Signatures of T Cells as Correlates of Immunity to Francisella tularensis

Kjell Eneslått¹, Monica Normark¹*, Rafael Björk¹*, Cecilia Rietz¹*, Carl Zingmark¹, Lawrence A. Wolfrain², Svenja Stöven¹, Anders Sjöstedt¹*

¹ Laboratory for Molecular Infection Medicine Sweden (MIMS), Department of Clinical Microbiology, Clinical Bacteriology, Umeå University, Umeå, Sweden, ² DynPort Vaccine Company, A CSC Company, Frederick, Maryland, United States of America

Abstract

Tularemia or vaccination with the live vaccine strain (LVS) of Francisella tularensis confers long-lived cell-mediated immunity. We hypothesized that this immunity depends on polyfunctional memory T cells, i.e., CD4⁺ and/or CD8⁺ T cells with the capability to simultaneously express several functional markers. Multiparametric flow cytometry, measurement of secreted cytokines, and analysis of lymphocyte proliferation were used to characterize in vitro recall responses of peripheral blood mononuclear cells (PBMC) to killed F. tularensis antigens from the LVS or Schu S4 strains. PBMC responses were compared between individuals who had contracted tularemia, had been vaccinated, or had not been exposed to F. tularensis (naïve). Significant differences were detected between either of the immune donor groups and naïve individuals for secreted levels of IL-5, IL-6, IL-10, IL-12, IL-13, IFN-γ, MCP-1, and MIP-1β. Expression of IFN-γ, MIP-1β, and CD107α by CD4⁺CD45RO⁺ or CD8⁺CD45RO⁺ T cells correlated to antigen concentrations. In particular, IFN-γ and MIP-1β strongly discriminated between immune and naïve individuals. Only one cytokine, IL-6, discriminated between the two groups of immune individuals. Notably, IL-2- or TNF-α-secretion was low. Our results identify functional signatures of T cells that may serve as correlates of immunity and protection against F. tularensis.

Introduction

There are two clinically important subspecies of Francisella tularensis, subsp. holarctica and subsp. tularensis. Strains of the former subspecies are found in many countries of the Northern Hemisphere whereas the latter only occurs in North America. Both are extremely infectious, and inhalation or intradermal inoculation of as few as 10 CFU is sufficient to cause disease in humans. A human live vaccine strain (LVS) of F. tularensis was derived from a strain of F. tularensis subsp. holarctica and developed in the United States during 1950s. It has been used in some countries for vaccination of at-risk staff and also widely used in experimental animal models.

Cell-mediated immunity (CMI) plays a crucial role in the host defense against F. tularensis [1]. Both natural infection and vaccination lead to long-lasting humoral and cell-mediated immunity. The development of an effective host resistance is paralleled by the appearance of F. tularensis-specific T cells [2,3,4]. Therefore, studies characterizing host CMI responses are important for understanding the host immunity to tularemia and, consequently, for the development of effective tularemia vaccines. In humans, specific T cells appear earlier than antibodies after the onset of disease or following vaccination with the live vaccine strain (LVS), and remain detectable for several decades thereafter [5]. As early as two weeks after vaccination, a majority of immunized volunteers exhibit an F. tularensis-specific proliferative response [6]. The T-cell responses after natural infection or vaccination in humans persist for at least 25 years, whereas levels of F. tularensis-specific antibodies are virtually undetectable two to three decades after primary infection or vaccination [4,7]. Both CD4⁺ and CD8⁺ T cells from individuals with a history of tularemia show proliferative responses and IFN-γ production to various membrane proteins of the pathogen [8,9]. The relative CD4⁺ and CD8⁺ T-cell responses were found to be equally potent [8]. This conforms well with recent studies in mice that have established the importance of both LVS vaccination-induced CD4⁺ and CD8⁺ T cells as well as of IFN-γ in protection against systemic and aerosol infection with F. tularensis subsp. tularensis strains [10,11]. These studies reaffirmed the notion that CMI is the critical determinant of F. tularensis immunity. Although IFN-γ production upon recall stimulation is a hallmark of F. tularensis immunity, it is not clear which subsets of cells are responsible for generating this cytokine and if the cytokine is necessary for effector cells. Moreover, although IFN-γ is necessary for protection in...
mice, it is not sufficient because vaccinated C57BL/6 mice that do not survive infection with *F. tularensis* subsp. *tularensis* still produce high levels of this cytokine [12].

Often T-cell responses have been characterized as the frequency of antigen-specific T cells and/or the expression of a specific effector function. However, this may be insufficient to describe their full potential. Therefore, other more multifaceted descriptions have been used recently to better describe the complexity of T-cell responses. One technique that allows more complex analyses of T-cell functions is multiparameter flow cytometry that can characterize multiple functions with regard to magnitude, phenotype, and functional capacity. A number of recent studies have elucidated cell-mediated immune responses as immune correlates of protection subsequent to various infections or after vaccination [13,14]. Collectively, the results demonstrate that the multifaceted descriptions of T-cell phenotypes show good correlation to protection and help to explain why certain functional populations of cytokine-producing T cells are critical to the host defense against infectious pathogens. Based on several models of infectious diseases, it has been suggested that proliferation of polyfunctional CD4+ or CD8+ T cells and their production of IFN-γ and IL-2 are crucial parameters to define protection [15]. One notable finding was that polyfunctional CD4+ T cells secreting IFN-γ, TNF-α, and IL-2 were found to constitute an important component of human and murine immune responses to *Leishmania* and the presence of the cell subset correlated with protection in mice [16]. However, a number of recent studies on tuberculosis have come to somewhat contradictory conclusions regarding the relevance of polyfunctional T cells [17]. Thus, much remains to be clarified about the significance of such T cells. There are no published reports describing the induction of polyfunctional T cells after LVS vaccination or after infection with *F. tularensis* in either animal models or humans.

