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TLR Activation Pathways in HIV-1–Exposed Seronegative Individuals

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TLRs trigger innate immunity that recognizes conserved motifs of invading pathogens, resulting in cellular activation and release of inflammatory factors. The influence of TLR activation on resistance to HIV-1 infection has not been investigated in HIV-1 exposed seronegative (ESN) individuals. PBMCs isolated from heterosexually ESN individuals were stimulated with agonists specific for TLR3 (poly I:C), TLR4 (LPS), TLR7 (imiquimod), and TLR7/8 (ssRNA40). We evaluated expression of factors involved in TLR signaling cascades, production of downstream effector immune mediators, and TLR-expression in CD4+ and CD14+ cells. Results were compared with those obtained in healthy controls (HCs). ESN individuals showed: 1) comparable percentages of CD14+/TLR4+ and CD4+/TLR8+ CD14+/TLR8+ cells; 2) higher responsiveness to poly I:C, LPS, imiquimod, and ssRNA40 stimulation, associated with significantly increased production of IL-1β, IL-6, TNF-α, and CCL3; 3) augmented expression of mRNA specific for other targets (CCL2, CSF3, CSF2, IL-1α, IL-8, IL-10, IL-12, cyclooxygenase 2) demonstrated by broader TLRs pathway expression analyses; and 4) increased MyD88/MyD88s(short) ratio, mainly following TLR7/8 stimulation. We also compared TLR–agonist-stimulated cytokine/chemokine production in CD14+ PBMCs and observed decreased IFN-β production in ESN individuals compared with HCs upon TLR7/8-agonist stimulation. These data suggest that TLR stimulation in ESN individuals results in a more robust release of immunologic factors that can influence the induction of stronger adaptive antiviral immune responses and might represent a virus-exposure–induced innate immune protective phenotype against HIV-1. ***The Journal of Immunology*****, 2010, 184: 2710–2717.**

The concept of resistance to HIV infection in individuals who have been repeatedly exposed to HIV-1, but who do not seroconvert (HIV-1–exposed seronegative [ESN]), and the search for immune correlates of such protection against HIV infection dates back 20 y to the first reports of this unexpected phenomenon (1, 2). These early studies proposed an immunologic advantage for these individuals, possibly conferring resistance against HIV-1 infection. The suggested multifactorial advantages included favorable genetic (3, 4) and innate immunity (5), resulting in the generation of HIV-1–specific systemic and mucosal cell-mediated immunity (6–8).

TLRs are a family of pattern-recognition receptors involved in the initiation of the immune response, allowing TLR-expressing cells to directly recognize pathogen-associated molecular patterns of pathogens such as bacteria and viruses (9). TLR3, 7, 8, and 9 have been implicated in antiviral defense (9, 10) and, in particular, ssRNA from HIV was found to trigger TLR7 and 8 in a MyD88-dependent manner (11, 12).

Despite reports that associate ESN individuals with adaptive immune responses (6–8), the initiating events leading to this state of resistance to HIV infection remain unknown. TLR activation represents the first line of defense against invading pathogens, and few reports describe a possible role for innate immunity on the modulation of susceptibility to HIV infection (5, 13, 14). Therefore, we investigated the effects of engagement of four different TLRs on cytokine and chemokine expression profiles in PBMCs from a cohort of ESN individuals, and we compared those findings with data obtained from an equal number of healthy control (HC) blood donors.

Materials and Methods

Study population and in vitro TLR stimulation

The study was reviewed and approved by the institutional review boards of the S. M. Annunziata Hospital; written, informed consent was obtained from all subjects. Blood samples were collected from 30 ESN individuals. Inclusion criteria were a history of multiple unprotected sexual episodes for more than 4 y at the time of the enrollment, with at least three episodes of at-risk intercourse within 4 mo before study entry, and an average of 30 (range, 18 to >100) reported unprotected sexual contacts per year. Thirty age-matched HCs, without known risk factor for HIV infection, were also included in the study.

We have been following this cohort of individuals for the past 12 y (the cohort was established in Tuscany at the beginning of 1997). This long period of time has allowed us to adopt stringent matching criteria between HC and ESN. Simply put, both our ESN and HC groups are involved in monogamous relationships, are part of long-lasting couples, and have similar sexual activities. HC and ESN individuals are from the same geographical area and share the same genetic background and the same

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Abbreviation used in this paper: COX2, cyclooxygenase 2; ESN, HIV-1–exposed seronegative; HC, healthy control; MFI, mean fluorescence intensity; pDC, plasmacytoid dendritic cell; poly I:C, polyinosic:polycytidylic acid.

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exposure to environmental factors. Part of the analyses performed on the individuals enrolled in this cohort are done by the Department of Obstetrics and Gynecology, Santa Maria Annunziata Hospital (Florence, Italy). This allows us to exclude from the study ESN and HC patients in whom sexually transmitted diseases or any other pathologic conditions are reported. The follow-up includes biannual visits to the Department of Obstetrics and Gynecology, during which an accurate clinical history is collected and vaginal swabs are performed.

Whole blood was collected from all subjects by venupuncture in Vacutainer tubes containing EDTA (BD Biosciences, San Jose, CA), and PBMCs were separated on lymphocyte separation medium (Organon Teknica, Malvern, PA).

