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Generation of heterogeneous memory T cells by live attenuated tularemia vaccine in humans

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Abstract

There is very limited evidence concerning the phenotype, function, and homing characteristics of memory T (T_M) cells elicited by vaccination against intracellular bacteria in humans. Here we studied T_M subsets elicited by exposure to *Francisella tularensis* in humans as a model of immunity to intracellular bacteria. To this end, T_M cells were evaluated in 2 groups: (1) subjects immunized with live attenuated tularemia vaccine by skin scarification and (2) tularemia naturally-infected subjects. In both groups the immune responses were mediated by $CD4^+$ and $CD8^+$ effector T_M cells, mostly $CD45RA^-CD62L^-$ and $CD45RA^+CD62L^-$. Based on the expression of CD27, integrins $\alpha_4\beta_7$, and $\alpha_4\beta_1$, it is likely that some of these T_M cells have lytic potential and the ability to enter both mucosal and non-mucosal sites. Thus, regardless of whether by immunization or natural exposure, tularemia antigens elicited a broad spectrum of specific T_M subsets with diverse homing characteristics.

Keywords

T cells; Bacteria; Vaccination

1. Introduction

One of the hallmarks of immunity against intracellular pathogens is the induction of memory T (T_M) cells. This process involves proliferation and differentiation of primed T cells into T_M cells with the ability to home to a variety of tissues. Based on the expression of defined surface molecules, T_M cells can be sub-divided into two main subsets: central memory T cells (T_{CM} , $CD45RA^-CD62L^+$) and effector memory T cells (T_{EM} , $CD45RA^-CD62L^-$ or T_{EMRA} , $CD45RA^+CD62L^-$) [1–3]. The T_{EM} re-circulate primarily through non- lymphoid tissues, whereas T_{CM} re-circulate primarily through lymph nodes, but both subsets are found in the blood [4]. Both subsets are equally good at producing IFN- γ and TNF- α , while IL-2 appears to be produced exclusively by T_{CM} [4,5]. However, there is significant phenotypic heterogeneity within each subpopulation; $CD8^+$ T_{EM} in mouse and human express a stronger direct *ex vivo* lytic activity than T_{CM} , whereas $CD4^+$ T_{EM} exhibit stronger effector cytokine

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production than T_{CM} [4]. The protective potential of each subset is also controversial; whereas studies in mice infected by *Lymphocytic choriomeningitis virus* (LCMV) show that T_{CM} confer the more effective protective immunity [6], protection against Sendai virus infection in the lung [7] requires T_{EM}. Moreover, when comparing T_M subsets obtained after vaccination, striking differences were also observed. Studies in humans have shown that proliferating vaccinia-specific T cells display the characteristics of T_{EM} decades after smallpox vaccination [8], while high rates of proliferating hepatitis B-specific T cells were found to be T_{CM} [9]. Although most of our knowledge of human memory-phenotype CD4⁺ and CD8⁺ T cells comes from analyses of lymphocytes from peripheral blood, the population dynamics and functional status of T_M subsets in the human blood remain poorly characterized.

Here, we evaluated the PBMC from subjects that were exposed to tularemia antigens by two different routes, 1) immunization with tularemia live attenuated vaccine (*F. tularensis Live Vaccine Strain*, LVS) by skin scarification, and 2) naturally infected-subjects with primary pneumonic tularemia, as a model to study T_M subsets in human blood. This model is particularly relevant because *Francisella tularensis* (*F. tularensis*), the causative agent of tularemia, is a CDC Category A threat organism of great public health importance. In addition, tularemia induces vigorous and long-lasting T-cell immune responses in humans. These responses are mediated by both CD4⁺ and CD8⁺ T cells and persist for up to 25 years, and are as vigorous as the responses to purified protein derivative (PPD) of *Mycobacterium tuberculosis* [10–12]. However, to our knowledge, the study of T memory subsets and their homing potential has not been explored in immunity to *F. tularensis* in either mice or humans. Specifically, we studied *ex vivo* IFN- γ -secreting and proliferating T_M cells in human peripheral blood following LVS immunization by scarification, as well as in tularemia naturally infected-subjects via aerosol by examining their phenotype, function and homing potential characteristics. We found that regardless of the site of exposure, *F. tularensis* elicited a broad spectrum of specific CD4⁺ and CD8⁺ T_M with diverse homing characteristics.

