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Identification of biomarkers to detect residual pertussis toxin using microarray analysis of dendritic cells

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ABSTRACT

In this study we aimed to identify genes that are responsive to pertussis toxin (PTx) and might eventually be used as biological markers in a testing strategy to detect residual PTx in vaccines. By microarray analysis we screened six human cell types (bronchial epithelial cell line BEAS-2B, fetal lung fibroblast cell line MRC-5, primary cardiac microvascular endothelial cells, primary pulmonary artery smooth muscle cells, hybrid cell line EA.Hy926 of umbilical vein endothelial cells and epithelial cell line A549 and immature monocyte-derived dendritic cells) for differential gene expression induced by PTx. Immature monocyte-derived dendritic cells (iMoDCs) were the only cells in which PTx induced significant differential expression of genes. Results were confirmed using different donors and further extended by showing specificity for PTx in comparison to *Escherichia coli* lipopolysaccharide (LPS) and *Bordetella pertussis* lipo-oligosaccharide (LOS). Statistical analysis indicated 6 genes, namely *IFNG*, *IL2*, *XCL1*, *CD69*, *CSF2* and *CXCL10*, as significantly upregulated by PTx which was also demonstrated at the protein level for genes encoding secreted proteins. IL-2 and IFN-γ gave the strongest response. The minimal PTx concentrations that induced production of IL-2 and IFN-γ in iMoDCs were 12.5 and 25 IU/ml, respectively. High concentrations of LPS slightly induced IFN-γ but not IL-2, while LOS and detoxified pertussis toxin did not induce production of either cytokine. In conclusion, using microarray analysis we evaluated six human cell lines/types for their responsiveness to PTx and found 6 PTx-responsive genes in iMoDCs of which *IL2* is the most promising candidate to be used as a biomarker for the detection of residual PTx.

1. Introduction

Pertussis disease (or whooping cough) is caused by infection with the gram-negative bacterium *Bordetella pertussis*. Since the course of this disease can be severe, especially in young children, immunization programs include pertussis vaccines. Pertussis toxin (PT), one of the major virulence factors produced by *B. pertussis*, is the principal antigen in all acellular pertussis vaccines in use today. PT is a typical A-B toxin, of which the enzymatic A protomer is responsible for ADP-ribosylation of the α subunit of G_i proteins while the B protomer is involved in binding and entry into the target cell [1,2]. The dose of biologically active PT (PTx) that would be required for effective immunization is toxic and therefore PTx is detoxified to produce pertussis toxoid (PTd) which is then used in the final formulation of the vaccines [3]. After the detoxification process however, there may still be low levels of PTx present, either because detoxification was not complete or because there was reversion of PTd back to PTx [4]. To ensure that any residual PTx is below safe levels, extensive safety testing for residual PTx in every new batch of vaccine is required by regulatory authorities [5–7]. To assess the safety of pertussis vaccines, two tests are specifically named in the European Pharmacopoeia, *i.e.* the *in vivo* histamine sensitization test (HIST) in mice and the *in vitro* Chinese Hamster Ovary (CHO) cell clustering assay [5]. Because the aluminum adjuvants in final vaccine formulations are toxic for CHO cells, the HIST is currently the only test that can be used for safety evaluation of final vaccine formulations. The HIST is based on the observation that mice injected with PTx are sensitized for histamine, which results in a decrease of the lethal dose of histamine [8]. The HIST is a lethal mouse test, although modifications have been proposed to reduce the lethality of this assay [9–11]. Additionally, the HIST suffers from large variations in inter-laboratory test performance caused by differences in test protocols used between testing sites [12,13]. Together, this makes *in vitro* alternative tests highly desirable. Several *in vitro* alternatives are currently under development, *i.e.* a cAMP assay in rat A10 cells [14], a fetuin binding assay [15,16] and an enzymatic HPLC method [15]. These assays reflect known

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activities of PTx, *i.e.* ADP-ribosylation of G proteins (HPLC method), the binding capacity of the toxin (fetuin binding assay) or a combination of the two including cellular entry of the toxin (cAMP assay). However, not all effector mechanisms of PTx are completely understood, nor are all of its clinical effects [17,18]. In search for biomarkers indicative of the presence of PTx, instead of focusing on known mechanisms by which PTx induces biological effects, we decided to use microarray gene expression analysis as an alternative approach to identify all genes affected by PTx in cell types that are implicated in the biological effects of PTx *in vivo*. *B. pertussis* infection, in which PTx is seen as a prime virulence factor, is associated with marked leukocytosis, hyperinsulinemia, bronchopneumonia and refractory pulmonary hypertension [18,19]. Cell types involved in these physiological effects in which PTx is implicated are pancreatic cells (islets of Langerhans), smooth muscle cells, barrier cells, *i.e.* epithelial and endothelial cells, and a variety of immune cells including neutrophils, macrophages, dendritic cells and T cells. Aiming to include all biological effects of PTx, cell types were selected in which both ADP-ribosylation-dependent effects of PTx have been described as well as ADP-ribosylation independent effects, *i.e.* receptor-mediated signaling *via* the B-oligomer. Both barrier cells and immune cells fit these criteria [20–23]. Six cell lines or primary cell types all of human origin (human bronchial epithelial cell line BEAS-2B, human fetal lung fibroblast cell line MRC-5, primary human cardiac microvascular endothelial cells (HMVEC), primary human pulmonary artery smooth muscle cells (HPASMC), human cell line EA.Hy926 (a hybrid of umbilical vein endothelial cells and epithelial cell line A549) and human immature monocyte-derived dendritic cells (iMoDCs)) were selected and exposed to PTx. A panel of PTx-induced genes was found in iMoDCs exposed to 250 IU/ml PTx for 2 h and the data were confirmed in another, more extensive microarray experiment. Upregulated gene expression was subsequently confirmed using quantitative RT-PCR and ELISA techniques were employed to measure protein levels of four candidate markers: XCL1, CXCL10, IL-2 and IFN-γ. From these data, the cytokines IL-2 and IFN-γ proved to be the most promising biomarkers and the assay was further optimized by determining the sensitivity, specificity and limit of detection. In conclusion, we show here that by meticulously unraveling the effects of PTx on gene expression, a relevant and promising biomarker assay can be developed to detect PTx. Before this assay can be employed for the detection of residual PTx in final vaccine formulations, several hurdles have to be taken concerning sensitivity and toxicity of aluminum adjuvants. However, we feel that the approach we employed here can have great promise to detect biomarkers that can be used for the development of new (vaccine) safety tests.