Based on data derived from a kinetic study (data not shown), 2.5×10^5 freshly isolated PBMCs were incubated for 6 (mRNA analyses) or 24 h (protein analyses) with medium, 10 μg/ml polyinosic:polycytidylic acid (poly I:C), 2 μg/ml LPS, 1 μg/ml imiquimod, or 2.5 μg/ml ssRNA40.

RNA extraction and reverse transcription

RNA was extracted from cultured PBMCs by using the acid guanidium thiocyanate–phenol–chloroform method. The RNA was dissolved in RNase-free water and purified from genomic DNA with RNase-free DNase (RQ1 DNase; Promega, Madison, WI). One microgram of RNA was reverse transcribed into first-strand cDNA in a 20 μl final volume containing 1 μM random hexanucleotide primers, 1 μM oligo dT and 200 U Moloney murine leukemia virus reverse transcriptase (Clontech, Palo Alto, CA). cDNA quantification for IL-1β, IL-6, TNF-α, CCL3, IFN-γ, MyD88, MyD88s (short), CD69, and GAPDH was performed by real-time PCR (DNA Engine Opticon 2; MJ Research, Cambridge, MA). Reactions were performed using a SYBR Green PCR mix (Finnzymes, Espoo, Finland) as described elsewhere (15). Results were expressed as ΔΔCt (where Ct is the cycle threshold) and presented as ratios between the target gene and the GAPDH housekeeping mRNA.

TLR signaling pathway

TLR signaling pathways were analyzed in a PCR array including a set of optimized real-time PCR primer assays on 96-well plates (SA Biosciences, Frederick, MD; Supplemental Material). This approach permits the monitoring of mRNA expression of 84 genes related to the TLR pathway activation, plus five housekeeping genes, following the procedures suggested by the manufacturer. Controls are also included on each array for genomic DNA contamination, RNA quality, and general PCR performance. The experiments have been run on all of the subjects included in the study, pooled into a unique ESN and HC sample. Thus, the results represent the mean value of the different targets analyzed in ESN and HC. Furthermore, those targets showing marked differences between ESN and HC have been retested by real-time PCR on each individual sample confirming the data obtained in the super array (data not shown).

Cytokine production

IL-6, TNF-α, and IFN-γ production was evaluated in the supernatants of the cultured PBMCs, using commercial ELISA kits (R&D Systems, Minneapolis, MN) and following the procedures suggested by the manufacturer. Cytokine concentration was calculated from a standard curve of the corresponding recombinant human cytokine.

TLR3, TLR4, and TLR8 expression in basal conditions and after specific agonist stimulation

PBMCs were analyzed under basal conditions and after 24 h stimulation with specific TLR 3, 4, or 8 agonists. PBMCs were resuspended in PBS and stained for surface mAbs CD14PECY7, CD4PECY7 (Beckman-Coulter, Fullerton, CA), and TLR4PE (eBioscience, San Diego, CA). After 15 min incubation at room temperature in the dark, cells were washed and fixed in 1% paraformaldeyde in PBS. Cells were then permeabilized with saponin 0.5% (Sigma-Aldrich, St. Louis, MO) and mAbs for TLR8 FITC (0.5 μg; Imgenex, San Diego, CA) and TLR3 FITC or PE (1 μg; eBioscience), IFN-β FITC (0.125 μg; PBI International, Piscataway, NJ), TNF-α FITC or PE, CCL3 APC, (R&D Systems, Minneapolis, MN), IL-6 APC (BioLegend, San Diego, CA) were added. Cells were incubated for 45 min at 4°C in the dark, washed, and fixed in 1% paraformaldeyde in PBS.

Flow cytometric analysis

All the cytometric analyses were performed using FC500 flow cytometer (Beckman-Coulter, Miami, FL) equipped with a double 15-mW argon ion laser operating at 456 and 488 interfaced with an Intercorp (Venice, Italy) computer. For each analysis 20,000 events were acquired and gated on CD4 or CD14 expression and side scatter properties. Green fluorescence from FITC (FL1) was collected through a 525-nm band-pass filter, orange-red fluorescence from R-PE (FL2) was collected through a 575-nm band-pass filter, red fluorescence from Cy5PE (FL4) was collected through a 670-nm band-pass filter, and far red fluorescence from PC7 (FL5) was collected through a 770-nm band-pass filter. Data were collected using linear amplifiers for forward and side scatter and logarithmic amplifiers for FL1, FL2, FL4, and FL5.

Statistical analyses

Statistical analyses were performed using SPSS (version 11; SPSS, Chicago, IL). Differences between the groups were assessed using nonparametric analyses (Mann-Whitney *U* test). All *p* values are 2-tailed.

