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Original Article

Temporal cytokine profiling of *Francisella tularensis*-infected human peripheral blood mononuclear cells

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Background and Purpose: *Francisella tularensis* is an intracellular bacterium known to replicate in monocytes and macrophages and cause tularemia in humans. Because of its infectious nature, *F. tularensis* is considered a biowarfare agent. Early cytokine profiles of *Francisella*-infected human peripheral blood mononuclear cells were evaluated.

Methods: Populations of human peripheral blood mononuclear cells were infected in vitro with *F. tularensis* live vaccine strain at a very low multiplicity of infection of 1:10 (bacteria:cells). A multiplex bead kit which analyzes 30 cytokines, chemokines and growth factors was utilized to measure secreted cytokines in cell supernatants 1, 4, 8, 16, and 24 h post-infection.

Results: Compared with uninfected controls, infected cells showed no increase in cytokine secretion at 1 and 4 h, implying a threshold for activation of immune responses. Starting at 8 h post-infection and continuing through to 24 h, an array of cytokines and growth factors was secreted by the infected cells. Some cytokines not previously associated with *Francisella* infection in humans were detected at 8 h, including interleukin-17 and interleukin-1 receptor agonist and vascular endothelial growth factor.

Conclusions: The cytokine profiles of *F. tularensis*-infected peripheral blood mononuclear cells indicate an intricate pattern of both pro- and anti-inflammatory responses, including early T-cell activation.

Key words: Cytokines; *Francisella tularensis*; Immunity, cellular; Macrophages; Tularemia

Introduction

Francisella tularensis is a facultative intracellular Gram-negative coccobacillus which causes tularemia in humans and, due to its infectious nature, is also considered a potential biowarfare agent [1]. Infection in humans is mostly due to two subspecies of *Francisella, tularensis* and *holoarctica*. *F. tularensis* live vaccine strain (LVS) is an attenuated strain of *Francisella* derived from *holoarctica,* and is utilized for laboratory studies [1]. Interaction of bacteria with peripheral blood occurs in ulceroglandular tularemia as a transient bacteremic phase in early infection, while a systemic phase exists for typhoidal tularemia [1]. Following infection of the host, bacteria are engulfed through receptor-mediated pathways of phagocytic cells, encapsulating them in the phagosome. *Francisella* disrupts the phagosome-lysosome fusion process and escapes into the host cell cytosol where it replicates [2].

After exposure to an infection, the initial immune response transitions to an adaptive response via the secretion of cytokines and upregulation of costimulatory molecules [3]. Because secretion of cytokines is one of the first responses by tissues to an invading pathogen, detailed knowledge of the type and role of these cytokines would provide important information

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for use in infection control. Early cytokine secretion via T-helper type 1 (Th1) or Th2 immune responses is thought to play an important role in determining the outcome of any infection [4]. Previous studies have established that stimulation of peripheral blood mononuclear cells (PBMCs) by pathogens could result in the production of cytokines, which polarize the immune response [5,6]. It has been well established that T cells producing interferon (IFN)-gamma and interleukin (IL)-2 are clearly protective against systemic intracellular replication of many human pathogens [6-8]. Previous studies have reported the production of cytokines and chemokines in vitro in isolated monocytes/monocyte-derived macrophages exposed to *F. tularensis* with a high multiplicity of infection (MOI) [9,10]. For example, human monocyte-derived macrophages secreted CXCL8 (IL-8), CCL2 (chemokine [C-C motif] ligand 2), IL-1beta and tumor necrosis factor (TNF)-alpha in response to live *F. tularensis*, and CXCL8 and IL-1beta were secreted by isolated monocytes [9]. A very recent report indicated that IL-23 was induced by peripheral blood monocytes infected with *F. tularensis*, and the secreted IL-23 induced IFN-gamma production from natural killer cells [10]. However, a detailed kinetic evaluation of early cytokine/chemokine secretion in total PBMCs exposed to *F. tularensis* has not been reported.