2. Methods

2.1. Subjects

Five healthy adult LVS vaccinees (volunteers 34, 36, 37, 40 and 42), three *F. tularensis* naturally-infected subjects (F0715V1, F0717V1 and F0716V1) and two healthy adult controls (CVD4000#89 and CVD4000#76) participated in this study. The LVS vaccinees involved laboratory personnel at an increased risk of infection with *F. tularensis*. These subjects were recruited from the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID), Frederick, Maryland. All vaccinees had positive “takes” (initial formation of small papule which ulcerates). Before blood collections, all volunteers were explained the purpose of this study and signed informed consents. The human experimentation guidelines of the US Department of Health and Human Services and those of the USAMRIID, were followed in the conduct of the present clinical research. The vaccinees donated blood before (day 0) and at days 15, 28 and 60 after immunization. The *F. tularensis* naturally-infected subjects, aged 45–60 years, were recruited at the Martha’s Vineyard Hospital at Oak Bluffs, Massachusetts. PBMC from these subjects were isolated at EpiVax, Inc., Providence, Rhode Island. Infection with *F. tularensis* was confirmed in all subjects by serology. Before blood collections, all subjects were explained the purpose of this study and signed informed consent. The human experimentation guidelines of the US Department of Health and Human Services and those of the EpiVax, Inc., were followed in the conduct of the present clinical research. They donated blood 2–>25 years after being diagnosed with tularemia (subject FV0715V1: 2–5 years, subjects FV0716V1 and F0717V1: >25 years). The diagnosis was pneumonic tularemia in subjects FV0716V1 and F0717V1 and unknown in subject FV0715V1. PBMC from the CVD4000#89 and CVD4000#76 adult healthy volunteers were collected at the Center for

Vaccine Development, U. Maryland, Baltimore, under the Blood Donor CVD 4000 protocol. The human experimentation guidelines of the US Department of Health and Human Services and those of the University of Maryland, Baltimore, were followed in obtaining these specimens. In all cases, PBMC were isolated from the blood of volunteers by density gradient centrifugation and cryopreserved in liquid N₂ following standard techniques.

2.2. Antigens

The heated and formalin-killed *F. tularensis* LVS strain antigenic preparation (NR-74) used in these studies was obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH. Briefly, the bacterial lawn was fixed in 3% formalin, heat-inactivated in a water bath at 60 °C for 30 min and washed 3 times with PBS. This antigenic preparation was used at a concentration of 10⁷ CFU/ml (CFU determinations were performed before killing). This heated and formalin-killed LVS preparation is the same as the one currently being employed to assess immune responses in phase 1 and 2 clinical trials with a new formulation of the LVS vaccine. A *Coxiella* particulate antigenic preparation (provided by Baylor College of Medicine, Houston, Texas) and *Staphylococcus* enterotoxin B (SEB) (Sigma, St. Louis, MO) were used as negative and positive controls, respectively.

2.3. Culture conditions

The cells were stimulated with LVS (10⁷ CFU/ml concentration) antigen at 37 °C, 5% CO₂. Cells stimulated with *Coxiella* (8.3 ng/ml) or SEB (Sigma, 10 µg/ml) were used as negative and positive controls, respectively. The culture medium consisted of RPMI (Gibco, Grand Island, New York) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamicin, 2 mM L-glutamine, 2.5 mM sodium pyruvate, 10 mM HEPES buffer, 1% non-essential aminoacids, and 2% heat-inactivated AB human serum.

2.4. Quantification of Cytokines by Cytometric bead array (CBA)

The IFN-γ, IL-2 and TNF-α cytokine production by PBMC was measured using the flow cytometry-based BD Cytometric bead arrays (CBA) human kit assays (Becton-Dickinson – B-D, San Diego, CA) as previously described [13]. Briefly, PBMC were stimulated with LVS and the supernatants harvested after 16–18 hours and kept at –70 °C until assayed. CBA assays were carried-out following the manufacturer's instructions. Based on linearity, the levels of sensitivity for the various cytokines ranged from 2.5–10 pg/ml. The cut-off for CBA assays was established as the median of the background obtained with the cells stimulated with *Coxiella*. Results with PBMC obtained from vaccinees were scored positive if after background (*Coxiella*-stimulated cultures) subtraction the data from at least one point after immunization was greater than two fold compared with those before immunization.