Our aim was to characterize *F. tularensis*-specific PBMC in individuals who had had tularemia or who had been vaccinated with LVS by determining proliferative responses and secreted cytokine profiles of PBMC upon re-stimulation with *F. tularensis* antigens. Our aim was to identify features of the T-cell responses present in immune individuals that may serve as correlates of immunity, i.e., correlates that uniformly characterize the *F. tularensis*-specific immune response.

**Results**

**Statistical evaluation of effects of age, gender, and duration after onset of disease on recall stimulation responses**

We characterized *F. tularensis*-specific human T cells that responded during recall stimulation. For this purpose we collected PBMC samples from donors who were either convalescent tularemia patients, had been vaccinated with LVS, or were naive individuals. We analyzed PBMC responses to recall stimulation with killed *F. tularensis* antigens (fLVS or fSchu S4) by measuring their proliferative capacity, cytokine secretion, and the presence of polyfunctional T cells within the PBMC population. All samples were characterized by all three methods to achieve co-linear data. The data were analyzed by pair-wise comparisons of the results from each donor group; naive donors vs. vaccinees (nv/vc), naive donors vs. patients (nv/p), and vaccinees vs. patients (vc/p). In addition to data from recall stimulation with specific antigen concentrations, we also compared the increases in the responses between each antigen concentration as an indicator of the antigen specificity of the measured immune response.

By use of Spearman’s correlation test, we analyzed if the reactivity as measured by each of the three methods was affected by the age of the individuals, or by the duration between onset of tularemia and time of blood sampling. However, no significant correlations were found, indicating that the measured immune responses are quantitatively and qualitatively long-lived. In addition, we tested differences for all parameters between male and female patients using Wilcoxon’s rank-sum test, but no significant differences were found.

Responses to a mitogen, ConA, were very similar (Spearman’s correlation *P* >0.60 for all pair-wise comparisons) between the two groups of immune individuals and naive individuals (naive: 27,900 cpm±16,000; patients: 32,700 cpm±31,800; vaccinees: 36,200 cpm±21,900, *n*=8 for each group), indicating that there were no inherent differences with regard to the T-cell reactivity of each of the groups.

**Proliferative responses to *F. tularensis* antigens**

Proliferative responses after recall stimulation of PBMC from immune donors, patients or vaccinees increased with increasing antigen concentrations and were significantly higher than those of cells from naive individuals [Fig. 1, Table S1]. PBMC samples from the majority of the immune donors were maximally induced by the medium antigen concentration of 0.1 cfu fLVS/PBMC and no significant differences were observed between the two highest antigen concentrations for any of the donor groups. In addition, we did not find any significant differences between the two groups of immune donors for any antigen concentration or antigen-dependent increase (Table S1). The proliferative responses to the fSchuS4 were somewhat lower than those to the fLVS antigen, but these differences were not statistically significant (not shown). In fact, the responses to the two antigens were correlated with a highly significant Spearman’s correlation coefficient of >0.7.

**Cytokine Secretion upon Recall Stimulation**

Previously, we found that PBMC from LVS vaccinated individuals secreted higher amounts of 11 cytokines in response to recall stimulation with fLVS than did PBMC from naive individuals, although only IL-5, IL-10, IFN-γ, and MIP-1β were significantly higher [7]. Most of these cytokines had also been...
shown to be expressed during *F. tularensis*-immune responses in a mouse model [18]. Therefore, we analyzed secretion of the same cytokines. Statistically significant differences between either of the immune donor groups and naïve individuals were consistently detected for eight out of the eleven cytokines, *i.e.*, IL-5, IL-6, IL-10, IL-12, IL-13, IFN-γ, MCP-1, and MIP-1β, and their secretion increased with increasing antigen concentrations (Figs. 2 and 3, Table S2). Surprisingly, this was not true for IL-2 and only partly true for TNF-α. Only IL-6 differed between the two groups of immune donors. Curiously, in the absence of antigen, there were somewhat higher levels of MIP-1β in PBMC cultures from immune donors than in those from naïve donors.

Cytokine levels were also determined using the ffSchu S4 antigen and they were lower compared to cultures stimulated with ffLVS, although the differences were not significant (Figs. 2 and 3). These discriminating cytokines were IL-5, IL-6, IFN-γ, MCP-1, MIP-1β, IL-10, IL-12 and IL-13. Again, the responses to the two antigens were highly correlated with a Spearman’s correlation coefficient of >0.7.

Thus, a majority of the tested cytokines discriminated between the responses of the immune versus the naïve individuals. However, only one cytokine discriminated between vaccinees and patients, indicating that the priming of the immune response after vaccination with LVS closely mimics that after natural infection with subspecies *holartica*.

