Using distinct molecular signatures of human monocytes and dendritic cells to predict adjuvant activity and pyrogenicity of TLR agonists

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Abstract We present a systematic study that defines molecular profiles of adjuvanticity and pyrogenicity induced by agonists of human Toll-like receptor molecules in vitro. Using P3CSK4, Lipid A and Poly I:C as model adjuvants we show that all three molecules enhance the expansion of IFNγ+/CD4+ T cells from their naïve precursors following priming with allogeneic DC in vitro. In contrast, co-culture of naïve CD4+ T cells with allogeneic monocytes and TLR2/TLR4 agonists only resulted in enhanced T cell proliferation. Distinct APC molecular signatures in response to each TLR agonist underline the dual effect observed on T cell responses. Using protein and gene expression assays, we show that TNF-α and CXCL10 represent DC-restricted molecular signatures of TLR2/TLR4 and TLR3 activation, respectively, in sharp contrast to IL-6 produced by monocytes upon stimulation with P3CSK4 and Lipid A. Furthermore, although all TLR agonists are able to up-regulate proIL-1β specific gene in both cell types, only monocyte activation with Lipid A results in detectable IL-1β release. These molecular profiles, provide a simple screen to select new immune enhancers of human Th1 responses suitable for clinical application.

Keywords Adjuvant screening · Human · Dendritic cell · Monocytes · Th1 cells

Abbreviations

CB  Cord blood
CT  Cholerae toxin
DC  Dendritic cells
IPSE  IL-4-inducing principle of SME
Mo  Monocytes
Osp A  Outer surface lipoprotein A
PI  Propidium iodide
PT  Pertussis toxin
SME  Schistosoma mansoni eggs

Introduction

Toll-like receptors are a family of pathogen recognition receptors, constitutively expressed on mononuclear phagocytes [1]. Up to 13 TLR molecules have been described in mammals, and for most of them a cognate natural ligand has been identified [2]. Thus, TLR2 senses bacterial lipopeptides [3], TLR3 recognizes viral particles [4] and TLR4 binds LPS from gram-negative bacteria [5].

Engagement of TLR on professional APC is required for the development of protective T cell responses in vivo [6]. Indeed, instruction of APC through TLR upon natural infection or administration of live or attenuated vaccines elicits long-term immunity, while exposure of antigen formulations that lack the capacity to signal through the TLR system are poorly immunogenic [7]. These observations have generated a strong demand for the rational design of
synthetic immune enhancers able to complement the activity of subunit vaccines. Given the instructive role of TLR signaling in the development of adaptive immunity, various synthetic analogs of TLRs have been identified and considered for clinical application [8]. As new compounds become available, there is need to develop applicable screening methods predicting adjuvanticity and safety of those molecules [9].

We present here a systematic in vitro study, which defines a correlation between molecular profiles induced by TLR agonists and their adjuvant activity. We studied three putative adjuvants: the synthetic bacterial lipopeptide analog P3CSK4, Lipid A from S. minnesota LPS and the synthetic analog of dsRNA, Poly I:C, each interacting with a different TLR complex. Since the expression pattern of TLR molecules is compartmentalized among functionally different subpopulations of APC [10–12], we compared the activation program induced by those TLR2, TLR4 and TLR3 agonists in DC and Mo, that are considered models of “professional” versus “non-professional” APC, respectively [13].

Our aim is to define cell-restricted molecular signatures induced upon TLR instruction in APC that correlate with the capacity of specific receptor agonists to act as safe immune enhancers for human Th1 responses.

Materials and methods

Chemicals and media

The synthetic bacterial lipopeptide analogs and the CSK4 peptide were produced as endotoxin-free compounds by EMC microcollections (Tübingen, D), dissolved in PBS and solubilized by sonication prior to use. Poly I:C was from Sigma (Buchs, CH). Lipid A from S. minnesota LPS, SME and IPSE were prepared as described elsewhere [14, 15]. Lip-OspA and MDP-OspA were kindly provided by M. Simons (Max Planck Institute for Immunobiology, Freiburg, D). PT and CT were from Calbiochem (San Diego, CA, USA). CFSE (5(and6)carboxyfluorescein diacetate succinimidyl ester) was provided by Molecular Probes (Eugene, OR, USA). Complete RPMI 1640 contained 1% sodium pyruvate, non-essential amino acids, glutamax and kanamycin and 0.1% β mercaptoethanol (Invitrogen, Barcelona, E). For cell culture, either 5% human AB serum (RPMI-HS; SRK, Bern, CH) or 10% heat inactivated FCS (RPMI-FCS; Invitrogen, Barcelona, E) was added.

