The Journal of Immunology

RESEARCH ARTICLE | MAY 15 2003

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J Immunol (2003) 170 (10): 5203–5209.
https://doi.org/10.4049/jimmunol.170.10.5203

Cumulative Toll-Like Receptor Activation in Human Macrophages Treated with Whole Bacteria[1]

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Purified components from bacteria selectively activate Toll-like receptors (TLR), leading to shared and unique responses in innate immune cells. Whole bacteria contain agonists for multiple TLR and induce a common macrophage activation program of transcription. It is not known, however, whether the stimulation of specific TLR by whole bacteria results in differential activation of the innate immune system. We evaluated gene expression data from human macrophages and found a unique gene expression profile induced by Gram-negative bacteria. In contrast, Gram-positive bacteria evoked few specific alterations in gene expression. LPS, a TLR4-specific ligand, was sufficient to elicit the distinct expression profile observed with Gram-negative bacteria. TLR4 activation regulated gene expression by both an IFN-dependent and an IFN-independent mechanism, illustrated by I-TAC and IL-12 p70, respectively. IL-12 p70 was produced by cells in whole blood exposed to Gram-negative bacteria, demonstrating faithful reproduction of the macrophage response in mixed populations of cells and identifying a potential diagnostic marker of infection. Our results show that the macrophage response to bacteria is dominated by the accumulated input from multiple TLR. For macrophages exposed to Gram-negative bacteria, gene expression changes encompass those induced by Gram-positive bacteria plus a distinct TLR4 response. This distinct TLR4 response may provide the basis to diagnose clinical Gram-negative infections. ***The Journal of Immunology*****, 2003, 170: 5203–5209.**

The innate immune system is responsible for identifying infectious pathogens and modulating immune responses. Toll-like receptors (TLR)[5] on the surface of cells of the innate immune system detect specific microbial products (1–3). The triggering of innate immune cells, such as macrophages and dendritic cells, through TLR initiates a well-defined signal transduction cascade that is conserved with components in the *Drosophila* Toll pathway, uses the adapter protein, MyD88, and culminates in the activation of NF-κB and Jun/Fos (1–3).

Purified components from microbes have been widely used to study activation of the innate immune system. Specific components have been associated with activation of particular TLR, such as LPS with TLR4 and peptidoglycan with TLR2 (4–10). Some components differentially activate innate immune cells through specific TLR. Purified bacterial components that activate TLR4 and its novel adapter protein, Mal/TIRAP, elicit characteristic gene expression changes in murine macrophages (11, 12). In human dendritic cells, *Escherichia coli* LPS activates TLR4 and induces IFN-β, IL-12 p35, and inducing protein-10 gene expression (13). In contrast, *Staphylococcus aureus* peptidoglycan activates TLR2 and preferentially induces IL-8 and IL-23 p19 (13).

During infection, humans are challenged by whole microorganisms that possess multiple components capable of activating the innate immune system. Activation of human macrophages by a variety of intact bacteria results in a common, patterned response of gene expression changes (14, 15) This macrophage activation program is comprised of genes that undergo similar changes in expression regardless of the inciting bacterial stimulus (14). The activation program is consistent with the idea that a common signal transduction cascade is induced by TLR (2).

Previous studies of human macrophage responses to whole bacteria did not evaluate whether the innate immune response has the capacity for differential gene regulation through specific TLR (14, 15). It is not known, for example, whether the responses to Gram-negative bacteria and Gram-positive bacteria are dominated by signals induced by an individual TLR. Alternatively, the response to whole organisms may result from the cumulative activation of multiple TLR (16). If TLR-specific responses exist, are they also seen in mixed populations of cells that are likely to encounter pathogens? To address these issues, we analyzed our gene expression data from human macrophages for specific responses to Gram-negative bacteria, Gram-positive bacteria, and TLR agonists. The results show a substantial response to Gram-negative bacteria through activation of TLR4 that is both dependent on and independent of type I IFN.

Materials and Methods

Cells and reagents

The gene expression data for macrophage responses to bacteria and bacterial components are from experiments previously reported (14). To generate macrophages, 2×10^7 human monocytes (Advanced Biotechnologies, Columbia, MD) were cultured in 10 ml of DMEM (Invitrogen, Carlsbad, CA) with 20% FBS (Intergen, Purchase, NY), 10% human serum (Nabi, Boca Raton, FL) and 50 μg/ml gentamicin (Invitrogen) in Primaria T-25 flasks (BD Biosciences, Franklin Lakes, NJ) for 5 days at 37°C in 5% CO_2. On days 5 and 7, half of the medium was removed and replaced with medium lacking FBS. Medium for the cultured macrophages was changed to 5 ml of DMEM with 1% human serum on day 9, 1 h before beginning experiments with bacteria. Medium was changed to 5 ml of 10% human serum 16 h before initiating experiments using bacterial components and

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Received for publication October 15, 2002. Accepted for publication March 3, 2003.

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[1] This work was supported by funds from the National Institutes of Health, Corning, Inc., Affymetrix, Inc., Millennium Pharmaceuticals, Inc., and Bristol-Myers Squibb Co.

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[5] Abbreviations used in this paper: TLR, Toll-like receptor; IFNAR2, IFN-α receptor 2; LTA, lipoteichoic acid; MDP, muramyl dipeptide.