Identification of antigen-specific polyfunctional T cells

To identify polyfunctional *F. tularensis*-specific T cells, *i.e.*, cells that produce several effector cytokines or chemokines simultaneously, we used polychromatic flow cytometry of PBMC that had been restimulated with *F. tularensis* antigen for 48 h. Memory T cells were identified as CD3⁺CD4⁺CD45RO⁺ or CD3⁺CD8⁺ CD45RO⁺ cells, and cytokine production was detected by intracellular cytokine staining. CD45RO is a marker of memory T cells in humans [19]. IFN-γ has long been recognized as a key characteristic of the *F. tularensis*-specific immune response [8,20,21]. Accordingly, we found that essentially all of the IFN-γ expressing T cells also were CD45RO⁺ (data not shown). We detected IL-2-producing cells in the CD4⁺ and the CD8⁺ T-cell populations of PBMC from vaccinees upon recall stimulation, but their numbers differed significantly (*P*<0.05) from those of non-stimulated control samples from the same donor only in the CD8⁺ subset (Fig. S1). The low number of cells expressing intracellular IL-2 at 48 h after recall stimulation likely reflected the regulation and turnover of IL-2 in response to antigen; we found IL-2-expressing cells also after other stimulation periods (24, 72 and 96 h), but their numbers did not correlate with antigen concentrations (not shown). For TNF-α, a small population could be identified (Fig. S1). However, similar to our observation for IL-2, there was no increase in the TNF-α⁺ cell populations with increasing antigen concentrations at any of the time points tested. Hence, for the recall response of human peripheral blood T cells to ffLVS, the intracellular expression of IL-2 and TNF-α appeared to be of minor importance and this was in agreement with the low levels of secreted IL-2 and TNF-α (Figs. 2 and 3).

We then analyzed IFN-γ⁺CD45RO⁺ cell populations for co-expression of MIP-1β, CD107α and/or CD127. The surface marker CD127 (also known as IL-7Rα) has also been examined in several studies characterizing memory T cells [22,23] but its expression did not increase in an antigen-dependent fashion (data not shown). MIP-1β (macrophage inflammatory protein-1β), CCL4 is a marker of recall responses in studies on tuberculosis patients and represents a type of polyfunctional T cell [24,25]. We identified MIP-1β as one of the secreted cytokines in response to *F. tularensis* (Fig. 3). Studies on vaccine-induced polyfunctional T cells showed that surface-relocated intracellular CD107α (also known as LAMP-1) together with granzyme B and perforin could be used as indicators of the cytotoxic capacity of CD8⁺ T cells [26,27].
frequency of CD45RO+ CD4+, or CD8+ T cells expressing at least one marker, i.e. IFN-γ, MIP-1β, or CD107a, increased with higher antigen concentrations (Figs. 4, 5, 6). This increase was more pronounced in PBMC samples from immune individuals than from naïve donors (Figs. 5 and 6 vs. Fig. 4). Moreover, among the 150,000 events recorded per PBMC sample, we reproducibly identified small groups of polyfunctional CD4+ or CD8+ T cells in immune individuals that simultaneously expressed two or three of the intracellular markers. We also identified CD45RA+ T cells, predominantly a marker of naïve T cells [19]. However, there exist short-lived effector cells that also express the marker CD45RA [28]. CD45RA+ T cells that expressed one, two, or three functional markers were relatively rare and frequencies were generally lower than those of CD45RO+ T cells (Figs. 4, 5, 6). In both cell subsets, frequencies of the polyfunctional populations showed a similar antigen-dependent increase. Thus, polyfunctional T cells appeared to be part of the recall response to the F. tularensis antigen.

Median fluorescence intensity (MFI) is a measure of the expression level of a given surface or intracellular marker in a cell population. Recently, the integrated MFI (iMFI) was introduced as a measure that in addition to cell marker expression levels takes into account the frequency of cells expressing a given marker [16]. The iMFI is calculated by multiplying the frequency of positive cells by the MFI of a given marker. Statistical analysis of iMFI data following restimulation with 0.1 cfu ffLVS/PBMC demonstrated no significant differences between the two groups of immune individuals, while most iMFI values (78 of 96 tests) from the immune donors differed significantly from those of naïve donors (Fig. 7). It is noteworthy that almost all of the non-significant P values were related to CD107a-expressing T-cell subsets. Similar observations were made with respect to antigen-dependent increases; we found significantly higher increases of iMFI with increasing antigen concentrations for most of the 24 variables, 208 of 288 comparisons, from the two groups of immune individuals as compared to iMFI data from naïve donors, while we did not detect any such differences between vaccinees and patients (Fig. 7). Again, we noted that most of the non-significant P values (65 of 80 tests) originated from the iMFI of CD107a or from the iMFI of IFN-γ or MIP-1β in a CD107a+ T-cell population. Collectively, IFN-γ and MIP-1β were very useful to discriminate between the responses of F. tularensis-immune and naïve individuals, whereas CD107a expression, although it increased in an antigen-dependent fashion, appeared to be less related to the Francisella-specific recall responses.

We also compared the iMFI values after stimulation with ffLVS or ffSCHU S4. iMFI values were higher after ffLVS than after ffSCHU S4 stimulation, nevertheless, the responses were very highly correlated (not shown).

Apart from the classical CD4+ and CD8+ single positive T cells, we identified a high percentage of CD3+CD4+CD8- cells in antigen restimulated cultures for all three donor groups (17–29% of CD3 T cells), but only small percentages of CD4+CD8+ cells (<1% of CD3+ T cells). Such CD4+CD8- double positive cells are increased in autoimmune disorders [29] and they produce cytokines like TNF-α, IFN-γ, or MIP-1β upon restimulation [30]. Although few, the frequencies of CD4+CD8+ T cells in recall stimulated PBMC from immune donors correlated to the concentration of recall antigen and so did a distinct subset that expressed IFN-γ (Figs. S2A and S2B). Similar observations were made for CD107a- and for MIP-1β-expressing CD4+CD8- cells (Figs. S2C and S2D).