Mo and DC cultures

Blood specimens from healthy donors were kindly provided by SRK (Bern, Switzerland), Dr. J. Esteves (Hospital Egaz Moniz, Lisbon, P) and Dr. T. Ghielmetti (University Hospital, Bern, CH) upon donor’s informed consent. PBMC were separated by Histopaque1077 gradient centrifugation and CD14+ Mo isolated by positive sorting using anti-CD14-conjugated microbeads (Miltenyi Biotech, Bergisch Gladbach, D). Mo-derived DC were generated from CD14+ Mo upon culture in RPMI-FCS medium supplemented with 50 ng/ml GM-CSF and 100 ng/ml IL-4 (RnD systems, Abingdon, UK) for 5 days. For activation, cells where stimulated with 100 ng/ml of P3CSK4 or equimolar (69 nM) concentrations of CSK4 peptide or any other lipopeptide analog, 100 ng/ml of Poly I:C or Lipid A, if not otherwise specified. Lip-OspA, MDP-OspA CT, PT, SMEA, IPSE were used at 1 µg/ml. Release of cytokines and chemokines in culture supernatants and cell phenotypes were assessed after 16 h of stimulation by specific ELISA assays and FACS analysis. Gene expression was measured after 5 h of activation by qRT-PCR.

ELISA assays

Cytokines and chemokines released in cell culture supernatants were determined by quantitative ELISA assays. Specific antibody pairs and standards for TNF-α, IL-6, CCL2, and CXCL10 were from BD PharMingen (San Diego, CA, USA), those specific for IL-1β were provided by RnD Systems (Abingdon, UK). Samples were read in a SpectraMax190 plate reader and analyzed using SOFTmax software (Bucher biotech AG, Basel, CH). Data are presented as mean results from different donors ± SD. Student’s t test was used to compare pared samples.

FACS analysis

Cell phenotype was evaluated by cell surface staining. FITC-conjugated mouse antibodies to human CD14, CD1a (BD PharMingen, San Diego, CA, USA) and TLR2 (eBioscience, San Diego, CA, USA) or isotype controls antibodies, were added to the cell pellet and incubated for 30 min at 4°C. Samples were then washed, diluted in PBS containing PI to label dead cells, and analyzed on a FACSCalibur (BD Biosciences, San Jose, CA, USA) using CellQuestPro (BD Biosciences, San Jose, CA, USA) software. PT+ cells never exceeded 10% of the total population.

Quantitative gene expression assays

Total cellular mRNA was isolated using RNAeasy kit (Qiagen, Valencia, CA, USA) following manufacturer’s instruction. Purified mRNA was reverse transcribed into cDNA using the TaqMan reverse transcription reagents from Applied Biosystem (Foster City, CA, USA). Expression
of selected genes was determined by qRT-PCR with specific primers and probes provided by Applied Biosystem (Foster City, CA, USA), according to provider’s guidelines. The following assays-on-demand were used: CCL3/Hs00234142_m1; CCL5/Hs00174575_m1; CCL20/Hs00171125_m1; CCL22/Hs00171080_m1; CX3CL1/Hs00171086_m1; CXCL10/Hs00171042_m1; EBI3/Hs_00194957_m1; IL27p28/Hs00377366_m1; IFNB1/Hs00277188_s1; IFNG/Hs00174143_m1; IL1B/Hs00174097_m1; IL4/Hs00174122_m1; IL7/Hs00174202_m1; IL10/Hs00174086_m1; IL12A/Hs00168405_m1; IL12B/Hs00233688_m1; IL15/Hs00542571_m1; IL18/Hs00155517_m1; IL23/00372324_m1; TGFB1/Hs00171257_m1; TNFα/Hs00174128_m1; TLR1/00413978_m1; TLR2/Hs00610101_m1; TLR3/Hs00152933_m1; TLR4/Hs00152939_m1; TLR6/00271977_s1. Samples were analyzed using ABI Prism 7700 or 7900HT Sequence Detection Systems. Average Cycle threshold (Ct) numbers of triplicate assays were derived from the exponential phase of PCR amplification. For each gene a ΔCt value was calculated by subtracting the Ct obtained for the reference 18S gene. The fold increase in genes expression was calculated as Relative Quantity (R. Q.) of gene “x” in the cell population A as compared to a calibrator cell population B derived by 2^ΔΔCt where ΔΔCt = ΔCtA−ΔCtB.