2. Materials and methods

2.1. Reagents

The WHO-standard pertussis toxin JNIH-5 (PTx) was purchased from NIBSC (Potters Bar, UK). Each vial contained 10 μg PTx corresponding to 10×10^3 international units (IU) PTx which was completely resuspended in 1 ml of PBS + 25% glycerol. The European Pharmacopoeia reference endotoxin from *Escherichia coli* 0113:H10.k (LPS) was obtained from EDQM (Strasbourg, France). *B. pertussis* lipo-oligosaccharide (LOS) was a kind gift from Dr. Watanabe (Kitasato University, Tokyo, Japan). Freeze-dried PTx, LPS and LOS were dissolved in PBS with 25% glycerol, aliquoted and stored at −80 °C until use. For every experiment, new vials were thawed to avoid repeated freeze–thaw cycles. To determine specificity of observed responses, pertussis toxin (PTx-GSK) and pertussis toxoid (PTd-GSK) were kindly received from GlaxoSmithKline (Rixensart, Belgium).

2.2. Cell culture

Six human cell types were used in this study. Human bronchial epithelial cell line BEAS-2B and human fetal lung fibroblast cell line MRC-5 were purchased from ATCC (Manassas, VA, USA). Primary human cardiac microvascular endothelial cells (HMVEC) were obtained from Lonza (Basel, Switzerland). Primary human pulmonary artery smooth muscle cells (HPASMC) were purchased from CellSystems (Troisdorf, Germany). The human cell line EA.Hy926, a hybrid of umbilical vein endothelial cells and epithelial cell line A549, was a kind gift from Sanquin Blood Supply Foundation (Amsterdam, The Netherlands). Finally, immature monocyte-derived dendritic cells (iMoDCs) were cultured from blood of healthy, anonymous donors who gave informed consent (Sanquin Blood Supply Foundation, Amsterdam, The Netherlands). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy donors by Ficoll-Paque (GE Healthcare) density gradient centrifugation and cultured in dendritic cell culture medium (RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 25 mM HEPES (N-2-hydroxyethylpiperazin-N′-2-ethane sulfonic acid), 100 U/ml penicillin, 100 μg/ml streptomycin (all from Gibco, Invitrogen, Carlsbad, CA, USA)). After isolation, PBMCs were seeded in culture plates. After 90 min, medium containing non-adherent cells was removed and DC culture medium supplemented with 100 ng/ml GM-CSF and 50 ng/ml IL-4 (both Immunotools, Friesoythe, Germany) was added. Cytokines were replenished on day three. On day six iMoDCs were used for incubation experiments.

2.3. Incubations for microarray experiments

Incubations were performed in 6-wells plates. Cells were incubated for 2 h with 1 ml of 250 IU/ml PTx, 1 IU/ml (=0.1 ng/ml) LPS, 0.1 ng/ml LOS or with PBS + 0.625% glycerol as a negative vehicle control. For the incubations in which the six different human cell types were exposed to vehicle or PTx, two independent experiments were performed with each experimental group consisting of four replicates. For additional experiments in which only iMoDCs were exposed to vehicle, PTx, LPS or LOS, four independent experiments were performed with each experimental group consisting of four replicates.

Cells were collected in RNA protect (Qiagen, Venlo, The Netherlands) to stabilize RNA. Total RNA was purified using the RNeasy plus mini kit (Qiagen) according to the manufacturer's instructions. The quantity of RNA in each sample was measured using a NanoDrop Spectrophotometer (NanoDrop technologies, Wilmington, DE) and RNA integrity was assessed on a 2100 Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands) using the RNA 6000 Nano Chip Kit (Agilent Technologies). RNA from the four replicates was pooled, resulting in two independent samples for each condition tested (vehicle and PTx) in the cell comparison experiment, and four independent samples for each condition (vehicle, PTx, LPS and LOS) in the iMoDC experiment.

2.4. Microarray experiments and data analyis

RNA was further processed for hybridization to Affymetrix HT HG-U133 + PM Array Plates at the Microarray Department of the University of Amsterdam, The Netherlands. RNA amplification, labeling and genechip hybridization, washing and scanning were carried out according to Affymetrix protocols.

Quality control and normalization were performed using the pipeline at www.arrayanalysis.org (Maastricht University, The Netherlands). Normalization was done using the Robust Multi-chip Average (RMA) algorithm [24] and the MBNI custom CDF (http://brainarray.mbni.med.umich.edu/) [25] version #14 for this chip. All slides passed the various quality control steps. Normalized output consisted of data for 18,909 probe sets each corresponding to unique Entrez GeneIDs. Statistical analyses such as ANOVA and Principal Component Analysis (PCA) were carried out using R software (Version 2.14.2, R Foundation for Statistical Computing, Vienna, Austria) unless indicated otherwise. Gene expression data of experimental groups (two or more) were compared using analysis of variance (ANOVA). *p*-values obtained were corrected for multiple testing by calculating the false discovery rate (FDR) according to Benjamini and Hochberg [26]. For the initial cell line comparison, probe sets with a False Discovery Rate (FDR) < 10% and a Fold Ratio > 1.5 compared to the matched vehicle control groups were considered significantly differentially expressed. For subsequent confirmation in the iMoDC exposures, more stringent criteria of a FDR < 5% and fold ration > 2 were applied. Gene expression data were further visualized as a heatmap combined with hierarchical clustering analysis using Euclidean distance and Ward linkage.