**Determining Correlates of Immunity**

To identify correlates of immunity that can be evaluated in animal models of tularemia, we analyzed our collected data for

---

**Figure 3. Levels of cytokines secreted by human PBMC after recall stimulation with ffLVS or ffSCHU S4 for five days.** Cytokine concentrations were measured in cell culture supernatants using multiplex analysis. Median values ± SEM from PBMC samples of 14–16 individuals per donor group are shown (black bars indicate convalescent patients; grey bars indicate LVS vaccinees; white bars indicate naïve donors). Statistically significant differences between immune and naïve donors are marked by asterisks (P<0.05). doi:10.1371/journal.pone.0032367.g003
features that accurately discriminated between PBMC responses of immune and naïve individuals (dimensional reduction). Hierarchical cluster analysis of the iMFI data for mono- and bifunctional subsets of CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells showed good separation of PBMC samples from naïve individuals from those of immune donors, since eight out of ten samples from naïve individuals clustered to one branch of the dendrogram (Fig. S3). Samples from predominantly vaccinees and patients formed another major branch in the middle and the left-most branch contained only samples from vaccinees and patients.

Problems with near co-linearity can arise in strict multivariate models with too many variables, i.e., strongly correlated variables contribute essentially the same information multiple times. Therefore, we asked whether some of the data could be disregarded without losing too much information. For each donor we compared the iMFI values of all three functional markers in all mono-, bi- and trifunctional T-cell subsets with each other and tested for correlation. Overall, iMFI values of IFN-γ and MIP-1β within the same polyfunctional T-cell subset were highly correlated (Fig. 8). Monofunctional IFN-γ⁺ or MIP-1β-expressing CD4⁺ and CD8⁺ T cells were highly correlated while this was not true for CD107a-expressing T cells. In fact, the CD107a⁺ cells were remarkably uncorrelated with the iMFI of other markers (Fig. 8). Since most polyfunctional cell subsets were gated based on their IFN-γ expression, the IFN-γ⁺ monofunctional T-cell subsets were highly correlated with a large number of bi- and trifunctional cells. Data for MIP-1β also showed this pattern, yet to a lesser degree. For the bi- and trifunctional cell subsets, most iMFI groups were strongly correlated to at least a few other groups. These observations suggest that it might be possible to select iMFI variables of a few cell subsets that contain essentially the same information as the complete data set, e.g., MIP-1β of the CD4⁺ IFN-γ⁺ subset was strongly correlated with almost all other variables and can be considered a good candidate for further modeling.

Most parameters measured from our three experimental methods, LPA, secreted cytokine analysis, and flow cytometry, each provided very good discrimination between immune and naïve individuals. The following features of human PBMC can therefore be considered correlates of immunity against F. tularensis: i) lymphocyte proliferation; ii) secretion of IL-5, IL-6, IL-10, IL-12, IL-13, IFN-γ, MCP-1 and MIP-1β; iii) IFN-γ and MIP-1β expression by CD4⁺ CD107α⁺ or CD8⁺ CD107α⁺ T cells. We asked how well these parameters predicted the immune status of a donor and whether any combination of parameters improved the predictive power. We used logistic modeling to investigate the multivariate relations between readouts from the three experimental methods. We based the modeling calculations on LPA,
secreted cytokines, and iMFI data from stimulation with 0.1 cfu fILV5/PBMC, with the aim to build models that with the smallest number of features and the highest accuracy correctly predict the immune status of a donor. We included the above listed correlates, but also those parameters that did not show significant differences between naive and immune donors, e.g. secreted levels of TNF-α in order to investigate whether the latter parameters could nevertheless contribute to increased predictive power. The models with the highest predictive power as measured by cross-validation were selected. Models with only one parameter, e.g., LPA or secreted IL-13, IFN-γ, IL-5 or MIP-1B, correctly identified the immune status of 90–97% of the individuals (Table 1). Flow cytometry data alone did not reach such accuracy, but could in combination with other data on secreted cytokines form highly accurate models. In addition, there were other combinations of two or three parameter-based models that performed as well as those listed. We also modeled the same parameters to distinguish between convalescent patients and LVS vaccinees, however, no clear pattern of separation could be found (not shown).

Thus, individual immune parameters, or combinations thereof, predicted with high accuracy the immune status of the individuals, however, they did not discriminate between the two groups of immune donors, indicating that the immune responses of these groups are very similar and may be functionally inseparable.

**Discussion**

Multi-parameter flow cytometry has been implemented recently to characterize memory T cells, and this technique has enabled detailed descriptions of the cells’ phenotypic characteristics and functional abilities. Generally, it has been argued that certain polyfunctional T cells show good correlation with improved host control of infectious disease. In several experimental models it has been found that simultaneous production of IFN-γ, TNF-α, and IL-2 by CD4⁺ or CD8⁺ T cells is crucial to protection. Recent studies of a recombinant tuberculosis vaccine based on Ag85A demonstrated that such trifunctional cells dominated the immune response [15,16,31]. In addition, a study on tuberculosis patients revealed higher numbers of such trifunctional CD4⁺ T cells in the patients compared to their exposed, but asymptomatic household contacts [32]. In contrast, another study found that cells expressing IFN-γ, TNF-α, and IL-2 were present in almost all patients with active disease, but in less than 15% of those donors with latent disease [17]. Instead, in patients with latent disease higher numbers of bifunctional T cells (IFN-γ/IL-2⁺) were found [17]. Moreover, studies of HIV and other chronic viral infections have associated trifunctional CD4⁺ and/or CD8⁺ T cells with non-disease progression [33,34]. Thus, although certain types of polyfunctional T cells play important roles as correlates of
immunity, there is no clear consensus for various diseases or for different modes of disease and more research is warranted in order to obtain a comprehensive picture.