Multiplex analysis measures multiple cytokines simultaneously in small sample volumes and thus provides a convenient tool for the detection of an array of secreted cytokines, in order to characterize cytokine profiles. We utilized the powerful high-throughput xMAP multiplex immunobead assay technology (Luminex Corporation, Austin, TX, USA) to simultaneously test for 30 cytokines, chemokines, angiogenic as well as growth factors, and soluble receptors from PBMCs infected with *F. tularensis* in vitro.

Methods

Study subjects

Seven male volunteers aged 21 to 44 years were recruited for the in vitro infection study after informed consent. These volunteers had not previously been exposed to *F. tularensis* (no history of natural disease nor receipt of a tularemia vaccine).

Isolation of PBMCs

PBMCs were isolated from whole blood of each volunteer using a Ficoll gradient method (Sigma, St. Louis, MO, USA). Briefly, whole blood was diluted 1:2 with Dulbecco's phosphate-buffered saline (Sigma), layered on Histopaque 1077 (Sigma), and centrifuged at 500 *g*. The resulting interphase was collected and washed twice with phosphate-buffered saline. The final cell pellet was resuspended in RPMI 1640 with 2 mM glutamine + 20% AB serum (Valley Biomedicals, Winchester, VA, USA). Concentration of cells for each sample (n = 7) was determined using a hemacytometer, and adjusted accordingly.

LVS for in vitro infections

The LVS vaccine was kindly provided by the United States Army Medical Research Institute for Infectious Diseases, Frederick, MD, USA. A live culture of *F. tularensis* LVS was grown in thioglycollate broth (BBL; Becton Dickinson Microbiological Systems, Cockeysville, MD, USA) with 2% IsoVitaleX™ (BD Diagnostic Systems, Sparks, MD, USA) supplementation at 37°C in a shaker incubator. The overnight stock cultures were aliquoted in the broth medium, and stored at –80°C for use in the infection of PBMCs.

Infection of PBMCs with *F. tularensis*

Infections were performed independently with PBMCs isolated from each volunteer. Cells were plated at a concentration of 1×10^7 cells/well in 6-well plates (Costar, Cambridge, MA, USA) and left to rest overnight in a 37°C incubator with 5% carbon dioxide. The next day, cells were exposed to LVS at an MOI of 0.1:1 (bacteria: cells), and incubated at 37°C, 5% carbon dioxide for 1, 4, 8, 16, and 24 h post-infection. Supernatants were collected from these cultures at the selected time points, together with the cell pellets for gene expression studies. Uninfected control supernatants were also collected at each time point, filtered through a Millipore 0.22 μm filter (Millipore Corporation, Billerica, MA, USA) and stored at –80°C until use.

Determination of intracellular bacterial cell count in infected PBMCs

PBMCs were plated in a 12-well plate at a density of 2.5×10^6 cells/well and infections were performed with 3 independent samples. At 1, 4, 8, 16, and 24 h post-infection, the cells were centrifuged at 500 *g* for 7 min and washed once with saline, and the cell pellet lysed with 0.1% deoxycholate. Appropriate dilutions were plated on chocolate agar plates, and after 3 days, colony-forming units were enumerated.

Multiplex quantification of cytokines and chemokines

Supernatants from LVS-infected PBMCs over five time points, and corresponding control supernatants, underwent multiplex cytokine assay. We utilized the human cytokine multiplex kit (29 Plex premix beads + regulated upon activation, normal T-cell expressed and secreted [RANTES]; LINCOplex™; Linco Research, St. Charles, MO, USA) to determine secreted proteins in the culture supernatants.