2.5. Confirmation of microarray results by quantitative PCR

To confirm the microarray data, mRNA of iMoDCs used for the microarray analysis and of two additional incubations performed similarly was converted to cDNA using the high capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Relative Expression of *IL2, XCL1* and *CXCL10* was quantified after normalization using reference genes *HPRT1* and *POLR2A* employing Taqman gene expression assays and Taqman Fast universal PCR master mix (both Applied Biosystems), according to supplied protocols on a 7500 Fast Realtime PCR system (Applied Biosystems).

2.6. Confirmation of microarray results and determination of sensitivity and specificity of response by ELISA

To verify the microarray data at the protein level and to determine the sensitivity and specificity of the observed responses, PBMCs were isolated and differentiated as described above and cultured in 24-wells plates. iMoDCs were incubated with PTx (0–250 IU/ml), LPS (1 or 100 IU/ml, *i.e.* 0.1 or 10 ng/ml, respectively), LOS (0.1 or 10 ng/ml), PTx-GSK (100 or 250 ng/ml) or PTd-GSK (100 or 250 ng/ml) for 24 h after which supernatants were stored at −80 °C until further analysis. Protein levels of interleukin-2 (IL-2), interferon-gamma (IFN-γ), Chemokine (C motif) ligand 1 (XCL1) and C-X-C motif chemokine 10 (CXCL10) were determined in supernatants using commercially available ELISAs (IL-2 and IFN-γ: eBiosciences, San Diego, CA and XCL1 and CXCL10: R&D Systems, Minneapolis, MN) with sensitivities of 2 pg/ml (IL-2), 4 pg/ml (IFN-γ), 62.5 pg/ml (XCL-1) and 31.2 pg/ml (CXCL10).

2.7. Statistical analysis of qPCR and ELISA data

Results are presented as mean ± standard deviation. Comparisons between different treatments were done using a one-way ANOVA with a Fisher's least significant difference (LSD) *post hoc* test using SPSS software (IBM SPSS Statistics version 20). A *p*-value < 0.05 was considered statistically significant.

Table 1
Human cell types included in study.

Name	Description	Cell line or primary culture
BEAS2B	Bronchial epithelial cells	Cell line
HMVEC	Cardiac microvascular endothelial cells	Primary cells
EA.Hy926	Hybrid of umbilical vein endothelial cells with epithelial cell line A549	Cell line
MRC-5	Fetal lung fibroblast cells	Cell line
HPASMC	Pulmonary artery smooth muscle cells	Primary cells
iMoDCs	Immature monocyte-derived dendritic cells	Primary cells

3. Results

3.1. Effect of pertussis toxin on mRNA expression levels in selected human cell types

Working toward finding sensitive markers to measure the presence of PTx, we examined the effect of PTx on gene expression in six different human cell types. The selected cell types are all implicated in the effects of PTx in humans (Table 1) and this strategy maximizes the chance of finding PTx-responsive genes. Cells were incubated for 2 h with either 250 IU/ml PTx or with the vehicle (PBS + 0.625% glycerol), after which microarray analysis was performed. Cell line expression data were corrected for their corresponding vehicle control group. Fig. 1 shows the results of the microarray analysis in which green indicates downregulated genes and red indicates upregulated genes. Furthermore, the shade of the color indicates the fold change compared to the control group as indicated in the legend. Statistical analysis showed that 65 genes were differentially expressed in at least one cell line (FDR < 10%, >1.5-fold regulated compared to the control group) (Fig. 1). The observed effects differed between cell lines, but were clearly most pronounced for iMoDCs. In fact, additional *post hoc* tests found no significantly regulated genes for any of the other cell lines (data not shown).

3.2. Pertussis toxin-responsive genes in iMoDCs

To confirm the data obtained with iMoDCs in the first experiment, we repeated the experiment with iMoDCs from four different donors. To extend the data from experiment 1 and to identify genes selectively induced by PTx, we included in this analysis incubations with LPS and LOS. PCA showed considerable differences between gene expression patterns in samples from the four different donors used. Therefore (based on our aim to identify PTx-induced biomarkers rather than donor-specific biomarkers) all data were corrected for the vehicle control exposure data of the respective donor. After this correction, exposure responses across the various donors showed similar trends in a principal component analysis (PCA). Statistical analysis of the data from this microarray found 42 genes differentially expressed by at least one exposure at a FDR < 5% ($p = 0.00039$) and >2-fold regulated relative to the control group (Fig. 2). To identify related biological pathways, functional enrichment analysis was performed. This analysis revealed that the regulated genes were enriched for immune and inflammatory pathways, most notably for chemokines, cytokines, and interferon-induced genes. Genes specific for PTx were selected using additional, more stringent criteria, namely a FDR < 2% and upregulation by a ratio > 4 of PTx exposure compared to any of the other exposures. This resulted in 6 genes: *IFNG*, *IL2*, *XCL1*, *CD69*, *CSF2* and *CXCL10* (Table 2). This confirms the initial microarray experiment, in which the same genes were found to be upregulated in iMoDCs treated with PTx compared to vehicle. Quantitative RT-PCR for *IL2*, *XCL1* and *CXCL10* using RNA from the four human donors