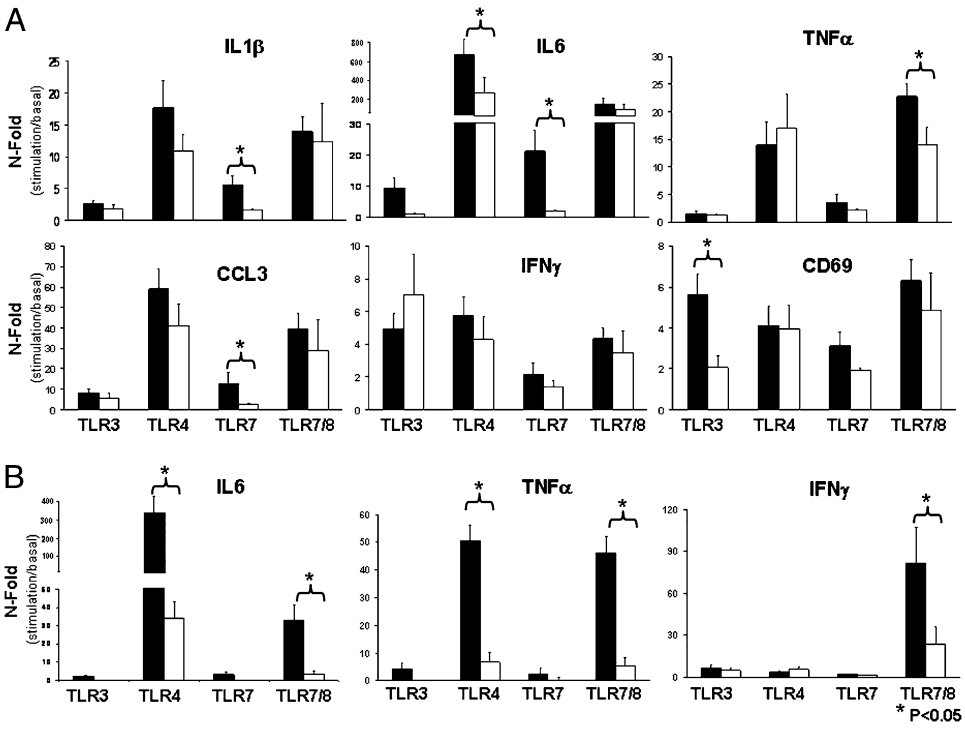

FIGURE 1. ESN (black bars) and HC (white bars) responsiveness following TLR3, TLR4, TLR7, and TLR7/8 stimulation with poly I:C, LPS, imiquimod, and ssRNA40, respectively. *A*, IL-1β, IL-6, TNF-α, CCL3, IFN-γ, and CD69 expression assessed by real-time quantitative RT-PCR after stimulation of ESN and HC PBMCs with poly I:C (TLR3), LPS (TLR4), imiquimod (TLR7), and ssRNA40 (TLR7/8) for 6 h, calculated relative to a housekeeping gene and shown as fold-change expression from the unstimulated sample. *B*, IL-6, TNF-α, and IFN-γ production assessed by ELISA after stimulation of ESN and HC PBMCs with poly I:C (TLR3), LPS (TLR4), imiquimod (TLR7), and ssRNA40 (TLR7/8) for 24 h, shown as fold-change expression from the unstimulated sample.

Results

ESN and HC PBMC responsiveness to TLR3, TLR4, TLR7, and TLR8 stimulation with poly I:C, LPS, imiquimod, and ssRNA40

To determine whether TLR-triggering results in a unique cytokine expression outline in ESN, we compared the cytokine/chemokine profiles in TLR agonist-stimulated PBMCs from 30 ESN and 30 HC subjects. As in PBMCs, a diverse TLR pattern is expressed by the different cellular subsets, and their percentage is unbalanced in HIV+ subjects; as a consequence of CD4+ cells depletion, this group was excluded from these analyses. Stimulation with poly I:C (TLR3), LPS (TLR4), imiquimod (TLR7), and ssRNA40 (TLR7/8) increased expression levels of mRNA specific for IL-1β, IL-6, TNF-α, CCL3, and IFN-γ in PBMCs from both ESN and HC, compared with medium alone. However, the mean responses were higher in ESN compared with HC for all parameters analyzed, except for TNF-α (TLR3 and TLR4) and IFN-γ (TLR3), particularly after stimulation of the MyD88-dependent TLR pathways (Fig 1*A*). Thus, compared with HC, we observed that ESN mRNA expression levels were significantly higher for: 1) IL-1β ($p < 0.02$), IL-6 ($p < 0.02$), and CCL3 ($p < 0.05$) following TLR7 agonist stimulation; 2) TNF-α ($p < 0.05$) following TLR7/8 agonist stimulation; and 3) IL-6 ($p < 0.03$) following TLR4 agonist triggering (Fig. 1*A*). We observed limited variability in the cytokine/chemokine profiles within members of the ESN and HC cohorts, suggesting uniformity within these groups.

mRNA expression levels of CD69, a surface marker specifically induced on early activated T-lymphocytes, was upregulated in both ESN and HC, particularly after TLR3-stimulation (ESN versus HC, $p < 0.005$; Fig. 1*A*).

IL-6, TNF-α, and IFN-γ protein production was measured by ELISA in the supernatants of the TLR agonist-stimulated PBMC cultures from ESNs and HCs. We found that all three cytokine proteins were significantly elevated in ssRNA40-stimulated (TLR7/8) cultures, and that IL-6 and TNF-α were significantly increased in LPS-stimulated (TLR4) cultures of PBMCs from ESN compared with HC (Fig. 1*B*).

The mRNA expression profile of the analyzed cytokines was partially different from the protein profile . For example, after TLR7/8 stimulation IFN-γ protein expression increased significantly without a corresponding raise in mRNA levels, suggesting the existence of a control mechanism operating at post-transcriptional level.

