nonlymphoid tissue (8, 10, 11). To investigate whether naive and memory CD4⁺ T lymphocytes become effector-like cells, we assessed CCR5 expression by flow cytometry. Data from Table II indicate that

Table II. CCR5 expression on naive and memory CD4⁺ T cells following treatment with the TLR2 agonist

	Naive $(\%)^a$	Memory (%) ^e CCR5	
Conditions	CCR5		
Isotype	1.0	1.0	
Mock	1.4	0.6	
OKT3	2.5	3.6	
Pam ₃ Csk ₄	2.6	10.0	
Pam_3Csk_4 +OKT3	5.2	15.5	
PHA-L +rhIL-2	10.1	13.7	

^a %, Percentage of cells expressing CCR5 was monitored by flow cytometry.

FIGURE 4. Replication of R5 virus is augmented following treatment of quiescent naive and memory CD4⁺ T lymphocytes with the TLR2 ligand. Purified naive (A) and memory (B) CD4⁺ T cells (1 \times 10⁵) were either left untreated (mock) or treated with OKT3 (1 μ g/ ml), Pam₃Csk₄ (5 μ g/ml), LPS (0.1 μ g/ml) (used as a negative control), Pam_3Csk_4 (5 μ g/ ml)/OKT3 (1 µg/ml), and LPS (0.1 µg/ml)/ OKT3 (1 μ g/ml) in the absence (*left panels*) or presence (right panels) of rhIL-2 (30 U/ml) for 24 and 72 h. The positive controls consisted of cells treated with PHA-L and rhIL-2 (upper and lower left panels). Next, cells were incubated with the R5-tropic strain NL4-3Balenv (10 ng of p24 per 1×10^5 cells) for 24 h. Cell-free supernatants were harvested at 3 days postinfection and production of infectious viruses was estimated through the use of the TZM-bl indicator cell line. Two days later, TZM-bl cells were lysed to monitor luciferase activity. Results are expressed as fold increase of luciferase activity of cells treated with the listed stimuli over mock-treated cells. The data shown represent the mean \pm SDs of quintuplicate samples and are representative of five separate experiments.



expression of the chemokine receptor CCR5 is increased upon exposure of the studied cell subsets to the TLR2 agonist Pam_3Csk_4 and this augmentation is more important when the TLR2 ligand is used in conjunction with OKT3.

Our next studies were aimed at defining whether the TLR2mediated increase in CCR5 expression could translate in a higher

permissiveness to R5 virions (i.e., NL4-3Bal*env*) given that untreated naive and memory $CD4^+$ T cells are not susceptible to a productive infection with NL4-3Bal*env*. Treatment of both cell subsets with Pam_3Csk_4 leads to a more important virus production with the tested strain of HIV-1 (Fig. 4). For example, a 1.4-fold increase in HIV-1 production was seen following treatment of



FIGURE 5. Cytokine profiles in stimuli-treated naive and memory $CD4^+$ T cells using a multiplex bead array test. Purified naive (*A*) and memory (*B*) $CD4^+$ T cells (1×10^5) were either left untreated (i.e., mock) or treated for 24 h at 37°C with Pam₃Csk₄ (5 µg/ml), LPS (0.1 µg/ml), and cross-linked OKT3 (1 µg/ml). Next, cell-free supernatants were harvested and analyzed with a Bio-Plex assay that can detect the 10 different listed cytokines. The results shown are representative of two separate experiments performed with different donors.

naive CD4⁺ T cells with the TLR2 ligand and a 2.7-fold enhancement in virus production was detected when Pam_3Csk_4 was also used in combination with OKT3. However, in the presence of rhIL-2, the augmentation reached 9.3- and 24-fold, respectively, with the TLR2 ligand and with both TLR2 and TCR/CD3 ligands. A more significant TLR2-mediated increase in HIV-1 production was seen in memory CD4⁺ T cells either in the absence or presence of exogenous rhIL-2. Taken together, our findings demonstrate that quiescent naive and memory CD4⁺ T cells acquire an effector-like phenotype following TLR2 engagement, a process resulting in a more important replication of R5-using virions.