Our results demonstrate that the immune responses of F. tularensis-immune individuals are quite distinct from those reported for tuberculosis or HIV patients. Although a previous study identified the presence of IL-2, IL-2R, and TNF-α as markers of the recall response against F. tularensis (12), we did not observe significant F. tularensis specific production of either IL-2 or TNF-α. It has been proposed that the phenotypes of identified memory T cells could be affected by the in vitro conditions utilized and that assays carried out for only 24 h would identify only recently primed cells, but not memory T cells in the resting state [35]. It has also been hypothesized that the latter cell population might be detectable only after prolonged incubation, for example six days [17,35]. Our protocol was based on the incubation of T cells for 48 h before flow cytometry analysis, and secreted cytokines were measured after five days of culture. Thus, our analyses were not based on very prolonged recall stimulation, but covered two different incubation periods. Moreover, in preliminary flow cytometry analyses we included incubation periods up to 96 h, but still did not detect any antigen-dependent increase in IL-2 or TNF-α. We believe that the length of the incubation period is an unlikely explanation for the lack of detection of IL-2, since it should precede the proliferation of the cells. It appears as if the IL-2 secreted by the T cells is minimal in this model, or has a very fast turnover. Therefore, we conclude that although we detected polyfunctional T cells after stimulation with F. tularensis antigen, these cells did not demonstrate polyfunctionality with regard to IL-2 or TNF-α, but instead produced IFN-γ, MIP-1β, and CD107a.

The importance of IFN-γ as an indicator of immunity against F. tularensis is well-documented [1] and IFN-γ secretion alone predicted the F. tularensis immune status with high accuracy. We here demonstrated that the majority of the IFN-γ producing T cells from peripheral blood are memory T cells as characterized by expression of CD45RO. This is in agreement with findings in our previous publication that characterized immune responses in vaccines [7]. In addition to the previous findings, we here demonstrate that the IFN-γ expressing cell population also displayed additional functions, i.e., expression of MIP-1β and/or CD107a, thereby showing polyfunctionality. CD107a expression was low in general, but showed an antigen-dependent increase in immune individuals. However, in several statistical comparisons we found that CD107a expression by T cells did not differentiate between immune and naïve subjects. CD107a was also less well, or not at all, correlated with expression of the other two intracellular
markers, IFN-γ and MIP-1β, which were highly correlated to each other. It is at present not clear whether there is biological significance in the observations regarding CD107a.

IFN-γ and MIP-1β were also secreted in high amounts and clearly discriminated between responses of immune vs. naive individuals. Together with the flow cytometry results, this implies that IFN-γ and MIP-1β are produced by *F. tularensis*-specific T cells as a result of recall stimulation. MIP-1β production was a parameter recurring in the mathematical models with high prediction accuracy. MIP-1β+ polyfunctional T cells have also been detected in other models of infectious diseases. For example, a tuberculosis vaccine based on modified vaccinia virus Ankara expressing antigen 85A, and the Yellow Fever vaccine induced T cells producing combinations of IFN-γ, TNF-α, IL-2, and MIP-1β [24,36]. In addition, Freel et al. demonstrated correlation between the combined expression of CD107a and MIP-1β by CD8+ T cells and anti-HIV-1 activity using a virus inhibition assay [37]. The mechanism whereby MIP-1β confers a protective effect has not been directly studied in conjunction with its proven role in the polyfunctional immune responses, however, in the case of viruses dependent on CCR5, it may act by blocking the receptor. Its role for control of bacterial infection is more elusive and a direct effector function has not been proven. However, it plays an important role for recruitment of monocytes in vitro and in vivo and thereby may contribute significantly to the protection in conjunction with other direct effectors, such as IFN-γ [38].

In agreement with the present results on the *F. tularensis*-specific T cells, expression of CD107a, IFN-γ, and MIP-1β therefore appears to be a rather ubiquitous feature of protective immune responses. Furthermore, logistic modeling revealed that several other parameters, i.e. the LPA test or secreted IL-13, MCP-1, IL-5 or IL-6, alone correctly identify the *F. tularensis*-specific immune status of >91% of the individuals. Thus, these parameters appear to be indicators of immunity to *F. tularensis* and further work will validate their utility as correlates of protection for vaccine licensing through the FDA’s Animal Rule.

Our analyses of the PBMC responses demonstrated clear discrimination between the two groups of immune donors versus...
the naïve donors for essentially all parameters. Moreover, there was an antigen-dependent increase of the PBMC responses from immune donors. Notably, for the majority of the results generated with the three methods, there were very few differences between vaccinees and convalescent patients. Altogether, our results strongly suggest that the immune responses of vaccinated individuals and patients are quantitatively and qualitatively more or less identical. Additionally, no significant differences were found between the ffLVS and ffSCHU S4 antigens. Thus, although the vaccine is a live subsp. holarctica strain, the ensuing immune response after vaccination closely resembles that after natural infection with subspecies holarctica and responses are equal to antigens from subspecies holarctica and tularensis strains [39]. With regard to the persistence of immunity upon vaccination, we recently performed an extensive analysis of CMI responses in long-term (27 years) and short-term (3 years) vaccinees and found that both donor groups exhibited essentially identical lymphocyte proliferation and secreted cytokine patterns in response to ffLVS [7]. This longevity of the T-cell memory to F. tularensis after vaccination is comparable to what has been found 25 years after tularemia [4]. Our results therefore indicate that vaccination with LVS may lead to essentially lifelong persistence of F. tularensis-specific T-cell immunity.

Collectively, our analyses demonstrate that LPA together with MIP-1β, IFN-γ, IL-5, and IL-10 secretion showed very high discriminatory power between naïve and immune donors. The flow cytometry analysis revealed that the responses of immune individuals were characterized by higher expression of MIP-1β and IFN-γ and this was true for all cell subsets. Therefore, all of these parameters are correlates of immunity and may be relevant as potential correlates of protection as well. For ethical reasons it is unlikely that challenge studies with virulent strains will be performed in humans to assess vaccine efficacy. We therefore
have to rely on relevant animal models in order to validate these parameters as correlates of protection since the FDA's Animal Rule allows for licensing of vaccines against rare but lethal pathogens by use of relevant animal models for efficacy testing.