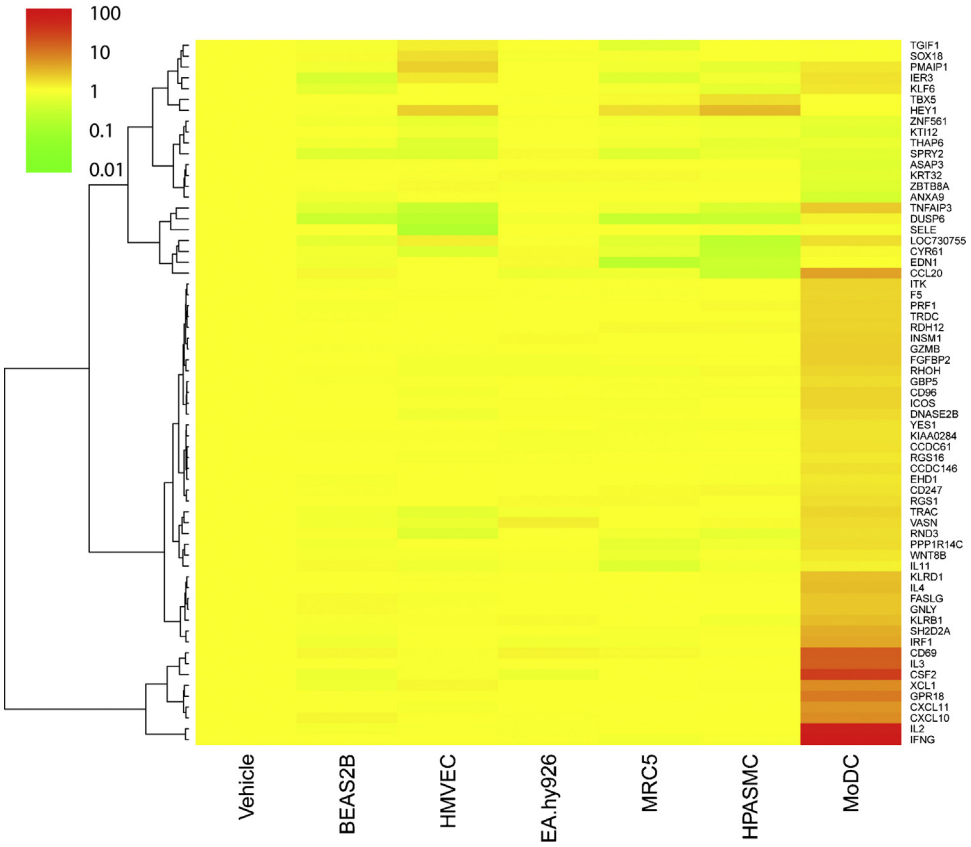

Fig. 1. Gene expression profiles obtained from selected human cell types exposed for 2 h to 250 IU/ml PTx. The color bar shows the expression ratio *vs.* a non-treated control of the respective cell line or type. Green indicates downregulated genes, whereas red indicates an upregulation. The shade of the color indicates the fold change compared to the control group as shown in the legend. Shown are the mean expression ratios of two samples from independent experiments, each consisting of four replicates. Red indicates upregulation, green indicates downregulation. 65 genes that were differentially expressed in at least one cell line are shown (FDR < 10%, >1.5-fold regulated to control group).

analyzed in the microarray experiment and from two additional donors confirmed the microarray results for *IL2*, *i.e.* a strong induction by PTx, and for *CXCL10*, *i.e.* a modest induction by PTx, but did not show induction of *XCL1* by PTx as found using microarray analysis (Fig. 3).

3.3. Effect of pertussis toxin on protein levels of pertussis toxin-sensitive genes in iMoDCs

In addition to gene expression analysis, we measured extracellular protein levels of four selected genes that encode secreted proteins in iMoDCs from two donors after 24 h incubation with vehicle, PTx, LPS or LOS. Fig. 4 shows that the PTx-induced gene expression of *IL2*, *IFNG* and to a lesser extent *XCL1* and *CXCL10* is also reflected in secreted levels of the proteins encoded by these genes after a 24 h incubation with PTx. IL-2, IFN-γ, XCL1 and CXCL10 levels in the supernatant were significantly higher than levels for any of the other treatments (Fig. 4). Of the selected responsive genes respectively proteins, IFN-γ and IL-2 responded strongest both at the gene expression and the protein level and were measured in subsequent experiments.

3.4. Sensitivity of pertussis toxin-induced IL-2 and IFN-γ secretion in iMoDCs

The microarray experiments and the experiments to verify the microarray data were performed by incubating iMoDCs with a high concentration of PTx, *i.e.* 250 IU/ml. To determine the sensitivity, we incubated iMoDCs for 24 h with a dose-range of PTx. IL-2 has a detection level as low as 12.5 IU/ml PTx (Fig. 5A). The IFN-γ response is less sensitive, with a detection level of 25 IU/ml PTx (Fig. 5B).

Table 2
PTx-responsive genes in iMoDCs.

Gene symbol	Gene name	GeneID	Vehicle	PTX	LPS	LOS	FDR
IFNG	Interferon, gamma	3458	1	92.0	1.2	1.3	4.54E−07
IL2	Interleukin 2	3558	1	50.3	1.0	1.1	2.03E−07
XCL1	Chemokine (C motif) ligand 1	6375	1	7.5	1.1	1.1	5.50E−05
CD69	CD69 molecule	969	1	8.5	1.2	1.3	1.16E−04
CSF2	Colony stimulating factor 2 (granulocyte-macrophage)	1437	1	11.1	1.9	1.3	1.16E−04
CXCL10	Chemokine (C-X-C motif) ligand 10	3627	1	7.1	1.5	1.3	1.25E−02

Genes responsive to PTx were selected using a False-Discovery Rate (FDR) < 2% and an upregulation by a ratio > 4 of PTx exposure compared to any of the other exposures. iMoDCs from 4 donors exposed for 2 h to vehicle, 250 IU/ml PTx, 0.1 ng/ml LPS or 0.1 ng/ml LOS.