TLR2-mediated effect on HIV-1 replication is not due to contaminating cells in the purified $CD4^+$ *T cell preparations*

Studies were next performed to address the possibility that stimulation of contaminating cells such as monocytes and DCs by the TLR2 ligand might have influenced HIV-1 expression and/or virus infection in the purified CD4⁺ T cell subpopulations. To this end, naive and memory CD4+ T cells were either left untreated (i.e., mock) or treated for 24 h at 37°C with the following stimuli: the TLR2 ligand Pam₃Csk₄, the TLR4 ligand LPS (which will activate monocytes and DCs but not CD4⁺ T lymphocytes because these cells do not express CD14) (used as a control to estimate contaminating cells such as monocytes and DCs), and cross-linked OKT3 (which will lead to T cell activation by engaging the TCR/ CD3 complex) (used as a positive control). Next, cell-free supernatants were harvested and analyzed with a Bio-Plex assay to quantify the following cytokines: IL-1*β*, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-15, TNF- α , and IFN- γ . As depicted in Fig. 5, ligation of TLR2 resulted in production of IL-8 from purified naive and memory CD4⁺ T cells. Engagement of the TCR/CD3 complex with cross-linked OKT3 led to secretion of various cytokines such as IL-1 β , IL-2, IL-4, IL-7, IL-8, IL-10, TNF- α , and IFN- γ . Interestingly, treatment with LPS did not result in the production of cytokines such as IL-6, IL-10, and TNF- α that are known to be secreted by human monocytes and DCs following TLR4/CD14 ligation (52). Altogether, these results suggest that our purified cell subpopulations are primarily constituted of CD4⁺ T lymphocytes and the observed TLR2-mediated effect on HIV-1 is thus not due to production of soluble factors by contaminating cells such as monocytes and DCs.

Discussion

Although many studies have reported the impact of TLR ligands on the activation of innate immune cells, the direct effects of microbial components that can bind to some TLRs in different T cell subsets is only beginning to be deciphered (53). Indeed, it has recently been reported that some bacterial cell-wall constituents can provide a cosignal and act in concert with a TCR/CD3-mediated signal, a phenomenon resulting in cell proliferation and secretion of IFN- γ and IL-8 in both naive and memory CD4⁺ T lymphocytes (54). These two processes are intimately linked with the acquisition of an effector-like phenotype by these cells (30). Considering the important role of effector CD4⁺ T cells in the pathogenesis of HIV-1 infection, we investigated whether a TLR2 agonist (i.e., Pam₃Csk₄) can directly modulate the function of quiescent naive and memory CD4⁺ T cells and affect permissiveness to infection with X4 and R5 viruses.

We first assessed the ability of the TLR2 ligand Pam_3Csk_4 to induce activation of the studied resting $CD4^+$ T cell subsets. We found that the tested TLR2 agonist triggers homotypic aggregation in both naive and memory $CD4^+$ T cells. The absence of aggregation in unstimulated cells proves the quiescent state of the purified naive and memory $CD4^+$ T cells. It has been established that

aggregation occurs upon T cell activation and involves adhesion molecules like CD43 (55), CD44 (56, 57), ICAMs (58-60), and the integrin LFA-1 (61). Activation and differentiation of CD4⁺ T cells are also characterized by an up-regulation of some activation markers such as CD69, CD25, and HLA-DR (43-46). We demonstrate here for the first time that exposure of resting naive and memory CD4⁺ T cells to the TLR2 agonist Pam₃Csk₄ is sufficient to increase expression of these cell surface markers on both cell subsets, but with a different time kinetics. For example, a portion of naive and memory cells becomes positive for CD69, CD25, and HLA-DR after 72 h of TLR2 stimulation. However, memory cells express these activation markers already after 24 h of stimulation. Again, the absence of the activation markers CD25 and CD69 at the surface of unstimulated naive and memory CD4⁺ T cells indicates that these cells were under a resting state. Cells stimulated with PHA-L/rhIL-2 and OKT3 were used as a positive control for activation. The low percentage of naive and memory CD4⁺ T cells expressing activation markers following OKT3 stimulation can be explain by the absence of costimulation via CD28. These data are in agreement with the previous demonstration that memory cells require a less stringent signal to be activated than naive cells, which are metabolically less active (62). The observed differences with respect to the responsive time could be linked to a greater expression of TLR2 in memory cells (63). We also measured LFA-1 expression and found that this cell surface constituent is not modulated by the studied TLR2 ligand (data not shown). Because LFA-1 is mainly regulated by changes in its affinity and avidity states, our data are thus not surprising. Further studies are needed to clarify the impact of TLR2 stimulation on the LFA-1 activation status, as well as on expression of other adhesion molecules that could be involved in homotypic cell adhesion. However, it can be postulated that the TLR2-mediated cell cluster formation is most likely attributable to the increased expression of ICAM-1.