Basal and stimulated TLR3, TLR4, and TLR8 expressing CD14+ and CD4+ cells in ESN versus HC

To determine whether the above-noted increases in cytokine/chemokine expression levels after TLR stimulation of PBMCs were associated with enhanced TLR expression in these subpopulations of cells from ESN individuals, we analyzed TLR3 and TLR8 expression in CD4+ cells and TLR4 and TLR8 expression on CD14+ cells of the two study groups by flow cytometry (TLR7 expression was not assessed because the specific Ab was not commercially available). The results showed no appreciable differences in the percentage of CD4+/TLR3+ and CD4+/TLR8+ cells, or of CD14+/TLR4+ and CD14+/TLR8+ cells in the PBMCs of ESN compared with HC under basal conditions (Table I). Following specific agonist stimulation, the percentage of TLR3- and TLR8-expressing CD4+ and of TLR4- and TLR8-expressing CD14+ cells increased in the PBMCs of both HC and ESN, without showing significant differences between the two groups.

The mean fluorescence intensity (MFI) indicated that TLR3 and TLR8 densities on CD4+ cells, as well as TLR4 and TLR8 on CD14+ cells, were comparable among the examined groups (Table I). The similarities observed between the two groups were maintained even after TLR-specific stimulation (Table I).

TLR signaling pathway mRNA expression in ESN and HC for TLR3, TLR4, TLR7, and TLR8 agonist-stimulated PBMCs

To determine whether the differences we observed for TLR stimulation in ESN versus HC are dependent on a differential modulation of transduction pathways, we used real-time PCR array, which screens for the expression of 84 genes that are involved in TLR pathway activation (Supplemental Material).

The data obtained following TLR3 stimulation (Fig. 2) show higher IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-12, TNF-α, CCL2, CSF3, and cyclooxygenase 2 (COX2) mRNA expression levels in ESN compared with HC individuals. These results suggest an association between a particular immunologic profile that may distinguish ESN from HC and is regulated by the TLR3 signaling pathway. Furthermore, expression of some negative regulators of TLR3-mediated immune response was downmodulated in ESN compared with HC. Thus, expression levels of: 1) NF-kBIL1, inhibitor of the NF-κB transcription factor; 2) the decoy receptor SIGIRR (single immunoglobulin IL-1R–related molecule); and 3) SARM1 (sterile α and TIR motif containing a negative regulator of the TIR domain-containing adaptor TRIF, were all markedly reduced in ESN compared with HC. Loss of expression of these negative regulators may contribute to the increased responsiveness observed in the ESN cohort. In ESN compared with HC, we also observed an increase in expression level of TANK-binding kinase 1, a kinase protein involved in TRIF-dependent signaling pathway.

The data collected following TLR7/8-stimulation (Fig. 3) resemble the immunologic profile observed in TLR3-stimulated PBMCs, but illustrate more pronounced differences in IL-1α, CCL2, CSF3, IL-6, COX2, and TNF-α mRNA expression levels. This comparison also shows that, unlike TLR3-stimulation, increased ESN-responsiveness is not associated with differential modulation of negative regulators controlling the TLR7/8 signaling pathways, but demonstrates an increase in MyD88 adaptor protein expression in ESN compared with HC. In response to TLR4 triggering, we observed a signaling expression profile comparable to that observed in response to TLR7/8

Table I. *Percentage (upper) and MFI (lower) of TLR3, TLR4, and TLR8-expressing CD4+ and CD14+ cells in basal condition and after stimulation of ESN and HC PBMC with poly (I:C) (TLR3), LPS (TLR4), and ssRNA40 (TLR7/8) for 24 h*

		Basal				Poly I:C	LPS	ssRNA40	
		CD4+ Cells		CD14+ Cells		CD4+ Cells	CD14+ Cells	CD4+ Cells	CD14+ Cells
		TLR3	TLR8	TLR4	TLR8	TLR3	TLR4	TLR8	TLR8
%	ESN	0.9 ± 0.3	0.6 ± 0.2	0.9 ± 0.3	1.7 ± 0.5	4.8 ± 1.0	5.3 ± 1.3	14.1 ± 3.7	49.1 ± 6.8
	HC	0.5 ± 0.1	0.4 ± 0.1	1.8 ± 0.7	2.4 ± 0.8	3.4 ± 0.9	4.9 ± 0.9	14.4 ± 3.5	51.9 ± 8.0
MFI	ESN	26.9 ± 2.7	26.0 ± 2.9	68.2 ± 6.8	83 ± 16.1	19.3 ± 2.1	55.9 ± 9.8	34.9 ± 6.9	84.3 ± 8.3
	HC	24.2 ± 2.1	27.3 ± 3.3	81.5 ± 5.3	84.6 ± 4.4	22.6 ± 3.6	64.3 ± 6.3	32.8 ± 4.3	79.2 ± 9.8

Mean values and SE are shown.