This test assays for thirty human cytokines and chemokines simultaneously and included the following: epidermal growth factor, eotaxin, fractalkine, granulocyte colony-stimulating factor (GCSF), granulocyte-macrophage colony-stimulating factor, IFN-gamma, IL-1alpha, IL-1beta, IL-1 receptor antagonist (IL-1Ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40, free form), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, monocyte chemotactic protein-1, macrophage inflammatory protein (MIP)-1alpha, MIP-1beta, RANTES, soluble CD40 ligand, transforming growth factor-alpha, TNF-alpha and vascular endothelial growth factor (VEGF). A 50-µL aliquot of supernatant from each sample was plated in a 96-well plate. The samples were then mixed with microbeads attached to fluorophores, and the reactions allowed to proceed according to the manufacturer's specifications. The plates were read on a Luminex 100 instrument system with appropriate standards and quality controls, and analyzed according to specifications.

Statistical analysis

Cytokine concentrations (pg/mL) were calculated from mean fluorescence intensity values using Mathematica 5.1 software (Wolfram.com, Boston, MA, USA). The standard fluorescence values were used to create a graph for each cytokine in the following manner. The fluorescence range for each cytokine was used to fit a degree three spline curve using trend analysis and regression analysis. The spline curve created for each cytokine was used to compute unknown cytokine values. To determine the differences between infected and uninfected samples, a paired Student's *t* test was used, and a value of $p \leq 0.05$ was considered significant.

Results

Temporal expression of cytokines/chemokines in infected PBMCs

The bacterial cell number increased exponentially, to 4 $\log_{10}$ by 24 h (Table 1). When PBMCs were exposed to LVS in vitro over a period of 24 h, an array of Th1- and Th2-related cytokines, chemokines and growth factors were induced from both monocytes and lymphocytes. Prior to the first eight hours of infection (1 h and 4 h time points), no significant increases in secreted proteins were detected in the infected samples. At 1 h and 4 h, the number of bacteria per cell was estimated as 0.006 and 0.019, or 6 and 19 bacteria per 1000 cells, respectively. However, by 8 h, we observed a ten-fold increase in intracellular bacteria compared to 1 h post-exposure, and the secretion of an array of cytokines, chemokines and growth factors. These include IL-1alpha, IL-1beta, IL-12p70, IL12-p40, TNF-alpha, IL-6, IFN-gamma, IP-10, MIP-1alpha, MIP-1beta and RANTES, which were detected in supernatants of infected cells in this study (Table 2). Some of these cytokines have been previously associated with *F. tularensis* infection of monocytes/macrophages, and primarily reflect a proinflammatory response [9,11, 12]. Out of a panel of 30 different cytokines/chemokines tested, most exhibited an increasing concentration trend with time, which correlated with the increasing bacterial load in infected cells.

The anti-inflammatory protein IL-1Ra was induced at 8 h post-infection at a concentration as high as 3000 pg/mL, and reached 8000 pg/mL by 24 h (Fig. 1). This cytokine may not have reached maximum expression by 24 h. The baseline level for IL-1Ra was already elevated in uninfected cells (1500 pg/mL) [Fig. 1].

The levels of IL-17 detected at 8, 16, and 24 h in infected samples were significantly higher than those in uninfected cells (p=0.004, p=0.048 and p=0.001,

Table 1. Growth of intracellular *Francisella tularensis* live vaccine strain in peripheral blood mononuclear cells (n = 3)

Variable	Time (h)				
	1	4	8	16	24
CFU	1.2×10^3	3.4×10^3	12.6×10^3	34×10^3	111×10^3
SEM	0.33	0.71	4.26	3.75	12.51

Abbreviations: CFU = colony-forming units; SEM = standard error of the mean

Table 2. Cytokines detected in peripheral blood mononuclear cells after infection with *Francisella tularensis* live vaccine strain (n = 7)[a]