2.5. Monoclonal antibodies for surface staining

The following mAb to surface molecules were used to stain PBMC: cutaneous lymphocyte antigen (CLA)(clone HECA-452), CCR4(clone 1G1), CD3(clone UCHT1), CD4(clone SK3), CD8(clone SK1), CD45RA(clone HI100) and CD62L(clone Dreg-56), all from B-D Pharmingen; CD14(clone RMO52), CD19(clone J4.119), CD27(clone 1A4CD27) and CD49d(clone IM1404), all from Beckman-Coulter (Miami, FL); and integrin α₄/β₇(clone Act-1)(kindly provided by W. Newman, Leukosite, Cambridge, MA) conjugated to Alexa 647 using an Alexa 647-labeling kit (Molecular probes, Eugene, OR). Monoclonal antibodies or their corresponding isotype controls conjugated to the following fluorochromes were used in these studies: FITC, ECD (Energy Coupled Dye), PE-Cy5, PE-Cy7, Alexa 647, allophycocyanin (APC)-Alexa 700, APC-Cy7, Pacific blue and biotin followed by streptavidin-Pacific Orange (Molecular Probes).

2.6. IFN- γ intracellular staining

After 16–18 hours of stimulation with LVS, protein transport was blocked by adding BD GolgiStop (B-D Pharmingen) and the cells were incubated for an additional 5 hours at 37°C as previously described [13–15]. Cells were then stained with ethidium monoazide (EMA) (Molecular probes), followed by staining with monoclonal antibodies against surface antigens, fixation/permeabilization by using cytofix/cytoperm solution (B-D Pharmingen) and stained intracellularly for IFN- γ (B-D Pharmingen, clone B27). Cells were then resuspended in fixation buffer (1% formaldehyde) and analyzed as soon as possible by flow cytometry. PBMC from healthy subjects (CVD4000#76 or CVD4000#89) were used as internal controls in experiments using specimens from naturally-infected subjects.

2.7. Proliferation assays by Ki67 intracellular staining

The ability of PBMC to proliferate was measured by intracellular staining with a mAb to Ki67 (PE, clone B56)(B-D Pharmingen), a protein expressed in dividing cells [16]. After 7 days of stimulation with LVS, cells were stained with EMA, followed by staining with mAb against surface antigens, fixation/permeabilization by using cytofix/cytoperm solution (B-D Pharmingen), and stained intracellularly for Ki67 as previously described [17]. Cells were then resuspended in fixation buffer (1% formaldehyde) and analyzed as soon as possible by flow cytometry.

2.8. Flow Cytometry Analyses

During sample acquisition, 100,000–500,000 events were collected in the forward and side scatter (FS/SS) lymphocyte gate. This large number of gated lymphocyte events was necessary to ensure that a sufficient number of positive cells for a defined subset would be collected for each tube analyzed. A “dump” channel was used to eliminate dead cells (EMA⁺), as well as macrophages/monocytes (CD14⁺) and B lymphocytes (CD19⁺) from analysis. Specimens were included in the analysis if (1) the cell viability was >50% after an overnight incubation (the viability of PBMC after overnight incubation ranged from 60 to 100% [median 91]), and (2) cells were shown to be functionally active as determined by the production of IFN- γ by at least 0.2% CD3⁺ cells after stimulation with SEB (the frequency of IFN- γ -producing cells ranged from 0.5 to 1.9% for total CD4⁺ [median 1.6] and from 5.1 to 18.4% for total CD8⁺ cells [median 9.7]) [18]. The list-mode data files were analyzed using the Win-List 3D software (Verity Software House, Topsham, ME). A response was considered specific if the differential in the number of positive events between experimental (LVS) and negative control (*Coxiella*) cultures was significantly increased by Chi-square tests. Vaccinees were considered responders if the results from at least one time point after immunization were greater than 2.0 fold from those observed before immunization.