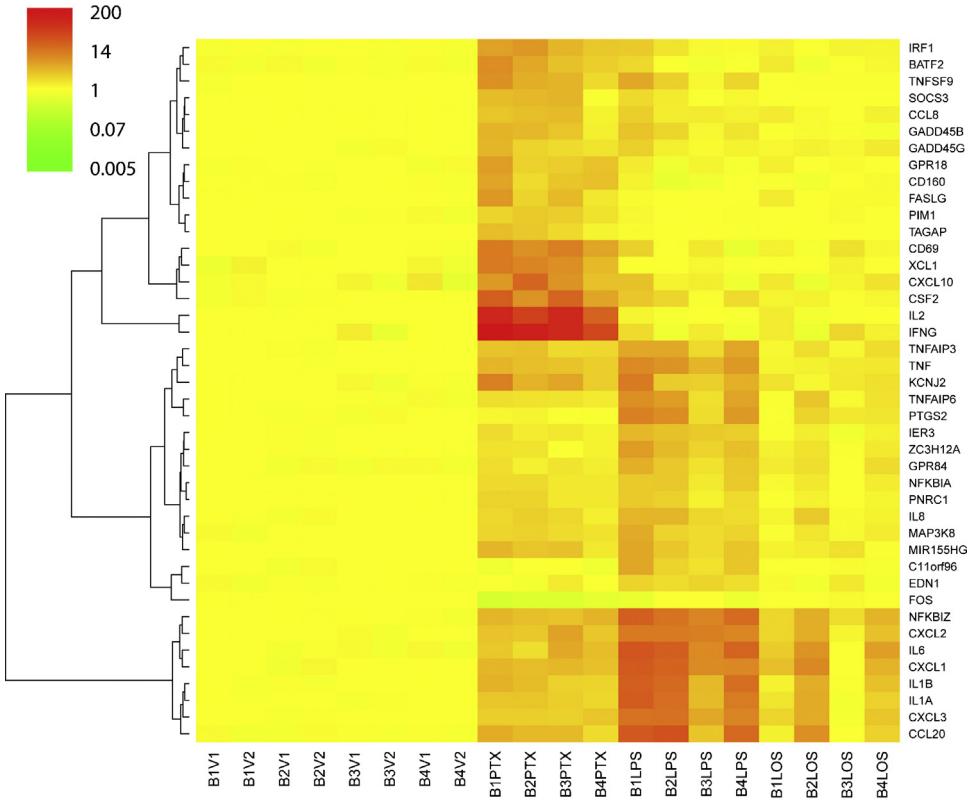

Fig. 2. Gene expression profiles obtained from iMoDCs from four donors (B1–B4) exposed for 2 h to vehicle (V), 250 IU/ml PTx, 0.1 ng/ml LPS or 0.1 ng/ml LOS. Red indicates upregulation, green indicates downregulation. The shade of the color indicates the fold change compared to the control group as shown in the legend. 42 genes are shown that are differentially expressed by at least one exposure (FDR < 5%, >2-fold regulated compared to the control group).

3.5. *Specificity of pertussis toxin-induced IL-2 and IFN-γ secretion in iMoDCs*

In addition to determining the sensitivity of the observed IL-2 and IFN-γ responses of iMoDCs to PTx, we also determined the specificity of these responses. We determined whether the responses can distinguish PTx from the detoxified variant, PTd. Whereas PTx-GSK induced a significant IL-2 or IFN-γ response in iMoDCs after 24 h incubation, PTd-GSK in similar concentrations did not induce any IL-2 or IFN-γ secretion indicating a clear distinction in response to the native or to the detoxified pertussis toxin (Fig. 6). Moreover, PTd-GSK in concentrations comparable to those used in vaccine formulations (25 μg/ml) also failed to induce an IL-2 response (data not shown). To verify more thoroughly that LPS and LOS do not elicit the same responses as PTx, we incubated iMoDCs with a 100-fold higher concentration of LPS or LOS than used in the microarray experiments, *i.e.* 10 ng/ml. Similar to the lower concentrations of LPS or LOS that were used in the microarray experiments, these higher concentrations of LPS or LOS did not elicit an IL-2 response (Fig. 6A). This was also the case for IFN-γ secretion induced by LOS; both 0.1 ng/ml and 10 ng/ml did not induce IFN-γ secretion (Fig. 6B). In contrast, while 0.1 ng/ml LPS (*i.e.* 1 IU/ml) did not induce IFN-γ release (Fig. 4), exposure to 10 ng/ml LPS significantly induced an IFN-γ response that was comparable to that induced by

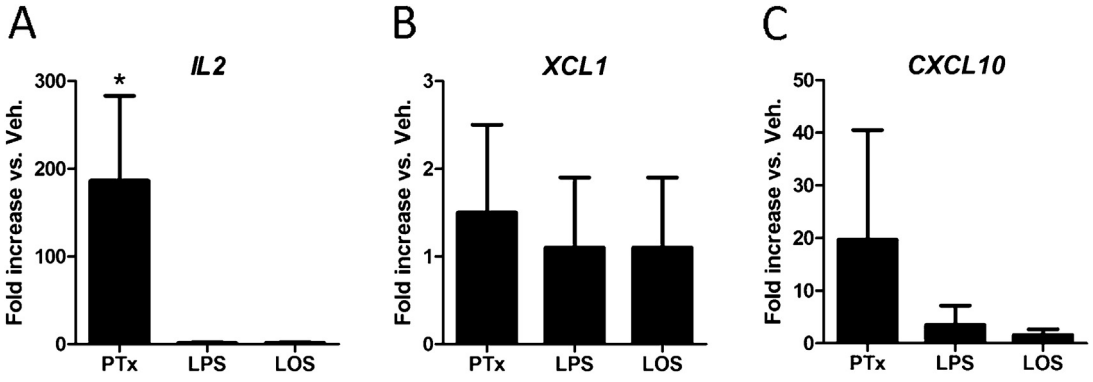

Fig. 3. Gene expression data of three genes specifically upregulated by PTx in iMoDCs. iMoDCs from six human donors were incubated for 2 h with vehicle, 250 IU/ml PTx, 0.1 ng/ml LPS or 0.1 ng/ml LOS. RNA expression of IL2 (A), XCL1 (B) and CXCL10 (C) was analyzed using quantitative RT-PCR. Data are presented as relative expression compared to the average expression of the vehicle treatment. * Indicates a statistically significant ($p < 0.05$) difference compared to cells of the same donor treated with vehicle, LPS or LOS.