Modulation of activation markers is controlled also by different transcription factors like NF-KB, NFAT, and AP-1 (47-49). Following their activation, these cytoplasmic proteins are translocated to the nucleus where they activate several genes participating in inflammation and to the establishment of an immune response. The presence of these factors in permissive cells also allows HIV-1 gene expression and virus production. NF-kB is considered as a key player in the virus life cycle because it binds to the two NF- κ B-binding motifs located in the HIV-1 enhancer region (64). Transcription of HIV-1 can also be modulated by NFAT and AP-1 (65, 66). The presence of certain proinflammatory cytokines in the extracellular environment (e.g., TNF- α and IL-1) and a number of T cell activators can induce these transcription factors and promote HIV-1 production. It is well-known that HIV-1 replicates preferentially in effector and memory CD4⁺ T cells and cells displaying a naive phenotype do not support virus gene expression (51). This inability to allow virus replication seems to be linked with the absence of NFAT in naive CD4⁺ T cells (65, 67-69). It was previously demonstrated that NFAT acts in synergy with NF-KB to promote HIV-1 replication in activated and memory CD4⁺ T cells. Moreover, naive CD4⁺ T cells are metabolically less active than memory cells and require a stronger stimulus to be fully activated and translocate AP-1 and NF- κ B to the nucleus (62, 70). Based on this information, we assessed whether a TLR2-mediated signal is sufficient to trigger nuclear translocation of NF-kB in naive and memory CD4⁺ T cells. NF- κ B was found to be activated in both T cell subsets following treatment with the TLR2 ligand Pam₃Csk₄. It can therefore be proposed that bacterial cell-wall components that can ligate TLR2 might promote HIV-1 replication by priming both naive and memory CD4⁺ T cells. The possibility that TLR-mediated signal transduction events can modulate virus

gene expression is not new. For example, it has been reported that TLR2 engagement either with Pam_3Csk_4 or *Neisseria gonorrhoeae* can directly enhance HIV-1 infection in DCs, a process leading to a more efficient virus transfer to CD4⁺ T cells (71). In contrast, engagement of TLR4 by LPS inhibits HIV-1 replication in primary human macrophages (72, 73). A similar observation has been made with the *N. gonorrhoeae*-derived lipo-oligosaccharide (74). In both cases, the inhibition of virus replication is linked to a TLR4-mediated secretion of the antiviral cytokines IFN- α and IFN- β .

More relevant to HIV-1, we observed that TLR2 stimulation increases virus replication in naive and memory CD4⁺ T cells and this effect was not due to soluble factor(s) made by contaminating cells such as monocytes and DCs. However, it cannot be totally excluded that the TLR2-mediated effect on HIV-1 replication in purified naive and memory CD4⁺ T cells might be partly due to contaminating cells through a cell-to-cell contact. It can be proposed that the TLR-2-mediated increase in cellular aggregation might be responsible to some extent for the enhanced HIV-1 production based on the idea that cell-to-cell virus infection is a much more efficient process than infection with cell-free virions. In both CD4⁺ T cell subsets, addition of exogenous rhIL-2 led to an augmentation of TLR2-mediated HIV-1 production. It seems that rhIL-2 provides a positive signal to naive and memory cells during virus infection and acts in synergy with the TLR2 agonist. Surprisingly, no synergic effect was observed when the TLR2 agonist was used in combination with a TCR/CD3 ligand (i.e., OKT3) in naive cells, which is in contrast to the situation prevailing in memory cells. This could be due to the absence of the CD28 costimulatory signal. Studies are currently underway to shed light on this matter.

It is now well-accepted that there is a selective transmission of R5 virions, a process occurring in mucosal sites like gastrointestinal and genital tracts (75). Effector memory CD4⁺ T cells, which are located in such mucosal tissues, are massively depleted very soon following primary infection (1, 2). This phenomenon is attributable to their high permissiveness to infection with R5 viruses because these cells express high levels of surface CCR5. In contrast, central memory CD4⁺ T lymphocytes in the absence of other stimuli can only be infected by X4 viruses because they do express CXCR4 and are negative for CCR5. Having established that TLR2 stimulation causes naive and memory CD4⁺ T cells to acquire an effector-like phenotype, we assessed whether they do acquire expression of the virus coreceptor CCR5. This chemokine receptor is expressed on effector T cells and allows them to reach inflamed nonlymphoid tissues and peripheral tissues (8, 11). Importantly, treatment of naive and memory CD4⁺ T cells with the TLR2 ligand resulted in an increased expression of CCR5 in both cell subsets. These data are in agreement with a previous work showing that the TLR2 ligand Pam₃Csk₄ can trigger secretion of IFN- γ (76), a cytokine produced by effector T cells. Interestingly, although virus production could not be detected in untreated cells, the TLR2-mediated induction of CCR5 rendered naive and memory CD4⁺ T cells susceptible to infection with the R5 strain NL4-3Balenv. Moreover, our results indicate that naive cells become permissive to infection with R5 virions only after a long stimulation time with the TLR2 ligand (i.e., 72 h) and the noticed increase in HIV-1 replication was further augmented in presence of rhIL-2. It is possible that TLR2 engagement is not powerful enough to allow productive infection with R5 viruses after only 24 h of stimulation. In fact, viral replication appears only after 72 h of stimulation either in absence or presence of rhIL-2. In addition, virus production is further augmented when TLR2 and TCR/CD3 complex are concomitantly engaged (i.e., 1.4- compared with 2.7-fold increase in absence of rhIL-2 in naive cells and 9.3- compared with 24-fold increase in presence of rhIL-2 in memory cells). The more important HIV-1 replication in memory compared with naive cells is most likely due to a higher surface expression of CCR5 and enhanced TLR2-mediated induction of NF- κ B.