T cell activation assays

CB samples were collected after vaginal birth in heparinized tubes, upon donors’ formal consent. To assess T cells proliferation, purified CB-PBMC were labeled with 1.0 μM CFSE for 8 min at 37°C, extensively washed, diluted in RPMI-HS medium and cultured at 4 × 10^5 per ml with CSK_4, P_2CSK_4, Lipid A or Poly I:C in the presence or absence of allogeneic DC (10^5/ml) or CD14^+ Mo (10^5–4 × 10^5 per ml), prepared from the same donor. After 6 days, cells were stained with PE-conjugated anti-CD3 and APC-conjugated anti-CD4 antibodies, diluted in PBS containing PI, and analyzed on a FACSCalibur. Proliferative responses were quantified by measuring the loss of CFSE staining on gated CD3^+CD4^+ lymphocytes. To assess T cell differentiation, co-cultures were set using unlabeled CB-PBMC. After 6 days cells were stimulated for 8 h with 50 ng/ml PMA and 0.5 μg/ml Ionomycin (Sigma, Buchs, CH), adding Brefeldin A at 10 μg/ml for the least 4 h. Cells were then collected and surface stained with PerCP-conjugated anti-CD3 and APC-conjugated anti-CD4 mAbs (BD PharMingen, San Diego, CA, USA). After washing, cells were fixed in 2% Paraformaldehyde and permeabilized with 1% Saponin (Sigma, Buchs, CH). Intracellular cytokines were finally detected by staining with PE-conjugated anti-IL4 and FITC-conjugated anti-IFNγ mAbs (BD PharMingen, San Diego, CA, USA) in the presence of mouse IgG at 300 μg/ml (Jakson Laboratories, Bar Harbor, ME, USA). After final washing samples were analyzed on a FACSCalibur by acquiring a total of 2 × 10^4 gated CD3^+CD4^+ lymphocytes.

Results

Effect of P_2CSK_4, Lipid A and Poly I:C on T cell activation

We first selected three different molecules known to act as specific TLR ligands: the synthetic bacterial lipopeptide analog P_2CSK_4 which is an agonist for TLR2/TLR1 heterodimers, Lipid A from *S. minnesota* LPS that binds to TLR4, and Poly I:C which depends on TLR3 for cell activation [3–5]. In addition, we used the biologically inactive CSK_4 derivative, as negative control of stimulation [16, 17].

We then compared the effect of these three molecules on naive T cell activation in vitro. To this aim, we first cultured CFSE-labeled CB-PBMC with CD14^+ Mo or DC, both prepared from the peripheral blood of the same allogeneic adult individual, in the presence or absence of TLR agonist, and assessed T cell proliferation by the loss of CFSE dye in proliferating CD3^+CD4^+ lymphocytes. In cultures devoid of allogeneic APC, ≤9.10% of T cells were CFSE^+ and represented the level of non-specific proliferation in vitro; similar results were obtained with Mo co-cultures in the presence of CSK_4 and Poly I:C (Fig. 1a). In contrast, stimulation of Mo co-cultures with P_2CSK_4 and Lipid A resulted in up to sevenfold increase in T cell proliferation (Fig. 1a). Allogeneic DC consistently induced T cell proliferation in the presence of CSK_4, and all TLR agonists enhanced this effect (Fig. 1a).