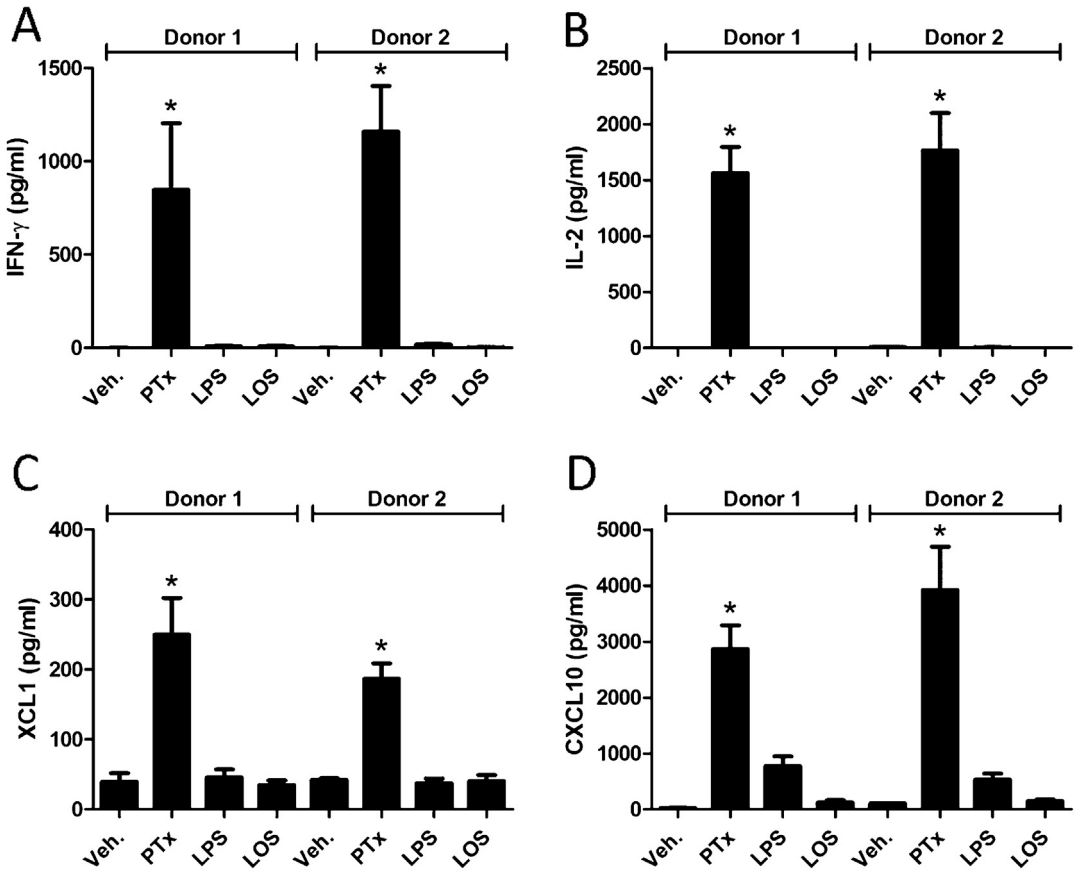

Fig. 4. Protein levels of four genes encoding secreted proteins that are specifically upregulated by PTx in iMoDCs. iMoDCs from 2 human donors ($n = 4$) were incubated for 24 h with vehicle, 250 IU/ml PTx, 0.1 ng/ml LPS or 0.1 ng/ml LOS. Supernatant was analyzed for IFN-γ, IL-2, XCL1, and CXCL10. Data from separate donors are presented as mean ± standard deviation of four replicates. PTx induced a statistically significant ($p < 0.05$) higher secretion of IFN-γ, IL-2, XCL1, and CXCL10 compared to all other incubations in iMoDCs from both donors, indicated by *.

100 ng/ml PTx-GSK. Finally, we also tested additional components that are present in the PTx preparation of the manufacturer, *i.e.* arginine, glucose, sucrose and lactose, all of which did not induce an IL-2 or IFN-γ response in iMoDCs (data not shown). In conclusion, we show that the IL-2 response is highly specific for PTx compared to PTd, LPS, LOS, arginine, glucose, sucrose and lactose, whereas the IFN-γ response is similarly specific except for a response to a high concentration of LPS.

4. Discussion

In this study we used comparative gene expression analysis of 6 cell types exposed to PTx, with the aim to identify PTx-responsive genes that might serve as biomarkers to measure the presence of PTx. Microarray analysis offered the advantage of examining all responsive genes instead of focusing on one known mechanism of PTx. This approach might be of interest for pertussis vaccine safety

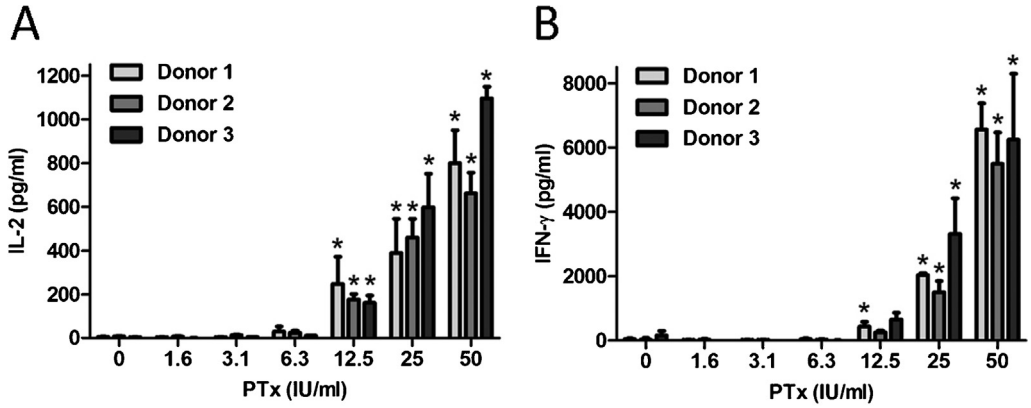

Fig. 5. Sensitivity of PTx-induced IL-2 (A) and IFN-γ (B) response in iMoDCs. iMoDCs from 3 human donors ($n = 3$) were incubated for 24 h with PTx and supernatants were analyzed for IL-2 and IFN-γ. Data from separate donors are presented as mean ± standard deviation of three replicates. * Indicates a statistically significant ($p < 0.05$) difference compared to cells of the same donor not treated with PTx.