Considering that circulating microbial products are responsible for a systemic immune activation, it is possible that these bacterial components are responsible for at least part of this activation via TLR stimulation of naive and memory CD4⁺ T cells. These products that originate from the microbial translocation might directly or indirectly, through the effects of cytokines and chemokines, result in a polyclonal T cell activation. It is of interest to note the recent study by Brenchley et al. (12) who have demonstrated that ongoing infection and depletion of CD4⁺ T cells throughout the chronic phase of HIV-1 infection prevents the re-establishment of a competent immunological control of microbial translocation. The complement to such events would be that microbial translocation in turn perpetuates viral replication through the provision and activation of CD4⁺ T cells that might serve as targets for HIV-1 (12, 77). This series of events can accelerate the deterioration of the immune system. In fact, it is known that the switch from R5 to X4 viruses takes place many years after primoinfection and that at this time, there is a rapid and dramatic decrease in CD4⁺ T cell counts. The results presented in the current study lead us to propose that the decline in the overall number of circulating CD4⁺ T lymphocytes might be accelerated in patients experiencing infections with pathogens that can engage some specific pathogen recognition receptors such as TLR2. Although the contribution of sexually transmitted bacterial infections to HIV-1 pathogenesis is established, the impact of community acquired bacterial infections is still unclear. However, it is recognized that these infections caused for example by Streptococcus pneumonia, Salmonella spp., Mycobacterium tuberculosis, and Haemophilus influenza are more prevalent in HIV-1-infected persons than in uninfected individuals, even if the former are under antiretroviral therapy (78-82). This phenomenon seems to be linked with the HIV-1-mediated immune dysregulation affecting both adaptive and innate immunity. Indeed, HIV-1-infected persons fail to control and eliminate bacterial infections that can become recurrent and invasive. Opportunistic infections are also commonly present in HIV-1-infected individuals due to their immunodeficiency status. When the number of CD4⁺ T cells decreases and viremia goes up, commensal bacteria can become pathogenic and cause opportunistic infections, a problem frequently seen in persons carrying HIV-1. Several diseases are commonly afflicting HIV-1-infected persons like pneumonia (caused by Pneumocystis carinii), tuberculosis (caused by M. tuberculosis), toxoplasmosis (caused by Toxoplasma gondii), CMV infections, encephalitis, and Kaposi's sarcoma. The majority of these human pathogens are present in the normal flora of the digestive tract. In individuals who have contracted HIV-1, the immune system is unable to control these opportunist microorganisms because memory CD4⁺ T cells are eliminated and other cell types suffer from several functional deficiencies (e.g., CD8⁺ T cells and macrophages). Treatment of these patients with potent antibiotics might lead to the release of microbial products like peptidoglycans, bacterial lipoproteins, lipoteichoic acid, and LPS. Based on our findings, it is possible that the liberation of these microbial products in the periphery can modulate HIV-1-mediated disease progression by activating naive and memory CD4⁺ T cells and rendering them more permissive to productive infection with both X4 and R5 isolates of HIV-1. Therefore, therapeutic approaches aimed at controlling bacterial infections in HIV-1 patients deserve to be re-evaluated in light of this new information.

In summary, bacterial infection and the ensuing microbial translocation might act as factors accelerating HIV-1 disease progression by promoting the transformation of naive and memory CD4⁺ T cells into effector cells and increasing the pool of cells permissive to HIV-1 infection. A better comprehension of this phenomenon and the various interactions between immune cells, bacterial commensal flora and pathogenic bacteria is needed to shed light on their possible impact on HIV-1-mediated immune dysregulation and pathogenesis.

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Disclosures

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