0022-1767/03/$02.00

cytokines. Some follow-up experiments were conducted in microtiter plates. For these experiments, monocytes were cultured for 7 days in 60-mm tissue culture dishes (Falcon 3002; BD Biosciences, Lincoln Park, NJ), and macrophages were removed using PBS containing 5 mM EDTA and 4 mg/ml lidocaine before seeding in Primaria microtiter plates (Falcon) at a density of $2.5 \times 10^4 - 1.0 \times 10^5$ macrophages/well. For whole blood experiments, peripheral blood was harvested in the presence of EDTA, washed twice with PBS, and resuspended to a 2× volume with DMEM containing 10% human serum. Two milliliters of these mixtures was plated per well of a six-well Primaria plate (Falcon). Supernatants were harvested after 24 h of culture with 2×10^7 bacteria. Phlebotomy protocols were approved by the Massachusetts Institute of Technology's institutional review board.

Human cells were activated with a variety of stimuli. Bacteria were thawed from frozen stocks for inoculation. *E. coli* strain sd-4 (ATCC 11143; American Type Culture Collection, Manassas, VA) was grown in Luria-Bertoni medium with streptomycin, and frozen stocks were created from stationary phase cultures as previously described (17). Glycerol stocks from overnight cultures of *S. aureus* strain ISP794, derived from strain 8325, *Listeria monocytogenes* strain EGD, *Salmonella typhi* (Quailes strain), *S. typhimurium* (ATCC 14028), and enterohemorrhagic *E. coli* O157:H7 (EHEC) were prepared from cultures grown in brain-heart infusion broth. A multiplicity of infection of 5–50/1 (bacteria/macrophage) was used. After 4 h flasks were washed with warm HBSS to remove extracellular bacteria, and 5 ml of fresh culture medium was added. Bacterial components were obtained from Sigma-Aldrich (St. Louis, MO): LPS from *E. coli* (L-2880; final concentration, 1 μg/ml), lipoteichoic acid (L-2515) and muramyl dipeptide (A-9519; final concentrations, 10 and 100 μg/ml, respectively), and FMLP (F-3506; final concentration, 100 nM). Human IFN-α was obtained from Schering-Plough (Kenilworth, NJ), IFN-β was purchased from Bioscience International (Camarillo, CA), and IFN-γ was obtained from R&D Systems (Minneapolis, MN). For experiments using bacterial components or cytokines, 5 ml of fresh medium was placed on flasks the night before the experiment. Components or cytokines were added directly to the flasks; these flasks were not washed at 4 h. Anti-IFN-α receptor 2 (anti-IFNAR2) mAb was purchased from Calbiochem (San Diego, CA), and isotype controls were obtained from R&D Systems.

cRNA target preparation and array hybridization

After incubation, supernatants were removed from the flasks and passed through a 0.2-μm filter. Macrophages were lysed with 2 ml of Tri-Reagent (Molecular Research Center, Cincinnati, OH), and total RNA was isolated with chloroform and isopropanol using the manufacturer's protocol, with Eppendorf Phase Lock Gel Heavy tubes (catalog no. 0032 005.152; Brinkmann Instruments, Westbury, NY) to aid isolation of the aqueous phase. First-strand cDNA was synthesized first by denaturing 20 μg of total RNA with 10 μM final T7-poly(dT) primer (Sigma-Genosys, The Woodlands, TX) in a volume of 11 μl for 10 min at 70°C. After cooling on ice and brief centrifugation, 7 μl of a master mix containing first-strand buffer (4 μl), 2 μl of 0.1 M DTT, and 1 μl of 10 mM deoxyribonucleotide triphosphates (all from Invitrogen, Carlsbad, CA) was added. The microcentrifugation tubes were warmed to 37°C for 2 min, 2 μl of Superscript II (Invitrogen) was added, and synthesis proceeded for 1 h at 37°C. Second-strand cDNA was synthesized by adding 130 μl of a master mix containing 91.3 μl of RNase-free water, 30 μl of second-strand buffer, 3 μl of deoxyribonucleotides (10 mM stock), 10 U of DNA ligase, 40 U of DNA polymerase I, and 2 U of RNase H (all from Invitrogen). After incubation for 2 h at 16°C, double-stranded cDNA was purified by phenol-chloroform extraction and precipitation with 20 μg glycogen (Invitrogen) and 75 μl of 7.5 M ammonium acetate. The entire cDNA product, resuspended in 3 μl of RNase-free water, was used for in vitro transcription with biotinylated-CTP and UTP (3.75 μl each; Enzo Diagnostics, Farmingdale, NY) and a Megascript T7 kit (Ambion, Austin, TX). The resulting cRNA was purified using a Qiagen RNAeasy kit (Valencia, CA). Typically, 60–80 μg of cRNA was obtained. Samples yielding <50 μg of cRNA were not used, and the process was repeated from total RNA.

For hybridization, 15 μg of cRNA was fragmented for the Affymetrix protocols, and 10 μg was incubated overnight to HuFL GeneChips. These microarrays contain >6800 unique human genes and established sequence tags. The total number of genes on the array ($n = 7070$) includes multiple representations of some genes. After removal of cRNA, microarrays were washed and sequentially incubated with streptavidin coupled to PE, biotinylated anti-streptavidin, and streptavidin-PE according to Affymetrix protocols. Fluorescence intensities were measured with Affymetrix scanners. Data from samples that had <1500 "present" calls (a measure of scan quality derived from Affymetrix software) were discarded, and cRNA was remade, or in some cases the experiment was repeated from the beginning.