2.9 Statistical analysis

All tests were performed using SigmaStat software (version 3.10, SSPS Science software products, Chicago, IL). Comparisons between groups were performed using One-way ANOVA tests. P values <0.05 were considered significant.

3. Results

3.1. Measurements of IFN- γ , IL-2 and TNF- α production as a surrogate marker of cell mediated immune (CMI) responses

Previous human studies showed that IFN- γ , IL-2 and TNF- α are secreted in response to inactivated *F. tularensis* [10,19]. Thus, in the present studies we used IFN- γ , IL-2 and TNF- α production as surrogate markers for CMI responses. PBMC were stimulated with LVS (10^7 CFU/ml) for 16–18 hours. The LVS concentration of 10^7 CFU/ml was selected based on

preliminary experiments showing that it induced the highest numbers of specific effector cells with the best viability, as indicated by their ability to exclude Trypan blue (data not shown). We selected 16–18 hours to minimize bystander stimulation while maintaining a close parallel with the *in vivo* cytokine response [20]. After 16–18 hours, supernatants were collected and IFN- γ , IL-2 and TNF- α production analyzed by using flow cytometry-based B-D Cytometric bead arrays (CBA). The cut-off for specific responses to LVS in CBA assays was established as the median of the background obtained when cells were stimulated with the control antigen *Coxiella* (2.5, 38.7 and 2.5 pg/ml for IL-2, TNF- α and IFN- γ respectively). Vaccinees were considered responders if the results from at least one time point after immunization were greater than 2.0 fold above those observed before immunization. In agreement with previous studies [10,19], we consistently observed increases in IFN- γ and TNF- α in all 5 subjects. However, substantial increases in IL-2 production were only detected in 3 subjects (Figure 1A). It is also important to note that there was considerable variation in the strength of responses among vaccinees but not among control subjects. This is not surprising since differences in the degree of responses among individuals is a common finding in human studies [13,15,21]. Finally, similar results were observed in *F. tularensis* naturally infected-subjects (Figure 1B and 1C). Having confirmed the presence of CMI responses in these specimens, we next focused our studies on the kinetics of induction of memory T cells.

3.2. Kinetics of ex vivo IFN- γ -secreting and proliferating memory T cells obtained from LVS vaccinees

To determine the kinetics of induction of CD4⁺ and CD8⁺ T cells implicated in the induction of the Th1 responses described above, we studied IFN- γ production by intracellular staining in PBMC stimulated with LVS 10⁷ for 16–18 hours. Because cell frequencies were expected to be low and because particulate antigens, like the ones used in this study (LVS), are known to induce non-specific responses, the specificity and sensitivity of the flow cytometric assays were first carefully established. As described in methods, specific responses were defined as those in which the differential in the number of positive cell events by flow cytometry between experimental and unrelated particulate negative control (*Coxiella*) was significantly higher by Chi-square tests. Cell number is another important parameter to consider when low frequency is expected. To this end, we first determined the median percentage of IFN- γ -producing cell frequency of specimens stimulated with the control *Coxiella* antigen using PBMC from all 5 LVS vaccinees evaluated at 4 time points (n=20). These frequencies were found to be 0.03% and 0.07% for CD4⁺ and CD8⁺ cells, respectively (data not shown). This threshold was used to define the sensitivity of the test. Finally, as a third criteria, the results from at least one time point after immunization needed to be greater than 2.0 fold compared to those recorded before immunization. Similar parameters were also used to define positive proliferative responses as determined by Ki67 staining. The median Ki67⁺ cell frequencies of specimens (n=20) stimulated with control *Coxiella* antigen were 1.18% and 2.31% for CD4⁺ and CD8⁺ cells, respectively (data not shown). It is also important to note that in positive control cultures (PBMC incubated with SEB) the frequency of IFN- γ -producing cells ranged from 0.5 to 1.9% for total CD4⁺ (median 1.6) and from 5.1 to 18.4% for total CD8⁺ cells (median 9.7), and the frequency of Ki67-producing cells ranged from 2.1 to 9.0% for total CD4⁺ (median 5.8) and from 4.6 to 11.6% total CD8⁺ cells (median 10.1) (data not shown).