We next examined the differentiation of naive lymphocytes to effector Th1 or Th2 cells, by assessing the expansion of IFN-γ versus IL-4 producing T cells, in CB-PBMC/APC co-cultures with or without TLR agonist [18]. In the absence of APC, cytokine-producing cells represented ≤0.95% of the total CD3^+CD4^+ T lymphocytes, irrespective of the TLR agonist used for stimulation (Fig. 1b, top panels). Upon co-culture with allogeneic CD14^+ Mo and TLR agonist the frequency of cytokine-producing T cells was ≤1.99% (Fig. 1b, central panels). In contrast, in cultures stimulated with allogeneic DC, all three TLR agonists promoted a consistent expansion of IFN-γ producing T lymphocytes over IL-4 secreting cells (Fig. 1b, lower panels). In P_2CSK_4-conditioned cultures, up to 18.9% of the CD3^+CD4^+ T cells produced IFN-γ, compared to 7.71 and 6.04% upon Lipid A and Poly I:C stimulation, respectively (Fig. 1b, lower panels). Mean results of independent experiments failed to demonstrate T cell differentiation in CB-PBMC/APC co-cultures with CSK_4 control peptide, irrespective of the type of APC used for stimulation (Fig. 1c).
The capacity of each TLR agonist to enhance T cell proliferation and differentiation appears to be conditioned by a high inter-individual variability (Fig. 1a, c), reflecting the known TLR polymorphisms or age-associated defects in human TLR function [19–22]. In spite of these variations, however, the adjuvant activity of each TLR agonist depends on the type of APC inducing T cell activation.

IL-6 and TNF-α are cell-restricted molecular signatures of TLR2/4 stimulation

We next asked whether Mo and DC similarly respond to the same TLR agonists. To this aim, we stimulated CD14+ cells, and Mo undergoing DC differentiation in vitro (according to ref. [23]) with P3CSK4, Lipid A and Poly I:C and measured the secretion of IL-6 and TNF-α cytokines, known to be induced by TLR engagement in human APC [11, 24]. Treatment with the CSK4 control peptide did not stimulate cytokines release at any time from day 0 to day 5 of culture (Fig. 2). Similar results were obtained upon stimulation of CD14+ cells with Poly I:C (Fig. 2, day 0). Monocyte, however, produced high amounts of IL-6 in response to TLR2 and TLR4 agonists, with poor detection of TNF-α (Fig. 2 day 0). Mo differentiated by culture with GM-CSF/IL-4 and then stimulated with TLR2 and TLR4 agonists, produced sevenfold less IL-6 and threefolds more TNF-α (Fig. 2 days 1 and 3). At day 5 of differentiation these cells responded to P3CSK4 and Lipid A by secreting even higher amount of TNF-α, but poorly detectable IL-6 (Fig. 2 day 5). At this time point the culture contained fully differentiated DC, as indicated by their increased cell size, lack of CD14 and de novo expression of CD1a, as compared to Mo cultures assessed from day 0 to day 3 of differentiation (Fig. 2c). The cell-restricted cytokine profile was also observed by determining the kinetic of IL-6 and TNF-α release from TLR-stimulated Mo and terminally differentiated DC from 30 min to 22 h upon stimulation (Fig. 3). Notably, TLR3 engagement by Poly I:C resulted in poor cytokine secretion in either cell type (Figs. 2, 3).

These results indicate that human Mo and DC respond distinctly to TLR2/TLR4 stimulation in vitro, and define IL-6 and TNF-α as cell-restricted molecular signatures of activation, by these two cell types, respectively.

CXCL10 signature in response to TLR3 triggering

We then asked whether diverse patterns of TLR expression in Mo and DC could explain the time-dependent switch in cytokines secretion observed above. By using specific monoclonal antibodies to monitor TLR2 expression, we found a higher cell surface expression of the specific protein on freshly purified CD14+ cells as compared to DC (Fig. 4a). Consistent with this, culture of Mo in medium promoting DC differentiation resulted in a time-dependent reduction of TLR2 specific mRNA (Fig. 4b). DC differentiation in vitro

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**Fig. 1** Effect of TLR2, TLR4 and TLR3 agonists on naive T cell activation. a CFSE-labeled CB-PBMC were cultured alone (white bars), or stimulated with allogeneic CD14+ Mo (hatched bars) or Mo-derived DC (black bars), in the presence of CSK4, P3CSK4, Lipid A, or Poly I:C, as indicated in the bottom line. T cell proliferation was assessed by measuring the frequency of CFSEdim/CD3+CD4+ lymphocytes by FACS analysis. b Unlabeled CB-PBMC were stimulated as in a and assed for T cell differentiation by detection of intracellular IL-4 versus IFN-γ on gated CD3+CD4+ lymphocytes; numbers indicate the percentage of cytokine-producing T cells. c Frequency of CD3+CD4+ IFN-γ+ cells upon priming with allogeneic CD14+ Mo (white bars) or DC (black bars) in the presence of CSK4, P3CSK4, Lipid A or Poly I:C. Data represent mean results ± SD obtained from four independent experiments.
was also characterized by a fourfold reduction of TLR4 gene transcription and a decreased transcription of TLR1 specific gene (Fig. 4b). In sharp contrast, mRNA encoding TLR3, was not detected in freshly isolated Mo, and first detected after 3 days of culture in medium containing GM-CSF/IL-4, rising to a fourfolds higher magnitude at day 5 (Fig. 4b).