Analysis of gene expression data

To compare the effects of Gram-negative and Gram-positive bacteria, the following analysis was conducted. Fluorescence intensities were normalized to median array intensities for all conditions tested on cells from a single donor and floored at 50, and the fold change was calculated relative to duplicate time zero controls. The average change in gene expression ($\log_2$) was then calculated at each time point for all time courses using Gram-negative organisms (nine time courses). A similar calculation was done for time courses using Gram-positive organisms (four time courses). Two or three time courses per bacterium were conducted on different donors. Genes were considered significantly different between the two average time courses if there was at least a 2-fold difference in expression levels and $p < 0.01$ by Students t test in at least one of the four time points measured (1, 6, 12, and 24 h). To test these selection criteria, datasets were randomized within each time point to create new groups of data regardless

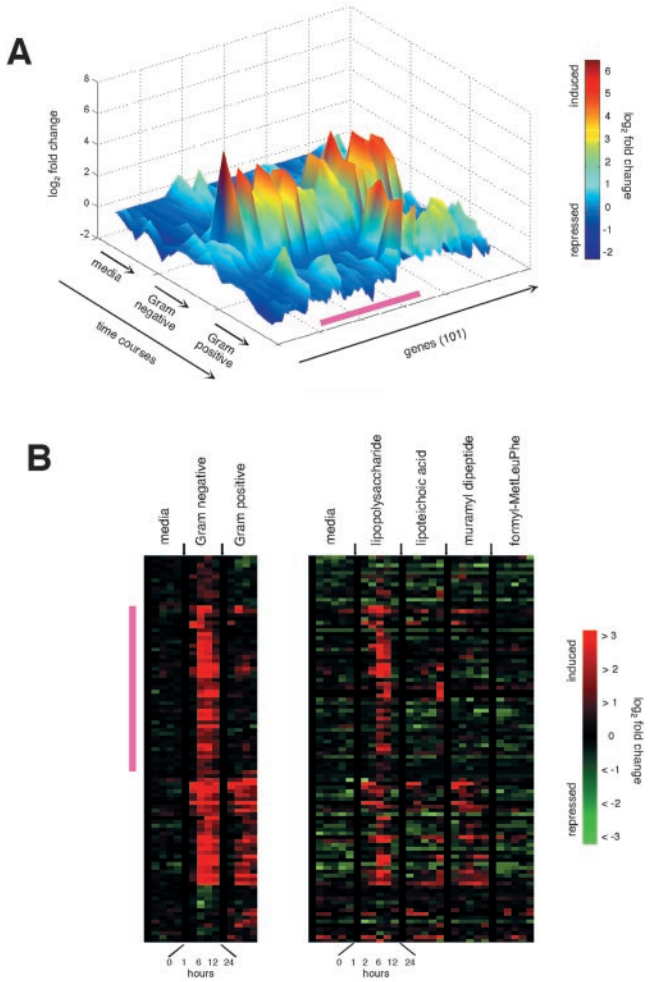

FIGURE 1. Class-specific differences in macrophage gene expression induced by bacteria and purified bacterial components. *A*, Three-dimensional plot of gene expression changes in macrophages exposed to medium, Gram-negative bacteria, or Gram-positive bacteria. The average expression changes of 101 genes are shown for macrophages exposed to Gram-negative bacteria (nine time courses), Gram-positive bacteria (four time courses), or medium (four time courses). Hierarchical clustering identified 43 genes induced most robustly by Gram-negative bacteria (pink bar). The data were clustered using previously described algorithms (18) and are displayed using MatLab (The MathWorks, Natick, MA). *B*, TLR-specific bacterial components recapitulate the differential expression patterns. Expression changes from *A* are represented in two-dimensional format with the TLR4-induced core cluster marked by the pink bar (*left*). Expression changes in macrophages exposed to TLR4 agonist LPS are compared with non-TLR4 agonists, LTA, MDP, and FMLP (*right*).

of the bacteria used to activate the macrophages. Application of the selection criteria to these shuffled datasets failed to identify significant differences in gene expression between the new groups of data.

ELISAs

Supernatants were harvested after 24 h of culture. For I-TAC, the capture Ab (MAB672) and detection antiserum (BAF672) were obtained from R&D Systems and were used at 2 μg/ml (capture) and 100 ng/ml (detection). The ELISA was conducted using the standard R&D Systems protocol for matched Ab pairs. IL-12 p40 was measured using the R&D Systems DuoSet. IL-12 p70 was measured with the R&D Systems High Sensitivity Quantikine Kit or DuoSet, as indicated in the figure legends.

Results

Differential gene expression in human macrophages

We explored the responses of human macrophages to a variety of whole bacteria to determine whether differential signals could be correlated with a particular host TLR. We previously exposed monocyte-derived macrophages to bacteria over a 24-h time course and measured macrophage gene expression using Affymetrix GeneChips (14). The same expression data were reanalyzed to identify macrophage gene expression changes in response to Gram-negative organisms (*E. coli*, enterohemorrhagic *E. coli* O157:H7, *S. typhi*, *S. typhimurium*) compared with those induced by Gram-positive organisms (*S. aureus*, *L. monocytogenes*). This analysis identified 101 genes that were significantly different in macrophages exposed to these two classes of bacteria. The time-course data for these genes were subjected to hierarchical clustering (18) and are displayed in Fig. 1*A*. For comparison, the average gene expression changes of four time courses of untreated macrophages are shown. Most differentially regulated genes were more highly expressed in macrophages exposed to the Gram-negative organisms (Fig. 1*A*). In some instances gene expression differed mainly in the magnitude of the response; Gram-negative organisms induced quantitatively higher expression levels of these genes than did the Gram-positive organisms. In other instances gene expression changes were qualitatively different between these two classes of organisms; Gram-negative bacteria modulated gene expression, and Gram-positive organisms did not (Fig. 1*A*, pink bar). The expression levels of six genes were repressed by Gram-negative organisms (Fig. 1*B*, *left*). Another nine genes appeared to be induced by Gram-positive organisms and were unchanged or repressed by Gram-negative organisms (Fig. 1).