Materials and Methods

Ethics Statement

Ethical approval, 05–166 M, was obtained from the Regional Ethical Review Board in Umeå, Sweden, and a written informed consent was obtained from all individuals included in the study.

Blood Donors

Individuals included in the study had either previously been diagnosed with tularemia caused by F. tularensis subsp. holarctica (convalescent patients, p), been vaccinated with LVS (vaccinees, vc), or had no anamnestic data on LVS vaccination, tularemia, or occupational exposure to F. tularensis (naive, nv). Convalescent patients had contracted tularemia in Sweden one to 32 months before blood donation (mean time 16.1 ± 10.6 months). All vaccinees had been given the same lot of LVS, designated NDBR 101, lot no. 11. The mean age and sex distribution of each group was the following: naive individuals 36.5 years (10 females, 5 males), vaccinees 44.5 years (10 females, 7 males), and convalescent patients 53.4 years (6 females, 10 males). A detailed description is provided in Table S3.

PBMC Collection and Cryopreservation

Venous blood from donors was collected using CPT-tubes (Becton Dickinson, NJ, USA) and PBMC were prepared according to the manufacturer’s recommendations. PBMC samples were cryopreserved as follows: the cells were resuspended in freezing medium (80% heat-inactivated human serum [Innovative Research, MI, USA], 20% dimethyl sulfoxide) at a concentration of 10^7 PBMC/mL. Cells were aliquoted into 1 mL cryo-vials, and transferred to long term storage in liquid nitrogen using a NALGENE cryo 1°C Freezing Container. To thaw PBMC samples, selected aliquots were quickly thawed at 37°C and diluted, first in washing media (10% heat-inactivated fetal calf serum, 40 μg/mL of gentamicin in RPMI 1640 medium [ Gibco/Invitrogen]), then in culture medium (10% heat-inactivated human serum, 40 μg/mL gentamicin in RPMI 1640 medium). Cells were allowed to recover overnight; cell viability and the cell recovery rate were determined prior to downstream functional assays.

Recall Stimulation and Lymphocyte Proliferation Assay (LPA)

For LPA and multiplex cytokine analysis, PBMC were seeded at 2 x 10^5 cells/well in 100 μL culture medium per well in 96-well plates. For each flow cytometry analysis, four wells of a 96-well plate with 4 x 10^5 cells per well were used. Cells were stimulated with formalin-fixed LVS (fLVS) or Schu S4 antigen at final concentrations of 0.02, 0.1, or 0.5 cfu/PBMC or without antigen and incubated for two (flow cytometry) or five days (LPA and multiplex cytokine analysis) at 37°C in a humidified atmosphere with 5% CO2. We used the mitogens Concanavalin A and Phytohemagglutinin as control antigens. PBMC was performed by thymidine incorporation in triplicates as previously described [4]. The mean of each triplicate for each antigen concentration was normalized by dividing it by the corresponding value for non-stimulated control cultures from the same PBMC sample to obtain a stimulation index.

Multiplex Cytokine Analysis

Cell culture supernatants, 80 μL, were collected from the same cell cultures as used for LPA and stored frozen at −80°C until analyzed using two custom-made multiplex kits and a Bio-Plex 200 system (BioRad Laboratories Inc, Hercules, CA, USA) according to the manufacturer’s instructions. A 5-plex kit and 30-fold diluted supernatants were used to determine the levels of MIP-1, MCP-1, IL-6, IFN-γ and TNF-α (high level cytokines), and a 6-plex kit in combination with two-fold diluted supernatants were used to measure IL-2, IL-3, IL-7, IL-10, IL-12p70 and IL-13 (low level cytokines). Estimated cytokine concentrations outside the range of the standard curve were censored to the nearest standard value. Samples were analyzed in duplicate.

Flow cytometry analysis of surface markers and intracellular cytokine staining

After 44 h of recall stimulation, 5 μg/mL of Brefeldin A was added to the PBMC cultures. Four h later, plates were centrifuged for 3 min at 500 x g and supernatants were removed. Cells were prepared for labeling with cell surface marker monoclonal antibodies (mAb) or conjugated intracellular cytokine mAb as recommended by BD Biosciences. The following mAb conjugates were used: CD3-AlexaFluor700 (cloneUCHT1, BD Biosciences), CD4-PE, Texas red (clone S3.5, Caltag/Invitrogen), CD8-PerCPCy5.5 (cloneSK1, BD Biosciences), CD45RO-PECy7 (cloneUCHL-1, BD Biosciences), CD45RA-APC57 (clone4KB5, Santa Cruz Biotechnology), IFN-γ-FITC (clone25723.11, BD Biosciences), MIP-1β-PE (clone D21-1351, BD Biosciences), CD107a-PE-Cy5.5 (cloneHI30, BD Biosciences), CD127-Alexa647 (cloneHIL-7R-M21, BD Biosciences), IL-2-PE (clone5344.111, BD Biosciences), TNF-α-APC (cloneMB11, BioLegend). Aqua Viability Dye (Molecular Probes/Invitrogen) was added to distinguish live and dead cells. PBMC from four wells were combined and 150,000 events were acquired for each analysis using an LSRII flow cytometer (BD Biosciences) with FACSDiva software (BD Biosciences). We developed software, which will be referred to as “Clust”, that performed semi-automated sequential gating to identify cell subsets of interest ([Fig. S4] and [Text S1]). In brief, Clust uses both cluster analysis and filtering strategies with rectangular filters to select positive cell subsets. The first three gating steps in all use cluster analysis to subsequently define live cells, T cells, and CD3+CD4+ or CD3+CD8+ cell populations, respectively. To identify cell subsets positive for the functional markers IFN-γ, MIP-1β and CD107a, we used a filtering strategy where the filter was rectangular and had fixed coordinates in the different channels. The gating procedure included a normalization step in which the lower filter coordinates were adjusted to the cell distribution in the non-stimulated control sample.