We then studied the biological consequences of the differential TLR expressions, by monitoring the time-dependent secretion of CCL2 and CXCL10 chemokines which are known to depend, at least in part, on TRIF signaling downstream from TLR4 and TLR3, particularly in DC [25, 26]. CCL2 was detected in culture supernatants of Mo undergoing DC differentiation in vitro stimulated with P3CSK4 and Lipid A, at any time point tested, although more consistently in the presence of the TLR2 agonists (Fig. 4d). Strikingly, detection of both CCL2 and CXCL10 upon cell stimulation with Poly I:C was detected only on days 3 and 5 of culture (Fig. 4c, d). Remarkably, CXCL10 release uniquely depended on TLR3 signaling, in sharp contrast to CCL2, which was released upon stimulation with all TLR agonists.

Altogether, our study confirms and extends previous findings [11, 27], suggesting that quantitative and qualitative differences in TLR expression on Mo and DC results in a cell-restricted activation program in response to the cognate ligand. In addition, CXCL10 represents a specific signature of TLR3 signaling, at least in our settings.

Cell-restricted and agonist-specific signatures in response to TLR2, TLR4 and TLR3 activation

To obtain a broader pattern of molecular profiles induced by TLR engagement in functionally different APC, we have extended our analysis to the expression of 21 genes, encoding soluble factors involved in inflammation (TNF-α, IFN-γ, IL-4, IL-7, IL-12p40, IL-12p35, IL-15, IL-23p19, IL-27p28, EBI3, CXCL10, CX3CL1, CCL5, CCL22) and immune regulation (TGF-β1, IL-10). Quantitative gene expression analysis was assessed in freshly purified CD14+ Mo and DC stimulated with P3CSK4, Lipid A or Poly I:C and relative gene expression values were calculated as compared to control cultures stimulated in the presence of PBS. Results obtained with three different donors are reported in Table 1. Because of the inter-individual variability, only gene expression profiles observed at similar magnitude in all donors tested are presented and discussed here below.
Stimulation with P3CSK4 resulted in the up-regulation of 9/21 genes in Mo and 10/21 genes in DC. Transcription of TNF-α and IL-12p35 specific genes was significantly increased only in DC, in contrast to IL-10 mRNA which was found more significantly increased in CD14+ Mo [24]. Moreover, the expression of eight genes, i.e. IL-1β, IL-7, IL-12p40, IL-23, IL-27p28, EBI3, CCL3, CCL5 and CCL20 was increased in both cell types. In none of the two cell types, P3CSK4 induced up-regulation of IL-4, TGF-β1 and CCL22 specific mRNA (Table 1, left panels).

Upon stimulation with Lipid A, 6/21 genes were significantly up-regulated in CD14+ Mo, and only 5/21 in DC. Increased transcription of TNF-α was measured only in DC, while increased IL-1β, IL-23p19 and CCL20 gene expression was detected in both cell types. Similarly to the TLR2 agonist just described, Lipid A stimulation never resulted in increased transcription of IL-4, TGF-β1 and CCL22 specific genes (Table 1, central panels).

Stimulation with Poly I:C induced the up-regulation of 10/21 genes in DC (TNF-α, IFN-β1, IL-1β, CCL3, IFN-γ, IL-12p35, IL-27p28, CXCL10, CCL5), but had no consistent effects on Mo gene expression. For a total of eight genes, including IL-4, IL-10, IL-18, IL-23p19, EBI3, CCL22, CX3CL1 and TGF-β1 no increased expression was observed in DC (Table 1, right panels).