LPS is present in the outer membrane of Gram-negative organisms and distinguishes the two groups of bacteria used in these studies. LPS is a TLR4 agonist, raising the possibility that the pattern of gene expression that differentiated Gram-negative and Gram-positive organisms was due to signaling by TLR4. We tested this possibility by measuring the responses of macrophages treated with LPS and other bacterial components. LPS recapitulated the expression pattern of the 101 genes that were differentially regulated by whole Gram-negative bacteria (Fig. 1*B*, *right*). In particular, expression changes in the genes that best discriminated between macrophages exposed to Gram-negative and Gram-positive organisms were reproduced by LPS, defining a TLR4-induced core cluster of genes. In contrast, muramyl dipeptide (MDP) and lipoteichoic acid (LTA) induced gene expression changes similar to the Gram-positive organisms. These two components, however, failed to induce the cluster of nine genes that appeared specific to exposure to Gram-positive bacteria (Fig. 1*B*). A non-TLR agonist, FMLP, failed to induce the expression changes that were characteristic of either class of bacteria. Together these results identify LPS and TLR4 as molecules that are important for the differential regulation of gene expression by Gram-negative bacteria.

Regulation of gene expression by IFN

Hierarchical clustering of the differentially regulated genes highlighted the TLR4-induced core cluster of genes (Fig. 1, *A* and *B*,

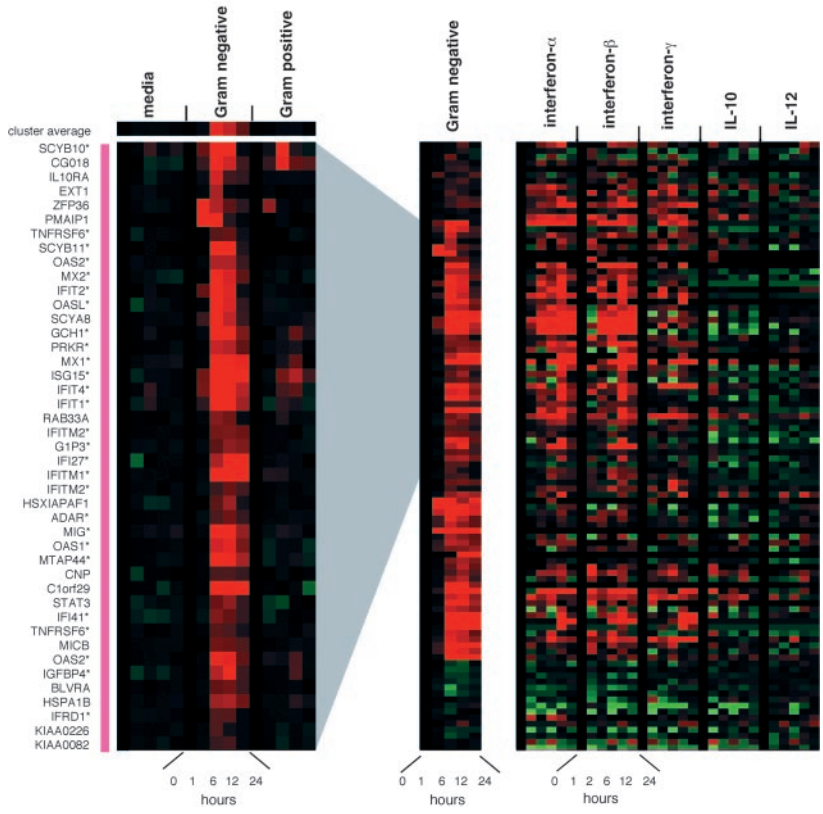

FIGURE 2. Identities of genes in core cluster induced by TLR4 agonists and regulation by IFNs. A core cluster of 43 genes is most different between macrophages stimulated with Gram-negative and Gram-positive bacteria (*left*). NCBI/LocusLink-compatible gene names are on the *left*. Those genes regulated by IFNs, based on PubMed search of gene name, are identified by an asterisk. Gene expression changes in macrophages are induced by IFNs, but not by IL-10 or IL-12 (*right*). The averaged response to Gram-negative bacteria is included for comparison. All IFNs were used at 1000 U/ml. The color scale depicting the magnitude of the expression change is the same as that used in Fig. 1*B*.

pink bar). This cluster contained genes most strongly induced by Gram-negative organisms and LPS that were most different between the two classes of bacteria. Twenty-six of the 43 genes in this cluster are known to be regulated by IFNs (Fig. 2, *left panel*). This association coupled with the fact that many genes of this cluster increased 6 h into the time course suggested that these genes were secondarily regulated by IFNs induced by Gram-negative bacteria.