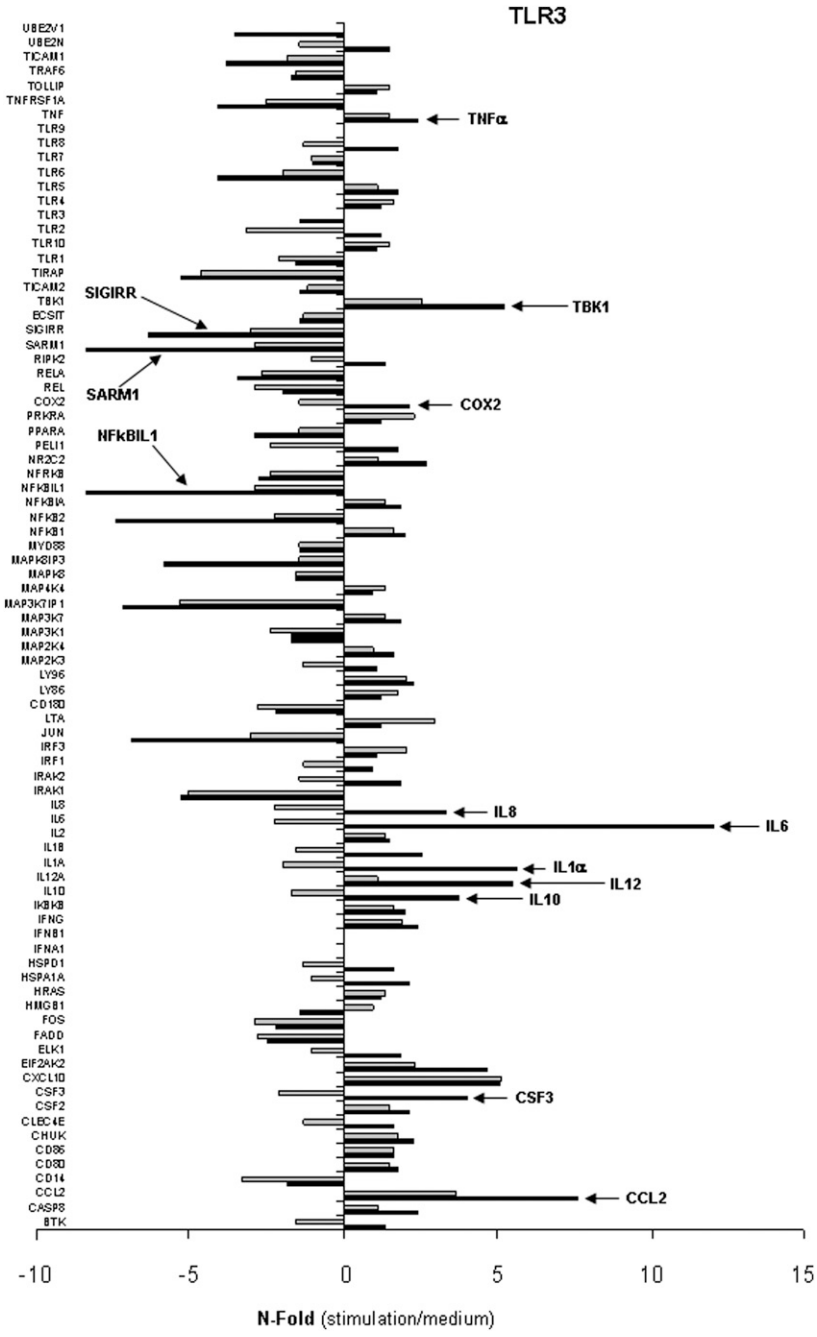

FIGURE 2. TLR signaling pathway mRNA expression in ESN (black bars) and HC (gray bars) for TLR3 stimulation with poly I:C. The expression of 84 genes involved in the TLR signaling pathway has been assessed by real-time quantitative RT-PCR, calculated relative to five housekeeping genes and shown as fold-change expression from the unstimulated sample. The targets showing different expression levels in ESN compared with HC are indicated by specific tags.

stimulation, except for MyD88 expression levels, which were equivalent in the two groups analyzed (data not shown).

IFN-α and IFN-β mRNA expression, after TLR3 and TLR7/8 stimulation, went from undetectable to detectable levels after TLR stimulation in both ESN and HC. Nevertheless, the increase could not be quantified by ΔΔCt calculation method (Figs. 2, 3).

MyD88/MyD88s ratio in ESN and HC TLR-stimulated PBMCs

To determine whether the increased ESN-responsiveness to TLR-stimulation is dependent on a difference in the ratio between the full-length and the negative-regulating shortened form of MyD88 (MyD88s) (16), we quantified mRNA expression levels of the two alternative forms of this adaptor protein following TLR-stimulation. MyD88s mRNA expression level was comparable in ESN and HC in response to each of the four agonists studied. In contrast, the full-length form of the adaptor protein showed an increase in mRNA expression levels in ESN compared with HC following TLR7 and 7/8 stimulation ($p < 0.05$). Thus, the balance between MyD88 and MyD88s in response to TLR7/8 agonists results in an increase of the full-length form in ESN compared with HC, possibly associated with higher responsiveness observed in the ESN group ($p < 0.05$; Fig 4).

Responses of CD4+ or CD14+ cells from ESN and HC to TLR3, TLR4, and TLR7/8 stimulation with poly I:C, LPS, and ssRNA40

We analyzed intracellular cytokine and chemokine production following TLR stimulation in CD4+ and CD14+ cells in PBMCs from ESN and HC individuals, because these subsets are primary targets of HIV-1 infection. Activation of TLR3 and TLR7/8 in CD4+ cells and of TLR4 and TLR7/8 in CD14+ cells confirmed a trend toward an increased responsiveness in ESN compared with HC for all the cytokines analyzed (CCL3, IL-6, and TNF-α), except for IFNβ whose production was reduced in ESN compared with HC (Fig. 5).