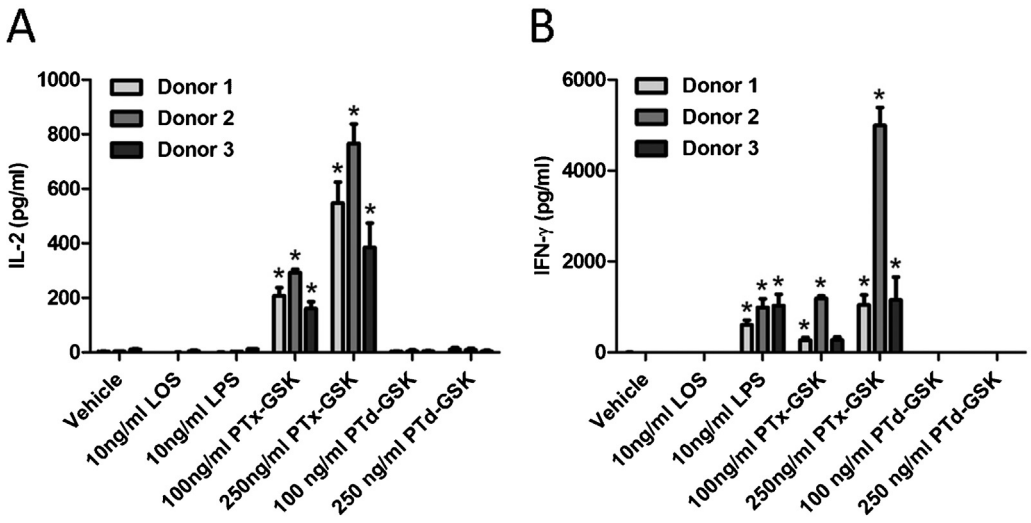

Fig. 6. Specificity of PTx-induced IL-2 (A) and IFN-γ (B) response in iMoDCs. iMoDCs from 3 human donors ($n = 3$) were incubated for 24 h with vehicle, LOS, LPS, PTx-GSK or PTd-GSK and supernatants were analyzed for IL-2 and IFN-γ. Data from separate donors are presented as mean ± standard deviation of three replicates. * Indicates a statistically significant ($p < 0.05$) difference compared to cells of the same donor not treated with PTx.

testing since not all effector mechanisms of PTx are completely understood, nor are all of its clinical effects [17,18]. Current *in vitro* alternatives under development to replace the HIST focus mainly on the binding and entry of PTx and consequent ADP-ribosylation of G_i proteins [14–16]. Although the enzymatic activity is a principal activity of PTx involved in many of its biological effects, over the past years several studies have described PTx effects independent of this enzymatic activity [21,22,27,28]. The latter kinds of effects are mainly attributed to direct recognition of PTx by pattern recognition receptors (PRR), such as TLR4.

The complexity of PTx signaling prompted us to study all effects of PTx at the gene level using a microarray-based approach. Since pertussis disease (and thereby the vaccine) pertains to humans, we decided to work only with cells of human origin. Although human cells are not a necessity for *in vitro* safety tests for products for human use, the biological responses observed in these human cells are more likely to be relevant and regulatory acceptance might be easier. Furthermore, we chose cell types whose involvement in the physiological effects of *B. pertussis* infection is evident, *i.e.* immune cells and barrier cells. Contrary to our expectations, the transcriptional activation of the selected cell types by PTx was limited; incubation of the 6 cell types with 250 IU/ml PTx for 2 h resulted in transcriptional activation of only iMoDCs in which we found merely six genes to be significantly upregulated compared to the other treatments, *i.e.* vehicle, LPS and LOS. Given the high PTx concentration of 250 IU/ml that we used, it is unlikely that the concentration is the reason for the low number of responsive genes in these cells. The result may indicate that PTx only has a modest effect on gene expression in these cell types, despite their suggested role in the *in vivo* effects of PTx. On the other hand, PTx may act predominantly through post-transcriptional events whose subsequent effects on a transcriptional level become apparent after a longer incubation period. In this study we used a relatively short incubation period of 2 h which may have precluded these effects from becoming apparent. Likewise, Belcher et al. [29], using BEAS-2B lung epithelial cells and a 3 h infection with *B. pertussis*, also reported only a modest number of genes, *i.e.* 33 of approximately 6800 genes that were analyzed, that showed significantly upregulated expression. Unfortunately, in the study of Belcher et al., no data were shown that compared the effects of PTx on gene expression in BEAS-2B cells to incubation with vehicle; therefore we cannot compare that study with our results. Despite the limited response, 6 genes, *i.e. IFNG*, *IL2*, *XCL1*, *CD69*, *CSF2* and *CXCL10*, were significantly upregulated after an incubation of iMoDCs with PTx for only 2 h. Analysis at the protein level indicated that the effect of PTx on expression of four selected genes that encode secreted proteins, is also reflected in increased protein secretion illustrating the biological relevance of the observed gene responses. Working toward possible application of these potential biomarkers for detection of residual PTx, we investigated the two strongest responders, *i.e.* IFN-γ and IL-2 using ELISA techniques. At least two parameters are critical to evaluate whether these two genes/proteins have the potential to be used for detection of residual PTx, *i.e.* the sensitivity and the specificity of the observed responses. For the microarray experiments a high concentration of 250 IU/ml PTx was used in order not to miss any effects on gene expression. With ELISA techniques, we show that the limit of detection that can be reached is 12.5 IU/ml PTx for IL-2 and 25 IU/ml PTx for IFN-γ. Notably, before any assay optimization has been performed, this sensitivity is already in the range of the acceptable detection limit of 1–10 IU/ml that was determined in a recent international workshop that discussed the requirements for an *in vitro* alternative for the HIST [30]. Specificity of the response compared to LPS and LOS, and especially to the detoxified form of pertussis toxin (PTd), is also critical for further applications. Concentrations of 0.1 ng/ml (1 IU/ml) LPS and 0.1 ng/ml LOS induced a specific transcriptional response (Fig. 2) but did not induce the 6 genes that were identified as PTx-responsive genes. However, in additional experiments using 100-fold higher concentrations, LPS but not LOS induced an IFN-γ response but no release of IL-2. This latter finding is important in developing a PTx-specific assay because vaccines contain bacterial components making it likely that trace amounts of compounds such as LPS and LOS are present in vaccines [31]. Indeed, concentrations up to 100 IU per single human dose, *i.e.* 10 ng for the LPS used in this study, are allowed for pertussis vaccines according to the European Pharmacopoeia [1]. This means that only IL-2 might be a suitable biomarker for detection of residual PTx. As shown in Fig. 6, both IL-2 and IFN-γ are induced by PTx-GSK but not by the same preparation after detoxification (PTd-GSK). Taken together, these initial experiments suggest that especially IL-2 is a promising candidate based on the sensitivity and specificity of the response.