Three important findings derived from the gene profiles described above. First, TNF-α gene expression is increased in DC upon stimulation with all TLR agonists under investigation, although TNF-α protein release does not represent a consistent signature of DC activation with Poly I:C (Fig. 2, ref. [12]). Second, the transcription of few genes is associated to specific TLR agonists. In particular, IL-23p19 specific mRNA, is up-regulated by both P3CSK4 and Lipid A, but not by Poly I:C. In contrast, CXCL10-specific gene is highly increased in DC only upon Poly I:C stimulation, thus confirming our protein expression data (Fig. 3). Third, all TLR agonists under investigation are able to increase the expression of IL-1β gene, irrespective of the responding cell type.

IL-1β protein release characterizes Mo activation by Lipid A

At a first sight, the detection of IL-1β specific gene transcription in Mo and DC upon stimulation with any TLR agonist under investigation was puzzling. IL-1β is produced as a cytoplasmic precursor that needs to be first processed and then secreted to act as endogenous pyrogen in vivo [28]. Indeed, biologically active IL-1β is responsible for the endotoxin shock induced by exposure to LPS and
Lipid A in vivo [29, 30]. In contrast, endotoxin-free preparations of P3CSK4 and Poly I:C are known to act as safe adjuvants when injected into animals [31, 32].

In order to understand the apparent discrepancy between the capacity of P3CSK4 and Poly I:C to induce IL-1β gene transcription, while lacking in vivo toxicity, we asked whether signaling through TLR2, 4 and 3 in Mo undergoing DC differentiation in vitro results in IL-1β protein secretion. To this aim we used the same cell cultures setting described in Fig. 2 and measured release of IL-1β in cell culture supernatants by ELISA. Remarkably, up to 135 pg/ml of IL-1β was only detected in culture supernatants of Mo stimulated with Lipid A, while any other condition of stimulation, irrespective of the cell differentiation stage, scored negative (Fig. 5a). We also found that the production of IL-1β by Mo in response to Lipid A was inversely correlated to the concentration of this specific TLR4 agonist, with maximum release of IL-1β at doses of Lipid A below 10 ng/ml.

Altogether, these results indicate that IL-1β production characterizes the activation of primary human Mo by the TLR4 agonist Lipid A and defines this cytokine as a molecular signature of pyrogenicity in vitro.

Applicability of molecular signatures for compounds selection

The definition of cell-restricted molecular signatures predicting adjuvanticity and safety of immune stimulatory factors can facilitate the large-scale screening of chemical libraries for the selection of putative adjuvants suitable for human vaccines.

In order to determine the applicability of our findings for compounds selection, we first determined the correlation between TNF-α release by stimulated DC and expansion of IFNγ+/CD4+ T cells in MLR cultures condition by various synthetic and natural agonists of TLR2, including analogs of the P3CSK4 lipopeptide and recombinant Lip-OspA lipoprotein from Borrelia burgdorferi. Results presented in Fig. 6a, show a significative positive correlation between those two parameters ($R^2 = 0.9023; P = 0.0093$). Thus, we assume that the amount of TNF-α produced in vitro is a measure of the adjuvant activity of each single compound tested.

Next, we studied the in vitro immune stimulatory effects of a larger chemical library, including all TLR-2 agonists tested above as well as pathogens molecules, i.e. CT from
Table 1 Expression of cytokines and chemokines specific genes in CD14+ cells and monocyte-derived DC upon stimulation with P3CSK4, Lipid A and Poly I:C