We next tested whether IFNs were sufficient to elicit the gene expression changes that were observed after macrophages were exposed to Gram-negative bacteria. The expression profiles of macrophages incubated with IFN-α, -β, or -γ demonstrated that IFNs were sufficient to induce the expression of the TLR4-induced core cluster of genes and the majority of changes seen in the other genes (Fig. 2, *right panel*). In contrast, IL-10 and IL-12 failed to induce comparable gene expression changes. These results implicated IFNs in the macrophage gene expression profile associated with Gram-negative bacteria and LPS.

IFN-dependent production of I-TAC after TLR4 activation

To confirm the findings of the array experiments and to investigate the mechanism of gene regulation, one member of the TLR4-induced core cluster was selected as a marker for expression changes specific to Gram-negative bacteria. CXCL11, also known as I-TAC, is a chemokine known to be induced by IFN (19). I-TAC gene expression was closely correlated with exposure of human macrophages to Gram-negative bacteria (Fig. 3*A*). Both Gram-positive and Gram-negative bacteria induced IL-12 p40 gene expression (Fig. 3*A*). Supernatants of macrophages exposed to Gram-negative organisms contained readily detectable levels of I-TAC (Fig. 3*B*). In contrast, Gram-positive organisms failed to elicit I-TAC secretion even though both classes of organisms induced IL-12 p40 (Fig. 3*B*). Consistent with the idea that TLR4 is stimulated by Gram-negative bacteria, I-TAC production was induced by LPS but not by MDP (Fig. 3*C*) or LTA (not shown). However, both LPS and MDP elicited IL-12 p40 secretion (Fig. 3*C*).