Time (h)		
8	16	24
IL-1beta[b]	IL-1beta[c]	IL-1beta[c]
IL-1alpha[c]	IL-1alpha[c]	IL-1alpha[c]
IL-1Ra[b]	IL-1Ra[c]	IL-1Ra[c]
IL-2[c]	IL-2[c]	IL-2[c]
IL-10[b]	IL-10[c]	IL-10[c]
IL-12p70[b]	IL-12p70[b]	IL-12p70[c]
IL-12p40[c]	IL-12p40-NS	IL-12p40[c]
IL-15[b]	IL-15[b]	IL-15[c]
IL-17[c]	IL-17[c]	IL-17[c]
IFN-gamma[c]	IFN-gamma[c]	IFN-gamma[c]
TNF-alpha[b]	TNF-alpha[c]	TNF-alpha[c]
MIP-1alpha[b]	MIP-1alpha[c]	MIP-1alpha[c]
MIP-1beta[c]	MIP-1beta[c]	MIP-1beta[c]
IP-10[c]	IP-10[c]	IP-10[c]
GMCSF[b]	GMCSF[c]	GMCSF[c]
RANTES-NS	RANTES-NS	RANTES[c]
VEGF[c]	VEGF[c]	VEGF[c]

Abbreviations: IL = interleukin; Ra = receptor antagonist; IFN = interferon; TNF = tumor necrosis factor; MIP = macrophage inflammatory protein; GMCSF = granulocyte-macrophage colony-stimulating factor; RANTES = regulated upon activation, normal T-cell expressed and secreted; NS = not significant; VEGF = vascular endothelial growth factor; IP = inducible protein

[a]Cytokine levels were assessed in the supernatants of live vaccine strain-infected peripheral blood mononuclear cells by multiplex bead array assay (LINCOplex™). Before 8 h, cytokines were not differentially secreted in infected or uninfected cells.

[b]$p \leq 0.05$.

[c]$p \leq 0.01$ by paired *t* test.

respectively) [Fig. 2]. The secretion profile of IL-17, reported as an inducer of GCSF [13], was similar to that of GCSF (Fig. 3), indicating a possible relationship between these two factors.

We also detected secreted IL-2, starting at 8 h post-infection (218 pg/mL) and increasing sharply to 616 pg/mL by 24 h (Fig. 4).

Discussion

Our findings expand upon previous published work on cytokines/chemokines associated with *Francisella*-infected monocyte/macrophages, and provide additional information on early cytokines in PBMCs exposed to infection. We infected PBMCs at a very low MOI of 0.1:1 (bacteria:cells) in order to study early systemic exposure of *Francisella* with PBMCs.

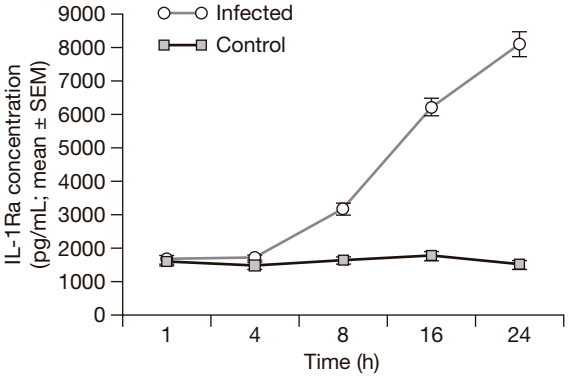

Fig. 1. Interleukin-1 receptor antagonist (IL-1Ra) secretion in live vaccine strain-infected human peripheral blood mononuclear cells (n = 7). SEM = standard error of the mean.

In this study, bacteria were continuously present in the culture medium and therefore cytokine secretion from PBMCs could not be attributed to intracellular bacteria alone. At the first two time points (1 and 4 h), with a low MOI, we did not observe a difference in cytokine production between infected and uninfected samples. At these times, the intracellular bacterial load was estimated to be roughly 6 and 19 bacteria per 1000 monocytes, assuming an average of 7% monocytes in the PBMC population. *F. tularensis* is known as a 'stealth' pathogen, and it would not be unexpected that in early infection it might not activate the signaling pathways of the host. After a certain bacterial threshold was reached, the host responded with a cascade of cytokines and chemokines.