|                | P3CSK4 | Lipid A | Poly I:C |
|----------------|--------|---------|----------|
|                | Donor 1 | Donor 2 | Donor 3  | Donor 1 | Donor 2 | Donor 3  | Donor 1 | Donor 2 | Donor 3  |
|                | Mo | DC | Mo | DC | Mo | DC | Mo | DC | Mo | DC |
| TNF-α          | 3.2 | 37.6 | 1.0 | 22.4 | 4.9 | 17.7 | 1.0 | 4.1 | 1.0 | 55.7 | 1.6 | 3.8 |
| IFN-β1         | 3.1 | 6.0 | 1.9 | 5.3 | 1.0 | 1.0 | 2.7 | 5.8 | 1.4 | 918.6 | 1.3 | 1.6 |
| IL-1β          | 28.2 | 213.0 | 88.7 | 73.3 | 11.9 | 139.6 | 9.0 | 17.9 | 27.6 | 376.0 | 8.4 | 20.1 |
| IL-18          | 1.2 | 2.5 | 4.2 | 1.9 | 1.7 | 2.0 | 1.0 | 1.2 | 2.7 | 3.2 | 1.0 | 1.2 |
| CCL3           | 11.7 | 18.5 | 13.6 | 20.9 | 6.5 | 8.7 | 4.5 | 4.4 | 8.2 | 27.9 | 5.8 | 2.8 |
| CCL20          | 101.9 | 277.9 | 209.9 | 84.6 | 19.3 | 39.5 | 17.8 | 28.6 | 45.9 | 118.6 | 12.5 | 4.5 |
| IFN-γ          | 1.0 | 1.0 | 14.6 | 1.0 | 1.0 | 1.0 | 843.1 | 1.0 | 53.9 | 10.8 | 1.0 | 71.7 |
| IL-4           | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| IL-7           | 12.6 | 12.5 | 12.9 | 10.9 | 8.1 | 2.3 | 3.3 | 4.9 | 10.7 | 9.9 | 9.4 | 1.8 |
| IL-12p40       | 55.7 | 149.5 | 130.9 | 134.8 | 50.7 | 36.2 | 14.4 | 10.3 | 156.3 | 267.5 | 28.5 | 2.7 |
| IL-12p35       | 1.5 | 7,582 | 1.0 | 158.3 | 5.8 | 11.1 | 2.4 | 311.7 | 3.3 | 258 | 14.2 | 1.2 |
| IL-15          | 3,182 | 12.6 | 1.0 | 2.8 | 5.7 | 2.5 | 1,502.0 | 14.1 | 1.0 | 1.3 | 31.0 | 7.1 |
| IL-23p19       | 166.1 | 392.5 | 44.5 | 228.0 | 343.3 | 174.0 | 25.2 | 39.9 | 32.7 | 12.2 | 83.4 | 534.9 |
| IL-27p28       | 5.4 | 1,906 | 4.3 | 753.0 | 6.2 | 52.9 | 1.8 | 661.6 | 26.3 | 45.4 | 122.2 | 1.0 |
| EBI3           | 43.5 | 9.1 | 7.6 | 7.3 | 13.8 | 13.1 | 5.2 | 1.9 | 9.0 | 1.0 | 8.9 | 33.2 |
| CXCL10         | 1.0 | 4.9 | 1.0 | 1.0 | 28.8 | 1.0 | 1.0 | 56.9 | 1.0 | 7.0 | 16.5 | 1.0 |
| CXCL11         | 1.0 | 4.4 | 1.0 | 3.5 | 1.0 | 1.6 | 1.0 | 3.4 | 1.0 | 2.6 | 1.3 | 1.0 |
| CCL5           | 5.2 | 7.9 | 5.8 | 21.3 | 4.0 | 3.4 | 3.9 | 2.9 | 20.1 | 141.6 | 9.1 | 1.9 |
| CCL22          | 1.0 | 1.2 | 1.0 | 1.8 | 1.0 | 1.3 | 1.0 | 1.0 | 1.1 | 1.0 | 1.0 | 1.0 |

Data are expressed in Relative Quantity (R.Q.) as compared to cell cultures stimulated in the presence of PBS (R.Q. = 1.0). A threshold of 1.0 was assigned to any gene with a R.Q. < 1.0. Values ≥3.0 are considered indicative of enhanced gene expression and are shown in bold. Results obtained from three independent donors are shown.

Discussion

Vibrio cholerae, PT from Bordetella pertussis, SKEA and IPSE from Schistosoma mansoni, that do not depend on TLR for cell activation. In our assay CSK₄, P₃CSK₄, Lipid A and Poly I:C were used as internal standards with the aim of selecting potential safe adjuvants from the chemical library. We set a cutoff for positive selection at 123 μg/ml for TNF-α and 11% for Th1 expansion, corresponding to the mean value obtained upon stimulation with Poly I:C, which scored as weakest adjuvant throughout our study (Fig. 6b left and central panels). A cutoff for negative selection was set above 105 μg/ml of IL-1β, corresponding to the mean value obtained upon stimulation with Lipid A (Fig. 6b right panel). Six out of 14 compounds tested were selected according to their capacity to induce TNF-α above the cutoff, i.e. PCPCS₄, P₃CSK₄, MtCSK₄, Ole₂CSK₄, MALP2 and Lip-OspA. When tested for the capacity to induce Th1 responses, P₃CSK₄, Ole₂CSK₄ and Lip-OspA scored above the cutoff of 11%. Among these three compounds, Lip-OspA also induced IL-1β secretion above the cutoff of 105 μg/ml, thus leaving P₃CSK₄ and Ole₂CSK₄ as the unique selected molecules. Indeed, these compounds are known to act as safe adjuvants in animal models [31], thus supporting the applicability of our method for the selection of potential vaccine adjuvants prior to in vivo studies.
By monitoring proliferation and differentiation of naïve CD4+ T cells as well as APC responses in short-term in vitro cultures, in the presence of nM concentration of standard TLR agonists we obtained three main findings. First, TLR2, TLR4 and TLR3 agonists induce distinct molecular programs in Mo as opposed to DC that correlate with their
potential to act as safe adjuvants in vivo. Second, TNF-\( \alpha \) and CXCL10 represent molecular signatures of adjuvanticity. Third, IL-1\( \beta \) represents an in vitro marker of pyrogenicity and this signature is restricted to primary Mo.