Data Analysis and Statistical Methods

We assumed that the data was not normally distributed and used Wilcoxon’s rank sum test, or for paired data, Wilcoxon’s signed rank test, to identify significant differences (P<0.05) between data sets. To test whether two data sets showed the same trend and whether they were correlated we used Spearman’s rank correlation test. A correlation with a coefficient ($R_d$) above 0.4 was considered high, and a coefficient above 0.7 was considered to indicate very strong correlation. To analyze the antigen-dependent increase in CMI responses, differences were calculated by subtracting the response to a low antigen concentration from the response to a high antigen concentration. Group-wise comparison between donor groups was performed using Wilcoxon’s rank sum test. Logistic regression, using log-
transformed data, and principle component analysis were used for modeling. Missing values in the LFA, secreted cytokines, or integrated median fluorescence intensity (iMFI) data, which occurred mainly in the naive donor group due to values below the detection limit, were imputed by repeated random sampling from non-missing values for the same donor group. In total, the analyses were based on 32 individuals; 11 vaccinees, 12 patients, and 9 naive donors. To reduce the complexity of modeling, iMFI analyses were based on 32 individuals; 11 vaccinees, 12 patients, from non-missing values for the same donor group. In total, the Francisella 1. Cowley S, Elkins K (2011) Immunity to (black bars) or CD4
CD8
CD8
CD8

Figure S2 Frequency of CD3

Figure S1 Frequency of IL-2

Supporting Information

Figure S1 Frequency of IL-2+(upper graphs) or TNF-α+(lower graphs) T cells and CD4+(left graphs) or CD8+(right graphs) T cells after stimulation with graded antigen concentrations. Median values ± SEM are shown for 1–5 naive individuals (white bars) or 7–11 LVS vaccinees (black bars).  

Figure S2 Frequency of CD3+CD4+CD8+ or CD3+CD4−CD8− T cells after stimulation with graded antigen concentrations. A, Percentage of CD4+CD8+ cells of the total CD3+ lymphocyte population. B–D, Percentage of CD4+CD8+ (black bars) or CD4+CD8−T cells (white bars) that express at least one intracellular marker, IFN-γ, CD107a, or MIP-1β, respectively. The bars indicate (from left to right) antigen concentrations of 0, 0.02, 0.1, and 0.05 ffLVS/PBMC. Mean values per donor group are shown.  

Figure S3 Hierarchical cluster analysis of iMFI values of intracellular markers in CD3+CD4+ or CD3+CD8− T-cell subsets in response to 0.1 cfu ffLVS/PBMC. Integrated MFI values were obtained for all three functional markers (IFN-γ, MIP-1β, CD107a) in all mono- and bifunctional T-cell subsets (18 values per PBMC sample) and for all donors using Clust semi-automated gating. 

Table S1 Probability values for the comparison of proliferative responses to ffLVS by PBMC from naïve individuals (nv), vaccinees (ve) and patients (p). 

Table S2 Probability values for the comparison of the antigen-dependent increase (0–0.1 cfu ffLVS/PBMC) in cytokine levels secreted by PBMC from naïve individuals (nv, 11–13 donors), vaccinees (ve, 11–15 donors) or patients (p, 14–15 donors). 

Table S3A–S3F Parameters for Clust semi-automated gating. Table S3A: Parameters for logicle transformation. Table S3B: Parameters for ‘mixFilter’ object for clustering live cells. Table S3C: Parameters for ‘mixFilter’ object for clustering CD3+ cells. Table S3D: Parameters for ‘mixFilter’ object for clustering CD3+CD4+. Table S3E: Parameters for ‘mixFilter’ object for clustering CD3+CD8+. Table S3F: Lower and upper boundaries for gating multifunctional populations. 

Text S1 Description of the semi-automated gating method. 

Acknowledgments

The work was performed in part at the Umeå Centre for Microbial Research (UCMR). We thank Dr. Patrik Rydén for insightful advice on the statistics and Dr. Shannon Martin for helpful comments on the manuscript. 

Author Contributions

Conceived and designed the experiments: KE CR LW AS. Performed the experiments: KE CR CZ. Analyzed the data: KE CR SS AS MN RB. Wrote the paper: AS SS. Performed the statistical analysis: MN RB. 

References

1. Cowley S, Elkins K (2011) Immunity to Francisella. Front Microbiol 2: 26. doi: 10.3389/fmicb.2011.00026. 
2. Kostiala AA, McGregor DD, Logie PS (1975) Tularemia in the rat. I. The cellular basis on host resistance to infection. Immunology 28: 855–869. 
3. Anthony JS, Kongsphavn PA (1987) Experimental murine tularemia caused by Francisella tularensis, live vaccine strain: a model of acquired cellular resistance. Microb Pathog 2: 3–14. 
4. Ericson M, Sandstrom G, Spjestedt A, Tarnvik A (1994) Persistence of cell-mediated immunity and decline of humoral immunity to the intracellular
Correlates of Immunity to *Francisella tularensis*