It was reported that *F. tularensis* LVS downregulates the initial TNF-alpha cytokine response after infection [10]. In this study, TNF-alpha was not downregulated by *F. tularensis* but was induced at a later time point (8 h) [Table 2]. This discrepancy

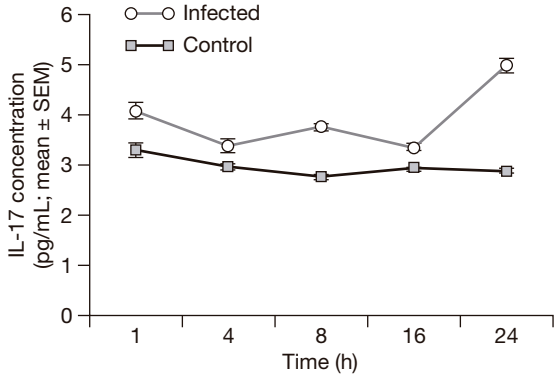

Fig. 2. Interleukin (IL)-17 secretion in live vaccine strain-infected human peripheral blood mononuclear cells (n = 7). SEM = standard error of the mean.

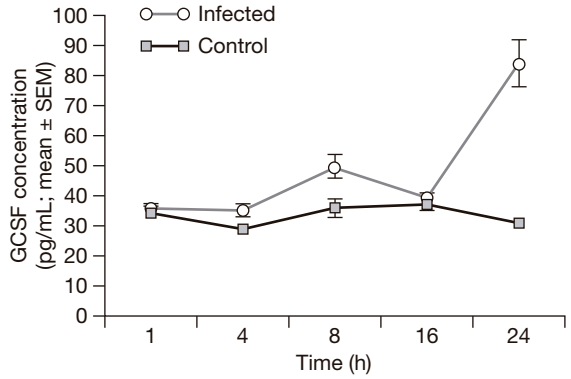

Fig. 3. Granulocyte colony-stimulating factor (GCSF) secretion in live vaccine strain-infected human peripheral blood mononuclear cells (n = 7). SEM = standard error of the mean.

may be explained by the fact that a very high MOI of 500:1 (bacteria:cells) was used by Telepnev et al [10]. The considerable increase in TNF-alpha in the culture supernatant 24 h post-exposure (689 pg/mL) may be attributed to extracellular bacterial multiplication, as reported by Bolger et al [9].

We found several proinflammatory cytokines, including TNF-alpha, IL-8, IL-12p70, and IFN-gamma, to be significantly elevated at 8 h exposure of resting PBMCs to the bacteria. The secretion of both IL-1alpha and IL-1beta was significantly greater in *Francisella*-infected samples. The production of IL-1beta by *Francisella* has been associated with the escape of bacteria into the cytoplasm [12], and from colony-forming unit counts at 8 h post-infection we could infer that the detected IL-1beta was due to intracellular bacteria. The main role of IL-1 is to mediate the early inflammatory reactions for protection against various stimuli, ranging from microbial colonization to infection, and both IL-1alpha and IL-1beta are involved in the innate and adaptive immune responses. Although the action of IL-1 protects the host by enhancing the response to pathogens, its overproduction can lead to pathological symptoms. We were surprised to detect IL-1alpha in the supernatant of the infected cells, as this cytokine is membrane-bound and usually intracellular [14]. The increase in IL-1alpha secretion occurred between 8 and 16 h, after which its levels remained constant until 24 h (data not shown). IL-1beta concentrations, albeit at lower absolute levels than IL-1alpha, continued increasing through to 24 h (data not shown).