The effect of PTx on dendritic cells (DCs) has been extensively described. There are several reports that demonstrate efficient maturation of DCs by PTx [20,23,28,32,33]. DCs are linked to the

development of the Th1/Th17 response that is observed in *B. pertussis* infection and that leads to the massive recruitment of neutrophils and macrophages [34]. The mechanism of how PTx induces DC maturation is still subject of debate. While Bagley et al. showed that the B subunit is insufficient to induce maturation markers CD83 and CD86 and suggested that maturation of DCs is induced by the ADP-ribosylating activity of the enzymatic A subunit with a consequent cAMP increase [32], others have shown that PTx-induced DC maturation can also be realized by the B subunit alone and have proposed TLR4-mediated signal transduction [20,22,23]. Although outside the scope of the current manuscript, the mechanism underlying the observed induction of IL-2 and IFN-γ by PTx and the failure of PTd to do so, needs to be established in future experiments to assess the value of the identified biomarkers. If the observed induction is independent of the ADP-ribosylation activity of PTx, an assay based on these markers might be a valuable addition to the current repertoire of assays that focus mainly on the ADP-ribosylation activity of PTx, whether or not including binding and entry of PTx to the used cells [14–16]. Ausiello et al. [20] showed in 2002 that PTx can induce IFN-γ secretion in human iMoDCs. Interestingly, they also showed that a mutated nontoxic PT variant (PT-9K/129G) that is devoid of ADP-ribosyltransferase activity was also able to stimulate IFN-γ release. Using a different cell type, *i.e.* murine splenic T cells, Ryan et al. [35] had previously shown similar effects. PTx and PT-9K/129G at low concentrations, *i.e.* 40 ng/ml, were both able to induce secretion of IFN-γ and IL-2, whereas a chemically detoxified pertussis toxin (PTd) failed to induce the effects in line with our own observations. Combined these data might suggest that the PTx-induced IL-2 and IFN-γ secretion is caused by an ADP-ribosylation independent effect of the B-subunit of pertussis toxin. Moreover, already in 1989, Stewart et al. [36] using the Jurkat T cell line, showed that the PTx holotoxin and the isolated B-subunit were both able to induce intracellular inositol-3-phosphate levels and intracellular Ca^{2+} concentrations without an increase of intracellular cAMP, which is a characteristic of PTx-induced ADP-ribosylation of G_i proteins. A recent review on IL-2 secretion by dendritic cells indicates the calcineurin/NFAT signaling pathway as the main driving force of IL-2 secretion in DCs, a signaling pathway activated by increased intracellular Ca^{2+} levels [37]. Experiments in which PRR such as TLR4 are not functional, experiments with pertussis toxin lacking enzyme activity, *e.g.* PTx-9K/129G [38] or experiments using forskolin to maximize adenylate cyclase activity, a key event after ADP-ribosylation of G proteins, are needed to elucidate the specific mode of action. In line with the fact that assays using cells from human blood are notorious for their donor variability, we found a moderate variation in the levels of IL-2 among donors, although every donor tested during these studies responded to PTx exposure by increasing the IL-2 secretion.

For further research, several aspects of the assay developed by us can potentially be elaborated on. First of all, to further explore the use of IL-2 as a biomarker, different kinds of cost-effective and practical assays could be studied in the future, such as a luciferase or GFP reporter assay with cell lines expressing constructs containing the responsive element of IL-2 cloned before the GFP or luciferase gene, or the use of quantitative RT-PCR techniques. Secondly, to circumvent donor variability as well as potential difficulties in purchasing human blood, the possibilities of performing the biomarker assay in a human cell line should be investigated, in combination with the changes in biomarker read-out suggested above. Also, the specificity of response should be expanded to other antigens and preservatives present in pertussis vaccines. Finally and importantly, if a (newly developed) assay is to be used for final vaccine formulations, the compatibility of this assay with the aluminum adjuvants present in vaccines should be investigated. In conclusion, using a microarray approach we evaluated gene expression in 6 relevant human cell lines/types exposed to PTx and found 6 PTx-responsive genes in iMoDCs of which *IL2* is a very promising candidate to be used as a biomarker to detect the presence of PTx.

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