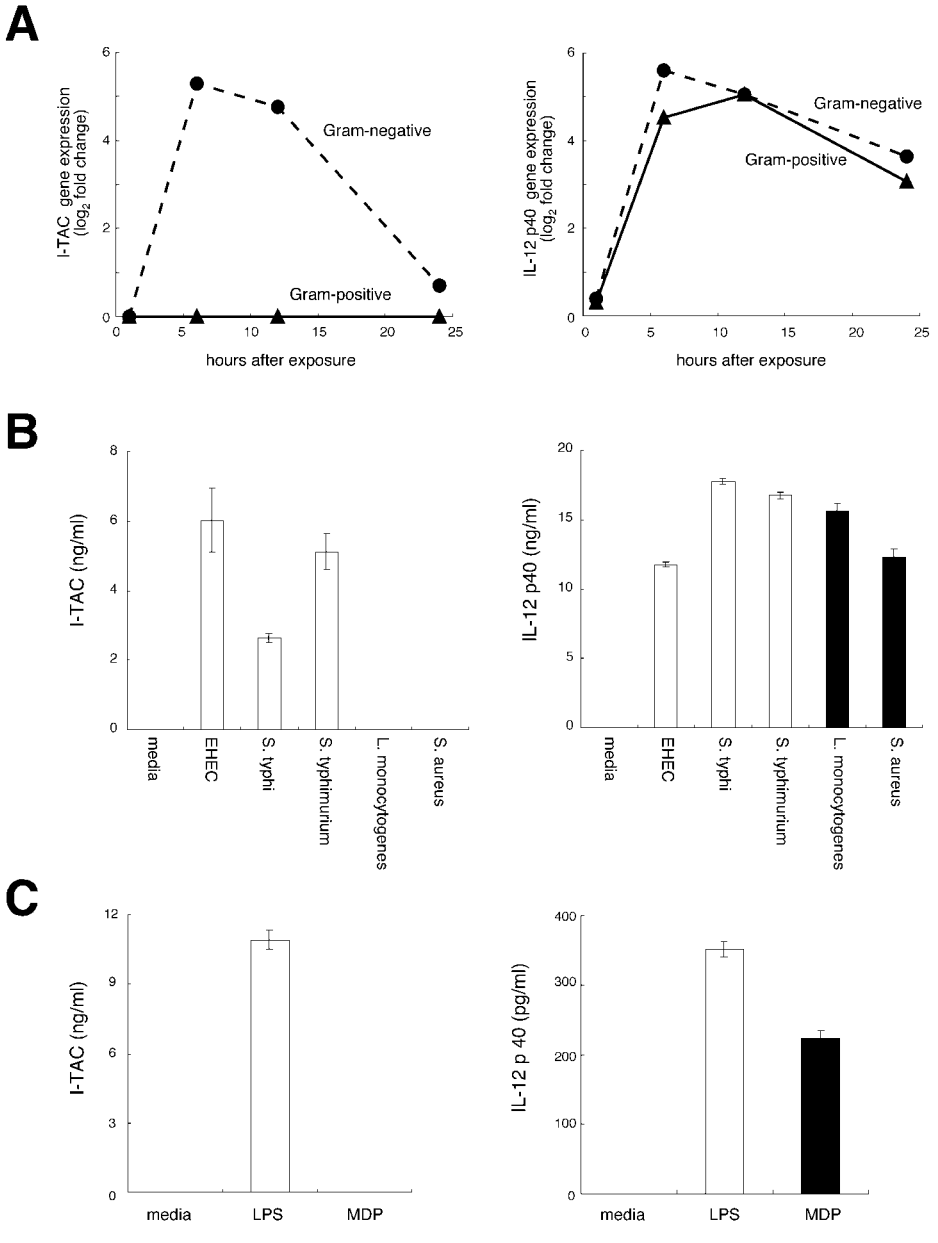

FIGURE 3. I-TAC gene expression and protein production correlate with exposure to Gram-negative bacteria, LPS, and IFN. *A*, Line graph of I-TAC gene expression derived from microarray data. The average $\log_2$ fold changes in I-TAC expression induced by Gram-negative (●) and Gram-positive (▲) bacteria are shown (*left*). Comparable levels of IL-12 p40 expression were induced by both classes of bacteria (*right*). *B*, Protein levels of I-TAC and IL-12 p40 induced by different bacteria. Gram-negative bacteria, but not Gram-positive bacteria, induce protein production of I-TAC (*left*). The mean ± SD of replicate measurements within the ELISA are shown. Supernatants were derived from macrophage cultures in 5 ml of medium at 10^7 cells/25 cm^2, as performed previously (14). Comparable levels of IL-12 p40 were produced by both classes of bacteria (*right*). Class-specific induction of I-TAC was observed in more than five experiments with different donors. *C*, I-TAC production in response to TLR4-specific LPS, but not other TLR agonists. *E. coli* LPS (1 μg/ml) induces I-TAC production, but synthetic MDP (100 μg/ml) does not (*left*). The results shown are the mean ± SD of replicate measurements within the ELISA. Similar patterns of I-TAC production were seen with *Salmonella* LPS and LTA in other experiments. Both LPS and MDP induce IL-12 p40 production (*right*).

Consistent with our array data, we found that IFN-α, -β, and -γ were sufficient to induce I-TAC production (Fig. 4*A*). An mAb that blocks the type I IFN receptor significantly reduced I-TAC production induced by *E. coli* (Fig. 4*B*). The production of IL-12 p40 was not affected by neutralization of IFNAR2 (Fig. 4*B*). We also observed phosphorylation of macrophage STAT1 in response to LPS, consistent with the activation of type I IFN receptors after TLR4 activation (not shown). These findings are consistent with the model in which IFN-β contributes to the differential responses induced by TLR4 stimulation in murine macrophages and human dendritic cells (12, 13, 20, 21).

IFN-independent production of IL-12 p70 after TLR4 activation

The p35 component of bioactive IL-12 p70 was not present in the cluster of genes associated with stimulation by Gram-negative organisms even though it has been associated with LPS stimulation of TLR4 (13, 22–24). Similar to the observations by Re and Strominger (13), inspection of the microarray data showed that p35 gene expression was low and changes were detected in only two of the eight time courses in which macrophages were exposed to Gram-negative bacteria. As a result, p35 failed to meet the cutoff criteria for statistical significance in our analysis. We reasoned that p70 protein in the supernatants from macrophages exposed to Gram-negative and Gram-positive bacteria might be a more reliable alternative assessment of p35 expression. By ELISA of culture supernatants, macrophages exposed to Gram-negative organisms produced substantially more p70 protein than those exposed to Gram-positive organisms (Fig. 5*A*). LPS also stimulated p70 production, although MDP did not (Fig. 5*B*). In contrast to I-TAC, however, IFNs were insufficient to induce p70 production (not shown). Moreover, the anti-IFNAR2 mAb that neutralizes the type I IFN receptor failed to block the production of IL-12 p70 by macrophages exposed to *E. coli* (Fig. 5*C*), identifying an IFN-independent mechanism of gene expression changes after TLR4 activation.

IL-12 p70 production by mixed cell populations

Because I-TAC and IL-12 p70 were produced primarily after macrophages encountered Gram-negative bacteria, these cytokines might be used to discriminate between infections caused by Gram-negative and Gram-positive bacteria. However, a bacterium is likely to encounter a mixed population of inflammatory cells during an infection. Therefore, we tested whether a mixed population of host cells would specifically produce I-TAC and IL-12 p70 after TLR4 activation. One source of inflammatory cells is unfractionated peripheral blood, which contains monocytes and neutrophils that bear TLR (25). When the cellular components of whole blood were incubated with *E. coli*, IL-12 p70 accumulated in the supernatants (Fig. 6). In contrast, *S. aureus* failed to induce p70. Both bacteria induced the production of IL-12 p40 that accumulated in the supernatants (Fig. 6). I-TAC, however, was not detectable in the supernatants of whole blood cells incubated with either bacterium (not shown). Thus, a mixed population of cells is faithful to the specific production of IL-12 p70 after exposure to *E. coli*, similar to purified macrophages.

Discussion

Understanding the innate immune response to infections is critical to understanding inflammation and induction of adaptive immunity. Studies of the innate immune response to purified bacterial components have identified TLR-specific signals and gene expression changes. Yet, it is crucial to understand how the innate immune system responds to whole organisms that cause infections.

Our results demonstrate that the innate immune response to whole bacteria is a consequence of the cumulative activation of TLR, as proposed by Underhill and Ozinsky (16). Gram-negative bacteria activate gene expression changes that encompass those induced by Gram-positive bacteria, defined as the macrophage activation program (14). We now show that Gram-negative bacteria induce an additional set of genes through IFN-dependent and IFN-independent mechanisms. The differences in expression profiles