5. Tarvik A (1989) Nature of protective immunity to *Francisella tularensis*. Rev Infect Dis 11: 440–451.
6. Tarvik A, Löfgren ML, Löfgren S, Sandstrom G, Wolf-Watz H (1985) Long-lasting cell-mediated immunity induced by a live *Francisella tularensis* vaccine. J Clin Microbiol 22: 527–530.
7. Erlank K, Rietz C, Rydén P, Stöven S, Housse RV, et al. (2011) Persistence of cell-mediated immunity three decades after vaccination with the live vaccine strain of *Francisella tularensis*. Eur J Immunol 41: 974–980.
8. Sjöstedt A, Eriksson M, Sandström G, Tarvik A (1992) Various membrane proteins of *Francisella tularensis* induce interferon-gamma production in both CD4+ and CD8+ T cells of primed humans. *Immunology* 76: 384–392.
9. Sercel K, Syrja H, Hartmann T, Tapaninaho S, Hurvea E (1991) Development of *Francisella tularensis* antigen responses measured as T-lymphocyte proliferation and cytokine production (tumor necrosis factor alpha, gamma interferon, and interferon-2 alpha) during human tularemia. Infect Immun 59: 1948–1953.
10. Cowley SC, McEvoy AE, Frelenger JA, Iwakura Y, Elkins KL (2010) Lung CD4+ CD69− double-negative T cells are prominent producers of IL-17A and IFN-gamma during primary respiratory murine infection with *Francisella tularensis* live vaccine strain. J Immunol 184: 5791–5801.
11. Elkins KL, Cowley SC, Bosio CM (2007) Innate and adaptive immunity to *Francisella*. Ann N Y Acad Sci 1105: 204–324.
12. Conlan JW (2008) Vaccines against *Francisella tularensis* - past, present and future. Expert Rev Vaccines 7: 307–314.
13. Millington KA, Innes JA, Hackforth S, Hinks TS, Deeks JJ, et al. (2007) Dynamic relationship between IFN-gamma and IL-2 profile of *Mycobacterium tuberculosis* specific T cells and antigen load. J Immunol 178: 5217–5226.
14. Winkler S, Nevek L, Winkler H, Adeglova AA, Perkann T, et al. (2005) Increased specific T cell cytokine responses in patients with active pulmonary tuberculosis from Central Africa. *Microbes and Infect* 7: 1161–1169.
15. Seder RA, Darrah PA, Roederer M (2008) T-cell quality in memory and potential for long-term survival of both CD62Lhigh central memory T cells and Th1 effector cells during Leishmania major infection. *J Immunol* 182: 5702–5711.
16. Conlan JW, Oyston PC (2007) Vaccines against *Francisella tularensis*. Ann NY Acad Sci 1105: 325–350.

Bacteria: *Francisella tularensis* 25 years after natural infection. J Infect Dis 170: 110–114.

5. Tarvik A (1989) Nature of protective immunity to *Francisella tularensis*. Rev Infect Dis 11: 440–451.
6. Tarvik A, Löfgren ML, Löfgren S, Sandstrom G, Wolf-Watz H (1985) Long-lasting cell-mediated immunity induced by a live *Francisella tularensis* vaccine. J Clin Microbiol 22: 527–530.
7. Erlank K, Rietz C, Rydén P, Stöven S, Housse RV, et al. (2011) Persistence of cell-mediated immunity three decades after vaccination with the live vaccine strain of *Francisella tularensis*. Eur J Immunol 41: 974–980.
8. Sjöstedt A, Eriksson M, Sandström G, Tarvik A (1992) Various membrane proteins of *Francisella tularensis* induce interferon-gamma production in both CD4+ and CD8+ T cells of primed humans. *Immunology* 76: 384–392.
9. Sercel K, Syrja H, Hartmann T, Tapaninaho S, Hurvea E (1991) Development of *Francisella tularensis* antigen responses measured as T-lymphocyte proliferation and cytokine production (tumor necrosis factor alpha, gamma interferon, and interferon-2 alpha) during human tularemia. Infect Immun 59: 1948–1953.
10. Cowley SC, McEvoy AE, Frelenger JA, Iwakura Y, Elkins KL (2010) Lung CD4+ CD69− double-negative T cells are prominent producers of IL-17A and IFN-gamma during primary respiratory murine infection with *Francisella tularensis* live vaccine strain. J Immunol 184: 5791–5801.
11. Elkins KL, Cowley SC, Bosio CM (2007) Innate and adaptive immunity to *Francisella*. Ann N Y Acad Sci 1105: 204–324.
12. Conlan JW (2008) Vaccines against *Francisella tularensis* - past, present and future. Expert Rev Vaccines 7: 307–314.
13. Millington KA, Innes JA, Hackforth S, Hinks TS, Deeks JJ, et al. (2007) Dynamic relationship between IFN-gamma and IL-2 profile of *Mycobacterium tuberculosis* specific T cells and antigen load. J Immunol 178: 5217–5226.
14. Winkler S, Nevek L, Winkler H, Adeglova AA, Perkann T, et al. (2005) Increased specific T cell cytokine responses in patients with active pulmonary tuberculosis from Central Africa. *Microbes and Infect* 7: 1161–1169.
15. Seder RA, Darrah PA, Roederer M (2008) T-cell quality in memory and potential for long-term survival of both CD62Lhigh central memory T cells and Th1 effector cells during Leishmania major infection. *J Immunol* 182: 5702–5711.
16. Conlan JW, Oyston PC (2007) Vaccines against *Francisella tularensis*. Ann NY Acad Sci 1105: 325–350.

23. Burgers WA, Kuus C, Mlotshwa M, Marnetje P, de Visser D, et al. (2009) Association of HIV-specific and total CD8+ T memory phenotypes in subtype C HIV-1 infection with viral set point. *J Immunol* 182: 4751–4761.
24. Bezuidenhout NC, Paizie DS, Venter J, Swanepoel R, Coetzee TD, et al. (2007) Immune stimulation with recombinant HIV-1 gag proteins induces long-lasting, polyfunctional *Mycobacterium tuberculosis*-specific CD4+ memory T lymphocyte populations. *Eur J Immunol* 37: 3089–3100.