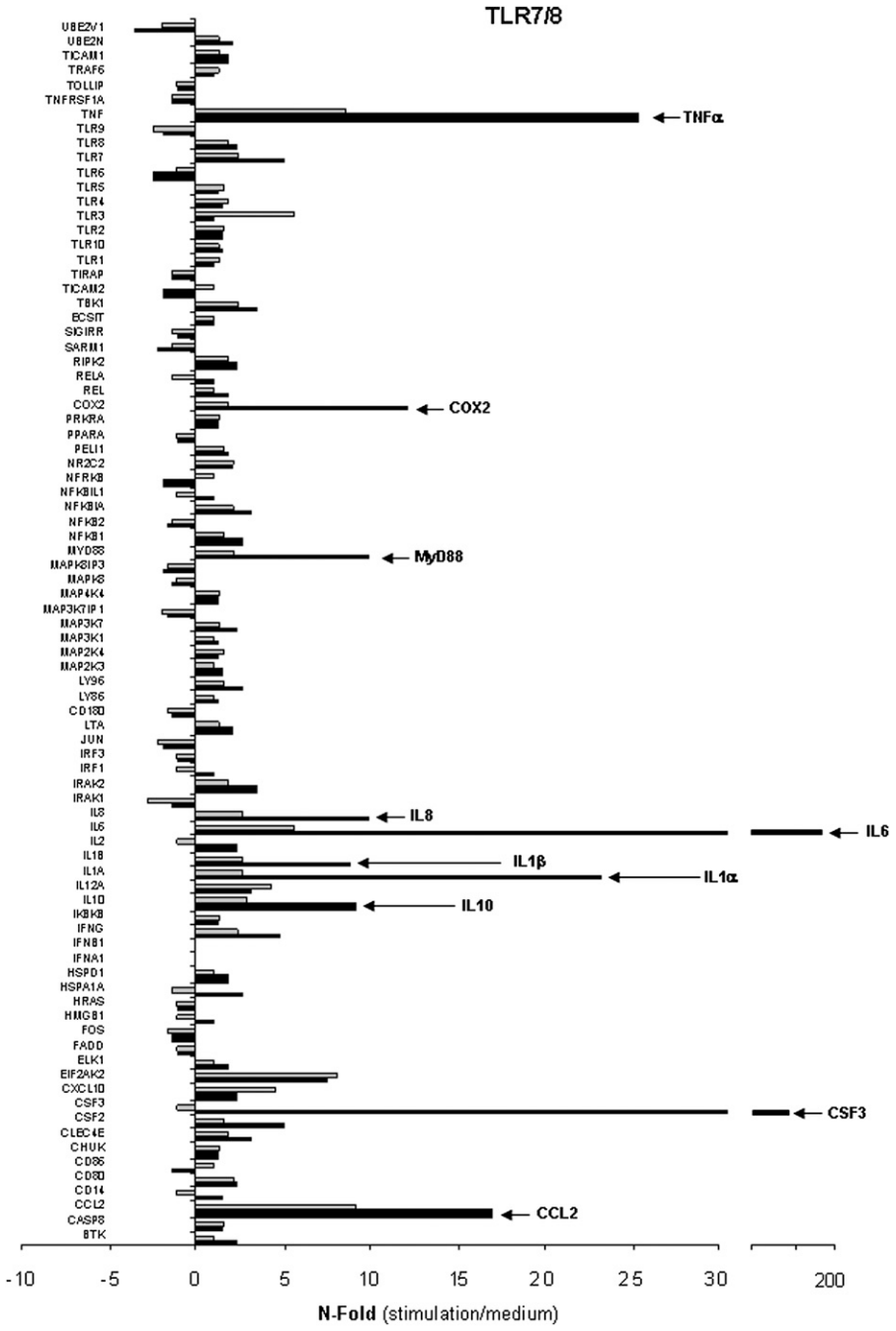

FIGURE 3. TLR signaling pathway mRNA expression in ESN (black bars) and HC (gray bars) for TLR7/8 stimulation with ssRNA40. The expression of 84 genes involved in the TCR signaling pathway has been assessed by real-time quantitative RT-PCR, calculated relative to five housekeeping genes and shown as fold-change expression from the unstimulated sample. The targets showing different expression levels in ESN compared with HC are indicated by specific tags.

Discussion

This study investigates the basal and TLR agonist-stimulated cytokine/chemokine profiles of PBMC from two cohorts of healthy individuals (n = 30 each), one composed of ESN individuals who are resistant to HIV-1 infection following repeated sexual exposure to the virus, and the other consisting of HC who have not been exposed to HIV-1. The data were generated using four TLR agonists: poly I:C for TLR3; LPS for TLR4; imiquimod for TLR7; and ssRNA40 for TLR7/8.

Our initial experiments indicated significant increases in agonist-induced TLR activation of proinflammatory molecules in PBMCs from ESN compared with HC for TLR7-stimulated IL-1β, TLR4 and TLR7 stimulated IL-6, TLR7/8-induced TNF-α, and TLR7-stimulated CCL3. TLR3 and TLR7/8 stimulation also resulted in elevated expression of the CD69 T cell activation marker in ESN. In addition, we observed increases in TLR4-stimulated IL-6 and TNF-α production, as well as TLR7/8-stimualted IFN-γ production. However, we did not observe differences in the percentage or MFIs of CD4+/TLR3+, CD4+/TLR8+ CD14+/TLR4+, and CD14+/TLR8+ cells between ESN and HC before or after TLR stimulation. These data suggest that the observed differences in TLR-responsiveness in ESN and HC individuals is not dependent on a disproportionate cell populations expressing TLRs and that the enhanced response to TLR triggering is involved in the activation of innate responses in ESN. Furthermore, the increased expression of CD69 after TLR triggering, observed in ESN, allows us to hypothesize an association between degree of responsiveness to TLR stimulation and activation of adaptive immune response. All the analyses were performed on PBMCs, because a mixed cell culture more accurately reflects what might happen in vivo; however, as different cell types express a peculiar TLR pattern, we intend to isolate the different cellular subpopulations to identify