Following immunization and after background subtraction, increases in IFN- γ -secretion were recorded in 2 of the 5 vaccinees (Figure 2). In contrast to the relatively low number of vaccinees with IFN- γ -secreting cell responses, after 7 days of *in vitro* antigen exposure, tularemia-specific proliferative responses were detected in the PBMC of 4 of the 5 vaccinees. Of note, negative responses for both IFN- γ -secreting cells and proliferative responses were observed in 1 of the 5 vaccinees (vol#42). The simultaneous evaluation of both functions showed that (a) when IFN- γ -secreting cell responses were detectable, they were associated with proliferative

responses [vol#34 and vol#36], and (b) 2 out of 5 vaccinees displayed only an expandable pool of memory T cells (i.e., proliferation in the absence of IFN- γ -secreting cell responses; vol#37 and vol#40). It is important to note that proliferative responses were somewhat lower among the group without IFN- γ -secreting cell responses. Both IFN- γ and proliferative responses were observed in CD4⁺ and CD8⁺ subsets, with the relative proportions of these responses differing among vaccinees. Peak responses were generally observed at days 28 and 60 after immunization (Figure 2). Taken together, these results suggest that IFN- γ -secreting T_M cells and the expandable pool of T_M cells had distinct patterns of long-term maintenance.

3.3. Identification of specific memory T cell subsets

To investigate the relationship between the observed kinetic patterns in antigen-specific responses and changes in the frequency and phenotype of the T_M cell subsets in peripheral blood, a detailed phenotypic analysis using 10-color flow cytometry was carried out in all individuals that responded to LVS at the peak of the response and then these measurements were compared with those recorded before immunization. Using the generally accepted definition to classify the T cells into the three main subsets, naïve (CD45RA⁺CD62L⁺), central memory T cells (T_{CM}, CD45RA⁻CD62L⁺) and effector memory T cells (T_{EM}, CD45RA⁻CD62L⁻ or T_{EMRA}, CD45RA⁺CD62L⁻) [1–3], we found that among vaccinees, most IFN- γ -secreting CD4⁺ and CD8⁺ exhibited a phenotype consistent with T_{EM} and T_{EMRA} subsets, whereas only a few IFN- γ + cells exhibited a phenotype consistent with naïve or T_{CM} cells. A representative example of vaccinees' responses is shown in Figure 3. To determine whether the distribution of these memory subsets was altered in our study population, we examined the *ex vivo* expression of CD45RA⁺ and CD62L⁺ on CD4⁺ and CD8⁺ T cell populations. After 16–18 hours of stimulation with *Coxiella* (negative control), no significant differences were observed in the % of CD4⁺ and CD8⁺ T_M subsets among PBMC obtained before and after vaccination (Figure 4). Of note, these % were similar to those previously reported in healthy controls [22,23].

We next investigated which cell subsets from LVS vaccinee's specimens were preferentially expanded after 7 days of *in vitro* re-stimulation with LVS. In agreement with IFN- γ -secreting cell results, significant increases in proliferative CD4⁺ T cell responses were observed in T_{EM} and T_{EMRA} subsets. However, significant increases in proliferative CD8⁺ T cell responses were only detected in T_{EMRA} subsets (Figure 5). Of note, the volunteers with lower proliferative responses and who did not exhibit increased IFN- γ -secreting cell responses (Vol#37 and Vol#40, Figure 2) exhibited frequencies of proliferating CD8⁺ T_{CM} subsets comparable to those observed in CD8⁺ T_{EM} and T_{EMRA} subsets (data not shown). In contrast, the volunteers with high proliferative and IFN- γ responses (Vol#34 and Vol#36, Figure 2) exhibited higher frequencies of proliferating CD4⁺ and CD8⁺ T_{EM} and T_{EMRA} subsets than those observed in CD4⁺ and CD8⁺ T_{CM} subsets, respectively (data not shown).