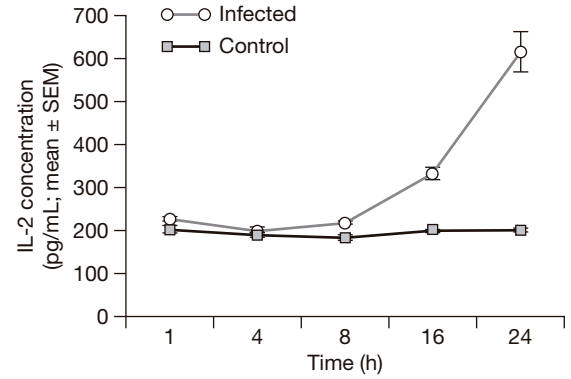

Fig. 4. Interleukin (IL)-2 secretion in live vaccine strain-infected human peripheral blood mononuclear cells (n = 7). SEM = standard error of the mean.

It is known that IL-1alpha is induced in a nuclear factor-kappa B-independent manner [15]. It has been suggested that the pattern of expression of IL-1alpha and IL-1beta in response to different stimuli indicates that the genes for IL-1alpha and IL-1beta are regulated independently [16]. Detection of both IL-1alpha and IL-1beta in the infected cells suggests that there might be more than one signal inducing proinflammatory cytokine responses in the host in response to infection by *Francisella*. Both IL-1alpha and IL-1beta bind to the IL-1 receptor, eliciting responses such as costimulation of T cells, fever, and the induction of acute phase responses [14].

We also detected very high levels of IL-1Ra in supernatants from infected cells, with an increasing trend by 24 h, while the uninfected cells had constant, but elevated levels of IL-1Ra. It has been reported recently [17] that serum factors contribute to some extent to IL-1Ra secretion in PBMC cultures, and it is possible that the presence of human serum (20%) in our cultures would have contributed to these higher basal levels observed in uninfected samples. IL-1Ra is stimulated under conditions where proinflammatory cytokines would be inhibited [18]. IL-1Ra has also been shown to inhibit the effects of IL-1 both in vitro and in vivo, and to reduce the severity of inflammation in several animal models of inflammatory disease [19,20].

In *Francisella* infection, the profound inflammatory response could by itself cause damage to the host [21]. Therefore, the presence of anti-inflammatory IL-1Ra would be an essential element for maintaining balance in the inflammatory immune response. The lesser inflammatory response to *Francisella* LVS in humans might be due to high IL-1Ra induction in the early stages of infection. It would be interesting to investigate the IL1-Ra/IL-1 balance for infections with more virulent strains of *Francisella*.

The role of T cells in *Francisella* infection has been investigated in protection against challenge, and T cells were required for clearance of infection [22,23]. In the infected cell supernatants, the concentrations of Th1 type cytokines INF-gamma (data not shown) and IL-2 (Fig. 4) increased between 8 and 24 h post-infection. IL-2 is associated with lymphocyte proliferation, and is a significant cytokine in secondary immune responses to *Francisella* infection in humans [24].

We detected significantly increased secretion of IL-17 in the supernatants of infected cells, albeit at low concentrations. This may be due to the very low MOI used in our infection of PBMCs. IL-17 is a cytokine secreted by CD4+ T cells and is thought to be an initiator of inflammation [25]. IL-17-producing CD4 helper cells have cell signaling pathways which are distinct from the classic Th1 and Th2 pathways [25,26]. Synthetic lipopeptides derived from the outer surface of *Borrelia burgdorferi* have been shown to produce IL-17 in both murine and human T cells [27]. At this time, the cellular component of *Francisella* LVS that results in the production of IL-17 is unknown.

IL-17A-producing cells regulate the production of GCSF, which promotes the maintenance of neutrophils, and in turn plays an important role in host defense against bacterial infections [28]. We observed that the secretion profile of GCSF along with its induction starting at 24 h post-infection was similar to that of IL-17, but at much higher concentrations (Fig. 3). IL-23, which also promotes T cells to differentiate into IL-17-secreting cells, was thought to be required for the secretion of IL-17 [29,30]. A recent report indicated that IL-23 plays a crucial role in the production of IL-17 by CD4 T cells during *Mycobacterium tuberculosis* infection, although the role of these IL-17-producing T cells in *M. tuberculosis* infection is not known [31]. Interestingly, we observed up-regulation of both IL-23p19 and IL-17A transcripts in the infected cells by 24 h post-infection in gene expression studies (unpublished data). In addition, the detected levels of secreted IL-12p40 were much higher than the total bioactive IL-12 (unpublished data), suggesting that IL-12p40 is consumed by another factor, possibly IL-23. Recently, it was reported that IL-23 was secreted by *Francisella*-infected human monocytes [11].