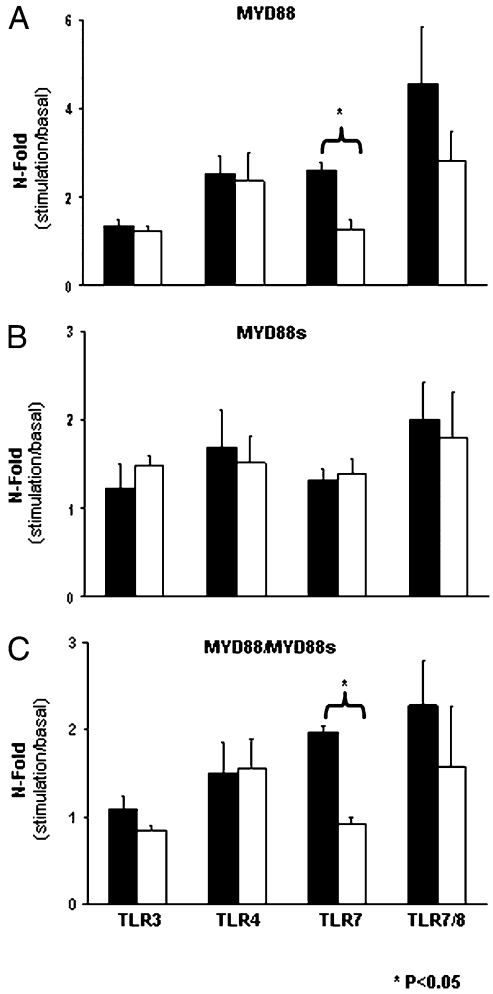

FIGURE 4. MyD88 (*A*) and MyD88s (*B*) expression assessed by real-time quantitative RT-PCR after stimulation of ESN (black bars) and HC (white bars) PBMCs with poly I:C (TLR3), LPS (TLR4), imiquimod (TLR7), and ssRNA40 (TLR7/8) for 6 h, calculated relative to the GAPDH housekeeping gene and shown as fold-change expression from the unstimulated sample. *C*, mRNA levels of MyD88/MyD88s ratio as fold-change expression from the unstimulated sample.

which one is mainly responsible for the increased responsiveness observed in ESN individuals.

Based on the differences that we observed between the TLR-activated cytokine/chemokine profiles of the ESN and HC cohorts, we expanded our comparison using gene array analysis. The array that we used profiles 84 genes that regulate TLR-mediated signal transduction, including TLR adaptors, key mediators, effectors, members of the NFKB, JNK/p38, NF/IL-6, and IRF signaling pathways downstream of TLR signaling. Interestingly, our results indicate differential control of TLR pathways not only between ESN and HC, but also in the TLR3 (MyD88-independent) versus TLR7/8 (MyD88-dependent) signaling pathways. Thus, in ESN individuals following poly I:C stimulation, PBMCs from ESN individuals compared with those from HCs exhibited increased expression of effector molecules (IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-12, TNF-α, CCL2, CSF3, COX2). Based on the results reported in this study and in previous work (17–19), our working hypothesis is that immune activation and inflammation are protective against infection, reducing susceptibility to HIV. This idea is supported by other data showing that $CD4^+/CD25^+$ and $CD8^+/CD38^+/CD45RO$-activated T lymphocytes are augmented in the peripheral blood (17, 18) and mucosal samples (19) of ESN individuals. The ultimate mechanism responsible for this protection is to be better investigated, but it is tempting to speculate that it could be related to a general immune activation mediated by proinflammatory cytokines or by COX2-induced prostaglandin, as well as CSF3-induced leukotrienes known to exert an anti–HIV-1 effects (20, 21). Furthermore, the increased expression of these effector molecules was accompanied by a decrease of some negative regulators of TLR-mediated immune response (NFkBIL1, SIGIRR, SARM) and to upregulation in TANK-binding kinase 1 expression, a kinase involved in TRIF-dependent signaling pathway. Although such modulation of the TLR3 signaling pathway might be associated with increased protective responsiveness in ESN individuals, the contribution of this pathway in resistance to HIV infection is not clear. In fact, TLR3 is not known to be directly involved in HIV-1 genome recognition, but its engagement has been repeatedly associated with the induction of a durable and protective anti–HIV-1 response (22, 23). Thus, it is possible that, following noninfectious HIV-1 exposure, the TLR3-pathway is activated through an indirect mechanism that is translated to a protective immune response in ESN individuals.