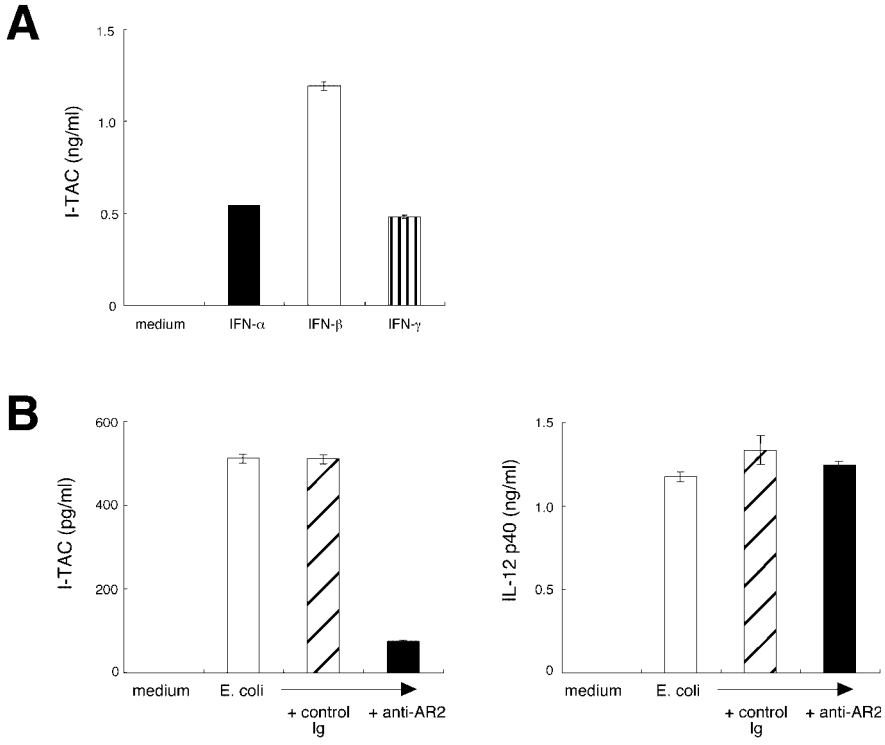

FIGURE 4. Type I IFN is sufficient and necessary for the production of I-TAC by macrophages stimulated with *E. coli*. *A*, I-TAC production in response to different IFNs. Macrophages in microtiter wells (2×10^4/well) were stimulated with 100 U/ml IFN. Results are the mean ± SD of replicate wells in the experiment. Similar patterns of I-TAC production were seen in three experiments with different donors; in two additional experiments IFN-β and IFN-γ induced comparable levels of I-TAC. *B*, An mAb that binds the second chain of type I IFN receptor (IFNAR2) blocks I-TAC production by macrophages stimulated by *E. coli*. An isotype control Ab fails to inhibit I-TAC production (*left*). The IFNAR2 Ab does not interfere with IL-12 p40 production (*right*). Data are the mean ± SD of replicate microtiter wells (2×10^4 cells/well) within an experiment. Similar results were seen in four donors.

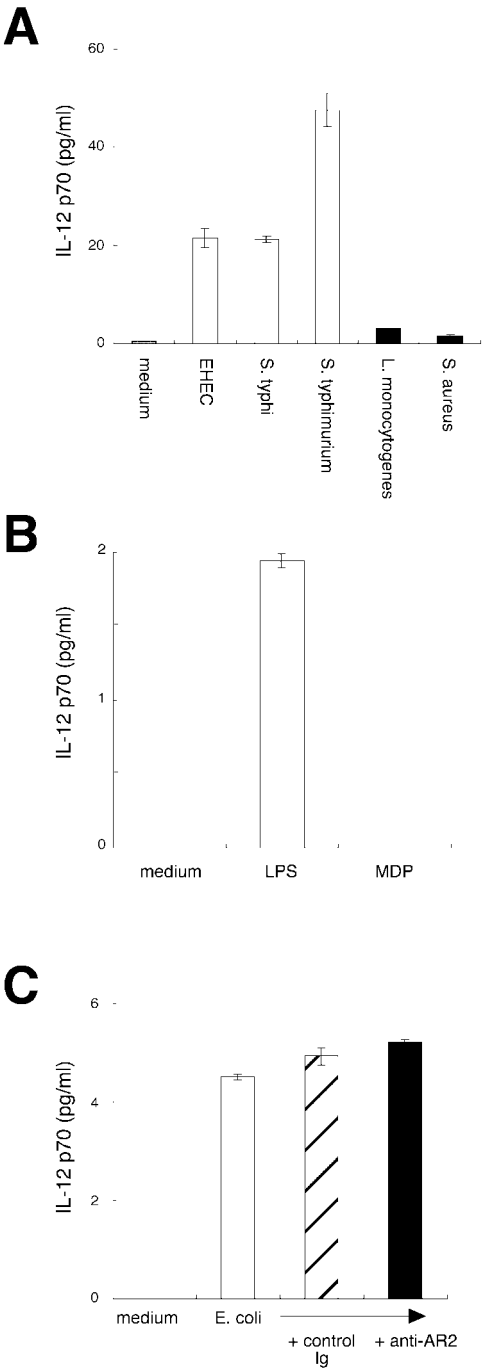

FIGURE 5. Regulation of IL-12 p70 production by human macrophages. *A*, More p70 was produced by macrophages exposed to Gram-negative bacteria. The same supernatants tested for I-TAC and IL-12 p40 displayed in Fig. 3*B* were used. Similar results were seen in three donors. *B*, p70 is produced by macrophages stimulated with LPS, but not by MDP. The same supernatants tested in Fig. 3*C* were used here. Similar results were seen in two other donors tested. *C*, Anti-IFNAR2 fails to inhibit IL-12 p70 production. The same supernatants tested in Fig. 4*B* were used in the high sensitivity ELISA. Similar results were seen in three donors tested. All results in this figure represent the mean ± SD of replicates within the high sensitivity ELISA.

induced by the two classes of bacteria are largely attributable to LPS, consistent with a specific role for TLR4. Thus, the differential response to the class of Gram-negative bacteria can be ascribed to the activation of an additional TLR.