We next evaluated whether the increases observed in the T_{EM} and T_{EMRA} subsets in LVS vaccinees were also present in the *F. tularensis* naturally-infected subjects. Increases in IFN- γ -secreting cell responses were recorded in 2 of the 3 subjects examined (F0715V1 and F0716V1) (Figure 6). The remaining subject (F0717V1) was a non-responder when IFN- γ -secreting responses were evaluated by intracellular staining. As observed with LVS vaccinees, these responses were mediated mostly by T_{EM} and T_{EMRA} cells in both CD4⁺ and CD8⁺ subsets. No increases in IFN- γ secreting cells were observed in a healthy subject (CVD4000#76) included as a control in these studies. Unfortunately, due to the limited number of PBMC available from naturally-infected subjects, we were unable to perform proliferative response T_M subset analysis in these subjects.

3.4. Function and homing potential of T memory subsets

To investigate whether the rise of IFN- γ secreting T_{EM} and T_{EMRA} subsets was correlated to specific surface molecules involved in lymphocyte cytotoxicity or migration into secondary lymphoid tissues, we investigated the expression of CD27, CD49d, integrin α_4/β_7 , CLA and CCR4 surface markers in these tularemia-specific T_M cell subsets. Since studies have shown that the lack of CD27 expression levels generally correlate with cytotoxic activity [24,25], we examined the lytic potential of IFN- γ -secreting CD4⁺ and CD8⁺ T_{EM} subsets by measuring their *ex vivo* expression of CD27. We found that a substantial % of the specific (i.e., IFN- γ ⁺) CD4 and CD8 T_{EM} subsets from the PBMC of both, LVS vaccines and *F. tularensis* naturally infected subjects do not express CD27 (Figure 7). Moreover, a considerable proportion of these specific IFN- γ ⁺ CD4⁺ and CD8⁺ T_{EM} cell populations also expressed CD49d⁺ (an adhesion molecule that helps direct lymphocytes from circulation into tissues by strengthening lymphocyte adhesion to endothelial cells) [26] and integrin α_4/β_7 ⁺ (a molecule that mediates lymphocyte migration into intestinal lymphoid tissues) [27]. Interestingly, skin-associated memory T cells as defined by expression of the cutaneous lymphocyte antigen (CLA) [28–30] and the chemokine receptor CCR4 [31,32], were not observed in either CD4 and/or CD8 T_{EM} subsets from the PBMC of LVS vaccines (Figure 7) or *F. tularensis* naturally infected subjects (Figure 8).

Similar trends appear to be present in specific IFN- γ ⁺ CD4 and CD8 T_{EMRA} subsets; however, these results are not definitive because of (1) the wide variations in CD49d, integrin α_4/β_7 , CLA and CCR4 expression levels among the T_{EMRA} subsets in PBMC of the subjects evaluated, and (2) the lower numbers of IFN- γ secreting cells in T_{EMRA} subsets, which typically represent a small proportion of cells among PBMC, these results are not definitive. Studies with additional volunteers should establish the validity of these results.

We also investigated *F. tularensis*-specific proliferating T cells for the expression of molecules required for lymphocyte homing. To this end, we re-stimulated the lymphocytes for 7 days in presence or absence of LVS antigen. After 7 days, the presence of proliferating lymphocytes was measured by intracellular staining with a mAb to Ki67, a protein expressed in dividing cells [16] in conjunction with the same surface markers used to identify IFN- γ secreting T_M subsets (CD27, CD49d, integrin α_4/β_7 , CLA and CCR4). Ten-color flow cytometric analysis showed that *in vitro* expanded T_{EM} and T_{EMRA} subset cells contained a greater proportion of cells expressing CD49d but lower expression of CD27. A variety of increases were observed in the percentages of CD4⁺ and CD8⁺ T_{EM} and T_{EMRA} subsets expressing integrin α_4/β_7 , CLA or CCR4 after *in vitro* re-stimulation. Representative examples of proliferative responses are shown in Figure 9 (LVS vaccinee #34). Overall, these results demonstrate the heterogeneity of these T_M subsets and highlight differences in the homing potential of specific cells that produce cytokines or proliferate in response to *F. tularensis* antigens.