This pattern of immune activity was observed using all four TLR agonists studied, but it was more evident following stimulation of the MyD88-dependent TLR and particularly TLR7/8, which are two receptors that are directly activated by HIV-1 (24, 25). Interestingly, the activation of this pathway, in ESN, was characterized by an increased expression of the full-length compared with the short form (MyD88s) of the adaptor protein MyD88, which acts as an intracellular negative regulator of the pathway (16). Thus, it is possible that this imbalance favoring the full-length form results in increased signal transduction, higher responsiveness, and resistance to HIV-1 infection. Furthermore, the observation showing that MyD88 knockout mice failed to control Friend murine leukemia virus because of the loss of the IgG Ab response to the virus (25) raises the possibility that MyD88 can regulate effective antiviral Ab responses. Hence, because ESN individuals display unconventional humoral immune responses that play a role in HIV neutralization (26, 27) and depletion of B cell lines was associated with loss of TLR-induced anti–HIV-1 infection (28), we plan to determine whether the altered MyD88/MyD88s ratio, characterizing ESN subjects, is related to their unusual humoral profile.

In general, our results suggest that following HIV-exposure in ESN individuals, TLR engagement results in the induction of enhanced innate and, possibly, adaptive immune responses that in turn interfere with HIV replication and productive infection. This hypothesis is consistent with recently published observations showing MyD88-dependent immune activation of plasmacytoid dendritic cells (pDCs) and monocytes by HIV-1–encoded uridine-rich ssRNA TLR7/8 ligands, which can be blocked by interfering with TLR signaling (29). Furthermore, HIV-1 RNA activates pDCs through endocytosis mediated by envelope-CD4 interactions, resulting in activation of TLR7 (30). These results raise the possibility that such events can occur in the absence of productive HIV-1 infection, as in the case of the ESN phenotype. Consistent with this idea is the report that engagement of TLR7/8 resulted in inhibition of HIV-1 replication before viral integration, which was accompanied by several changes in the cytokine milieu (28, 31). Thus, it appears that the antiviral effect induced by TLR activation occurs irrespective of productive infection and is activated rapidly after HIV-1 exposure, two features that are consistent with the resistant ESN phenotype.

However, it should be noted that a series of immunopathogenic events can also be induced by noninfectious interactions between HIV-1 gp120 and CD4-expressing pDCs (32, 33). Thus, although the

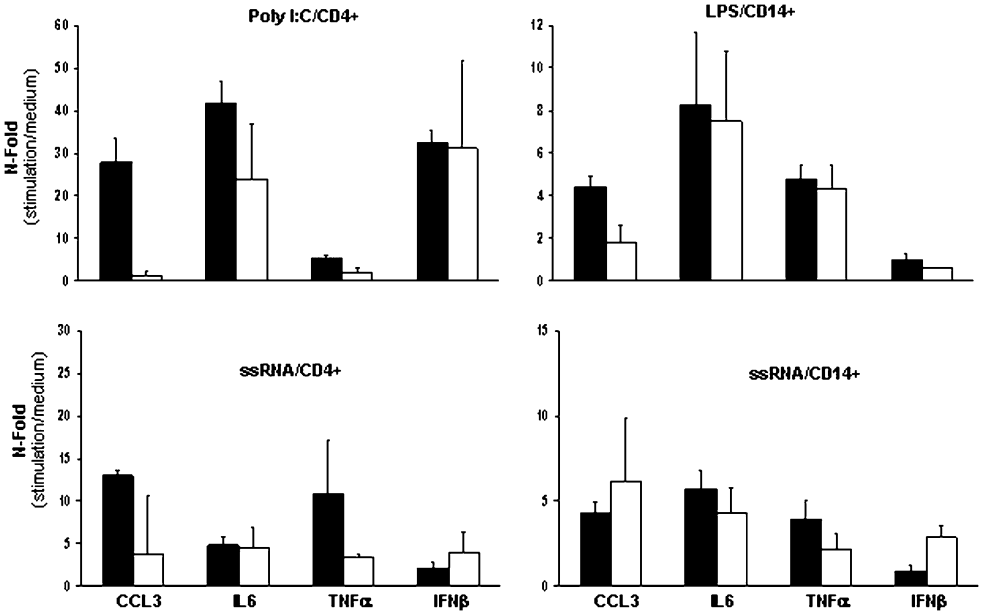

FIGURE 5. Intracellular cytokine production by CD4+ and CD14+ cells in response to poly I:C, LPS, and ssRNA40 stimulation. The graphs show the mean fold-change production (stimulation/medium) ± SE of CCL3, IL-6, TNF-α, and IFN-β CD4+ or CD14+ cells in ESN (black bars) and HC (white bars).

ESN phenotype can be defined by multiple nonproductive infectious exposures to HIV-1, which have been estimated to occur in ~10% of the exposed population (34), we observed certain immunologic similarities in the current study between ESN and HIV+ patients. ESN individuals exhibited increased MyD88 and CD69, resulting from HIV-1 exposure (this study). Similarly, HIV-1 infection results in increased MyD88 (35) and increased CD69 expression (36). In fact, in vitro exposure to RT-deficient AT-2 HIV-1 also induced MyD88 (35) and CD69 (35, 36), which are immunopathogenic markers, even in the absence of productive HIV-1 infection. Therefore, TLR7-mediated innate immune similarities exist resulting from productively noninfectious encounters detected in ESN individuals and those in which PBMCs from uninfected blood bank donors were briefly cultured with AT-2 HIV-1, which binds to CD4 on pDCs and induces a series of immunopathogenic events (32, 33). Studies are in progress to resolve the apparent paradox between these ESN and immunopathogenic innate immune profiles, which might be related to our current preliminary finding that TLR7/8 activated CD14+ PBMCs from ESN individuals produced less IFN-β than did the HC subjects.

Disclosures

The authors have no financial conflicts of interest.

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