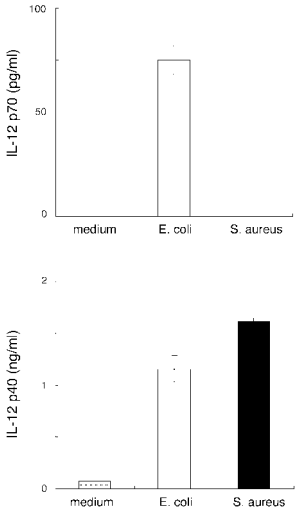

FIGURE 6. IL-12 p70 is produced by whole blood cells exposed to *E. coli*, but not *S. aureus*. Washed whole blood was diluted 1/2 with 10% human serum/DMEM and incubated with 5×10^7 bacteria/ml blood for 24 h. Data are the mean ± SD of replicates within the IL-12 p70 DuoSet ELISA (*top*). Both *E. coli* and *S. aureus* induced production of IL-12 p40 (*bottom*). Similar responses to TLR4 stimulation were seen in five experiments using three donors.

Several lines of evidence argue that the Gram-negative-specific expression profile is activated by TLR4. The gene expression changes induced by Gram-negative organisms were also induced by *E. coli* LPS, a known agonist for TLR4. The TLR4 core cluster contains inducing protein-10 (CXCL10, Fig. 2) and GARG16 (IFIT1, Fig. 2), genes that have been identified in other studies of LPS activation of macrophages and dendritic cells (11–13). I-TAC production, a marker for the expression changes induced by Gram-negative organisms, is dependent on an IFN autocrine feedback loop similar to that observed in murine macrophages stimulated with LPS (12).

We identify an alternative mechanism by which TLR4 regulates differential gene expression. Toshchakov and colleagues (12) have recently demonstrated an IFN-dependent pathway that leads to specific gene expression changes after TLR4 stimulation. Consistent with this mechanism, the majority of the expression profile observed after TLR4 activation was recapitulated by IFNs, and type I IFN was necessary and sufficient for I-TAC production. In contrast, our data show that IL-12 p70 was produced in response to Gram-negative bacteria and *E. coli* LPS, but the regulation of this cytokine was different from that of I-TAC. IFNs were not sufficient, and IFNAR2 was not necessary for IL-12 p70 production after TLR4 stimulation. Thus, IL-12 p70 production after TLR4 activation appears to be independent of IFN.

Although LPS recapitulated the majority of the response induced by Gram-negative bacteria, the gene expression changes induced by LPS were less robust in magnitude and duration. This may be related to differences between free LPS and LPS presented on the surface of whole bacteria. Whole Gram-negative bacteria may deliver quantitatively more LPS to the macrophage. However, it is also likely that whole Gram-negative bacteria synergistically activate multiple TLR compared with purified LPS, thereby eliciting a more potent inflammatory response (26).

While a robust TLR4-specific pattern of expression changes was observed, our analysis identified few genes that were induced uniquely by Gram-positive bacteria. It was possible that Gram-positive organisms would generate a specific pattern of expression changes, because IL-8 has been specifically associated with TLR2

signaling in human monocyte-derived dendritic cells (13). In our datasets, however, IL-8 is a gene in the macrophage activation program that is induced by diverse bacteria (14). Because whole bacteria possess molecules such as peptidoglycan that activate TLR2, IL-8 is not likely to discriminate between the different classes of bacteria studied here. The few expression changes that were induced by Gram-positive bacteria were of low magnitude. Nevertheless, the TLR2-specific agonists, MDP and LTA, were insufficient to induce this small cluster of genes, suggesting that these expression changes are induced by some other molecule(s) in Gram-positive bacteria.

Our data demonstrate that Gram-negative bacteria are more potent than Gram-positive bacteria at inducing IL-12 p70. While others have shown that *S. aureus* can induce this critical regulator of adaptive immune responses (27–29), we have seen little or no p70 production by human macrophages stimulated by Gram-positive organisms and bacterial components that activate TLR2. Titrating the *S. aureus* multiplicity of infection over 5 orders of magnitude and using the same strain of fixed *S. aureus* (Pansorbin; Calbiochem) employed by previous investigators (27–29) failed to enhance p70 production (not shown). While *S. aureus* and *L. monocytogenes* can induce low levels of this cytokine (Fig. 5*A*), the production of p70 appears most robust after TLR4 activation. IL-12 p70 is an important modulator of host defenses. It remains to be determined how differential production of cytokines, including IL-12 p70 and I-TAC, influences the host inflammatory and adaptive immune responses to infection by Gram-negative organisms.

Differential production of IL-12 p70 by cells in whole blood suggests a simple means to discriminate between clinical infections caused by Gram-negative and Gram-positive bacteria. Ultimately, the specificity of candidate diagnostic markers such as I-TAC and IL-12 p70 must be evaluated in clinical specimens to determine their utility as diagnostic markers. In the case of I-TAC, several IFNs induce I-TAC expression, which may limit its specificity for Gram-negative infections. In addition, molecules such as I-TAC might be consumed shortly after production, which could account for the failure to detect I-TAC in supernatants from whole blood cultured with *E. coli*. Further investigation of expression profiles from in vitro infections and ex vivo from patients should help to identify additional candidate diagnostic markers.

Acknowledgments

We thank Christine Ladd for technical assistance.

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