4. Discussion

In this study we evaluated PBMC from subjects who were exposed to tularemia antigens by two different routes, i.e., (1) immunization with the tularemia live attenuated vaccine (*F. tularensis* Live Vaccine Strain, LVS) by skin scarification, and (2) natural infection with *F. tularensis*, as models to study the induction of T_M subsets in human blood. We observed that the immune responses elicited by LVS vaccination, as well as by natural infection were mediated mostly by T_{EM} and T_{EMRA} cells in both CD4⁺ and CD8⁺ subsets. These specific cells were able to produce IFN- γ and proliferate. Remarkably, two of the three naturally infected subjects that showed CMI responses to *F. tularensis* antigens had been infected >25 years before PBMC were collected (#F0716V1 and #F0717V1). Although we can not exclude that volunteers were subsequently exposed to *F. tularensis* after their initial infection, these results suggest that CMI to *F. tularensis* persist for extended periods of time. These results

further support previous observations demonstrating long term CMI responses to *F. tularensis* antigens in humans [10–12]. It is worth noting that IFN- γ responses may be more easily detected by CBA than by flow cytometry. Using CBA, positive IFN- γ responses were detected in 5/5 vaccinees and in 3/3 naturally infected subjects; whereas using flow cytometry, IFN- γ responses were detected in only 2/5 vaccinees and 2/3 naturally-infected subjects. These differences could be due to differences in the levels of sensitivity or the timing of both assays. CBA measures the cumulative levels of cytokines in culture supernatants over 16–18 hours, whilst intracellular staining measures cumulative intracellular levels during the last 5 hours in culture. Interestingly, proliferative responses were relatively lower among the vaccinees without IFN- γ -secretion detected by flow cytometry. These results argue in favor of the possibility that multifunctional cells able to secrete high levels of cytokines, as well as proliferate, may play an important role in the host's response to tularemia infection. In this regard, previous human study have demonstrated a relationship between the quality of functional T cell responses and the AIDS disease course [33]. This study showed that non-progressors preferentially maintain HIV-specific T cells that are able to mount multiple cytokine/chemokine and degradation responses on a cell-by-cell basis. Similar results were observed in a human vaccinia virus immunization model [34]. It is also important to highlight that significant increases in proliferative CD8⁺ T cell responses in PBMC of LVS vaccinees were only detected in T_{EMRA} subsets (Figure 5), indicating either that *in vitro* stimulation with formalized-LVS is not ideal for optimal CD8⁺ T_{EM} cell stimulation or that CD4⁺ T_{EM} cells remain in circulation for longer periods of time. It should be emphasized that proliferative responses may reflect the loss of some T cell subsets (e.g., by apoptosis death, loss of specificity) and their replacement by others able to better expand *in vitro* in response to antigen, a phenomenon frequently observed in T cell cultures.

Based on the expression of CD27, integrin α_4/β_7 and integrin $\alpha_4\beta_1$, some of these memory T cells have lytic potential and the ability to enter intestinal mucosal and non-mucosal sites such as the lung. Interestingly, skin-associated memory T cells as defined by expression of the CLA and the chemokine receptor CCR4, were not observed in IFN- γ -secreting cells from LVS vaccinees or from *F. tularensis* naturally-infected subjects. Of note, a considerable proportion of cells from LVS vaccinees that were found to proliferate to *F. tularensis* antigens (Fig. 9), expressed CLA and CCR4, which presumably endow them with the potential to home to the skin. We hypothesize that cells that proliferate in response to *F. tularensis* antigen might represent a heterogeneous population of cells, some of which are endowed with the capability to home to the skin but are not capable of secreting IFN- γ . However, we can not exclude the possibility that the up-regulation of CCR4 and CLA markers in Ki67⁺ cells represent the selective expansion of a few T cell clones that cross-react with the *F. tularensis* particulate antigen used in these studies, a phenomenon unlikely to be observed in short incubation periods such as our culture conditions to measure intracellular IFN- γ production. It is widely accepted that a short period of incubation minimizes bystander stimulation. Taken together, these observations appear to indicate that regardless of the site of exposure, *F. tularensis* elicited a broad spectrum of specific cells with diverse homing characteristics. These results support the contention raised by others that it is possible that there are homing characteristics shared between mucosal and non-mucosal sites [35]. Moreover, recent studies have demonstrated plasticity in the memory T cell homing potential. Migrating memory cells may modify their migration behavior depending on their location at the time of the antigen reencounter [4]. It should be emphasized that, although cells and phenotypes may be present and may even increase in number or functional potential following exposure to *F. tularensis* antigens, these increases by themselves do not prove that they play a role in protection. However, since human mucosal biopsies are extremely difficult to obtain, the use of peripheral blood cells constitutes a reasonable alternative approach that allowed us to observe increases in defined T_M cell subsets that might be directly relevant for human resistance to infection.