A comparison of human Mo and DC as examples of functionally different APC, show that DC but not Mo do effectively prime CD4\(^+\) T cells in the presence of TLR agonists in vitro. The molecular signatures of each of these two APC subsets to TLR signaling were then analyzed in terms of induction of relevant cytokines and chemokines. Thus, based of the knowledge that DC are professional APC endowed with full T cell priming capacity, we define TNF-\( \alpha \) and CXCL10 as molecular signatures of the adjuvant activity of TLR2/4 and TLR3 agonists, respectively.

Molecular patterns of adjuvanticity do not necessarily correlate with the safety of an immune enhancer. Indeed, the risk of inducing endotoxic shock in vivo represents a limitation for the transfer of adjuvants in practical medicine, even when the compounds tested lack toxicity in animal models. Although TNF-\( \alpha \) and IL-6 can act as pyrogenic molecules when injected in animals, the most potent fever-inducing factor in vivo is IL-1\( \beta \), being active at sub-nanomolar doses in humans [28]. We found that Lipid A stimulation of Mo, but not DC, resulted in IL-1\( \beta \) release at doses which could be pyrogenic in vivo. In contrast, stimulation of both cell types with the synthetic TLR2 agonist P\(_2\)CSK\(_4\) and the TLR3 ligand Poly I:C resulted in induction of proIL-1\( \beta \)-specific gene transcription, in the absence of bioactive IL-1\( \beta \) release. These results are consistent with animal models documenting the lack of toxicity of P\(_2\)CSK\(_4\) and Poly I:C, as compared to the lethal effect of Lipid A [30–32]. Our results also confirm previous finding on the capacity of Lipid A to stimulate IL-1\( \beta \) induction, and, importantly, reveal that primary human Mo are sensitive to a lower concentration range of pyrogens as compared to murine Mo or immortalized macrophage cell lines in vitro [33]. The latter stresses the importance of studying the relevant cellular model, in this case, human primary lymphocytes.

Based on the above findings, we used TNF-\( \alpha \) and IL-1\( \beta \) signatures to predict adjuvant activity and pyrogenicity of a series of natural microbial molecules or synthetic analogs thereof. This simple method enabled us to select the only two compounds contained in the chemical library, known to act as safe adjuvants in vivo, thus demonstrating the applicability of our findings to identify potential adjuvants for humans. Although only few parameters of an immune response were modeled here, the method described presents a number of positive features. These include the simplicity and sensitivity of the biological responses assessed, the broad applicability of the assay and the fact that the system is based on human primary cells that require standard isolation procedures and in vitro culture. The high inter-individual variability observed represents an important concern, which requires the establishment of accurate value of reference for the selected parameters, standardized procedures to perform these assays but also age-matched blood donors [22]. For this implementation, there is need to identify adjuvants and pyrogens of reference, develop calibrated ELISA and MLR assays and select suitable blood donors.

In conclusion, the applicability of our method of screening to large-scale chemical libraries will facilitate the selection of candidate adjuvants prior in vivo testing, thus reducing the need of costly, demanding and potentially irrelevant animal studies. In a possible scenario, depicted in Fig. 7, pre-screening tests assessing the production of TNF-\( \alpha \) and/or CXCL10 by human DC, and IL-1\( \beta \) release by CD14\(^+\) Mo will narrow down the number of compounds that need to be assessed for enhanced immune protection and lack of toxicity in vivo.

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