The IL-23/IL-17A immune pathway has been shown to be important for the rapid recruitment of neutrophils to sites of acute injury and infection [32]. Indirect evidence from our study may indicate a role of IL-23/IL-17 in host response to *Francisella*, and warrants further investigation. Another cytokine associated with IL-17, namely IL-15, was increased in infected samples at 8 and 24 h post-infection (Table 2) and has been shown to induce the production of IL-17 in human PBMCs in vitro [33].

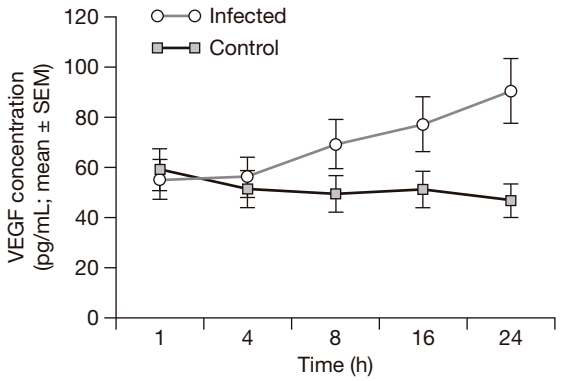

Fig. 5. Vascular endothelial growth factor (VEGF) secretion in live vaccine strain-infected human peripheral blood mononuclear cells (n = 7). SEM = standard error of the mean.

The concentrations of VEGF in the supernatant of infected cells was also elevated, increasing from 69 pg/mL at 8 h post-infection to 90 pg/mL at 24 h (Fig. 5). Studies have shown VEGF to be a significant mediator for intracellular bacteria such as mycobacteria [34] and *Bartonella henselae* [35] infection in macrophages and monocytes. Angiogenic activity in conditioned medium from *B. henselae*-infected THP-1 cultures resulted in endothelial cell proliferation comparable with recombinant VEGF treatment. Toll-like receptors 2, 4, 7, and 9, together with activating ligands, synergize with adenosine, resulting in the increased synthesis and release of VEGF [36]. At present, the role of VEGF in *Francisella* infection is not known.

In conclusion, we looked at the early inflammatory changes in total PBMCs infected with *Francisella* LVS. Our results are consistent with previous reports which have observed a dominant proinflammatory cytokine response in blood mononuclear cells exposed to *F. tularensis* in vitro [9,10]. This work indicates that a certain threshold of bacterial numbers is needed for signaling to be initiated in PBMCs, and results in a complex, regulated cytokine response, starting at 8 h post-infection.

Further work is planned to identify the source of these cytokines and compare cytokine profiles of PBMCs exposed to virulent strains of *Francisella*. The stimulus for secreted IL-17 in *Francisella*-infected

peripheral blood cells is unknown, and possible interactions of IL-17 with IL-23, GCSF and IL-15 remain to be elucidated. In the future, we plan to investigate the role of IL-17/IL-23/GCSF/IL-15 interaction, IL-1Ra/IL-1, IL-2 and VEGF in host defenses against a primary infection with *Francisella*.

We present results demonstrating that, in addition to the well-founded host proinflammatory (Th1 type) responses to *Francisella* infection, exposure of PBMCs to *Francisella* in vitro activates novel pro- and anti-inflammatory cytokines and growth factors. Our findings indicate that an intricate cytokine network is induced early in the host peripheral blood cells by *Francisella* infection.

Acknowledgments

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