In summary, we have shown that T_{EM} and T_{EMRA} cells may play an important role in the host immune response against *F. tularensis* and that both specific CD4⁺ and CD8⁺ subsets are elicited following *in vivo* exposure to this organism. An unexpected observation was that these effector cells are heterogeneous in their homing characteristics, whether these measurements were performed in PBMC from LVS vaccines or naturally infected subjects. These effector cells may concomitantly express homing markers for mucosal and non-mucosal sites. This observation contributes important information for the development of vaccines that rely on the induction of T_M cells.

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Fig. 1.

Measurements of cytokine production as a surrogate marker of the cellular immune response. (A) PBMC from LVS vaccinees were collected before (day 0) and after LVS vaccination (days 15, 28 and 60) and stimulated with LVS at 10^7 CFU/ml. Vaccination days are shown in the figure legend. (B) PBMC from naturally-infected subjects were stimulated with LVS 10^7 CFU/ml. Bar graphs represent means + SE of the pooled data of 3 different subjects. Pre-vaccination (day 0) specimens were used as controls. (C) Individual data from the 3 naturally-infected subjects depicted in panels “B”. In all cases, the supernatants were harvested after 16–18 hours of incubation and IL-2, TNF- α and IFN- γ production levels were measured using cytometry bead arrays. The dashed line represents the cut-off for positive responses (median of the background obtained with cells stimulated with *Coxiella* antigen).

Fig. 2.

Kinetics of specific memory T cell subsets in LVS tularemia vaccinees. PBMC from 4 LVS vaccinees (vol. # 34, 36, 37, and 40) were collected before (day 0) and after vaccination (days 15, 28 and 60) and stimulated with LVS 10^7 CFU/ml. After either 16–18 hours or 7 days, the samples were stained intracellularly for IFN- γ or Ki67, respectively. To this end, cells were first surface stained with a combination of mAb to CD3, CD4, CD8, CD14, CD19, as well as ethidium monoazide (EMA). After fixation and permeabilization, cells were then stained intracellularly for IFN- γ or Ki67 and analyzed by multichromatic flow cytometry. Lymphocytes were gated based on forward and side scatter (FS/SS) and then through a “dump” channel used to eliminate dead cells (EMA⁺), as well as CD14⁺ and CD19⁺ cells from analysis.

Following this, CD4⁺ and CD8⁺ T cells were gated as CD3⁺CD4⁺CD8⁻ and CD3⁺CD4⁻CD8⁺ respectively, and then analyzed for their IFN- γ or Ki67 content. Results are expressed as % positive cells. (*, positive (p<0.05) responses when compared with pre-vaccination levels).

Fig. 3.

Specific IFN- γ producing T memory subsets in LVS tularemia vaccinees. PBMC from vaccinee # 34 collected at days 0 and 28 were stimulated with LVS 10^7 CFU/ml for 16–18 hours. Cells were then surface stained with a combination of mAb to CD3, CD4, CD8, CD14, CD19, CD45RA and CD62L, as well as ethidium monoazide (EMA). After fixation and permeabilization, cells were stained intracellularly for IFN- γ and analyzed by multichromatic flow cytometry as described in the legend to Fig. 2. An example of the sequential gating used to identify the populations of interest is shown in the first row. First, a “dump” channel was used to eliminate dead cells (EMA⁺) as well as CD14⁺ and CD19⁺ cells from analysis. This was followed by additional gating on CD3, CD4 and CD8, as well as CD45RA versus CD62L (to analyze the various effector and memory T cell subsets). Numbers correspond to the % of positive cells in the indicated quadrants in each scattergram. Major increases are bolded.

Fig. 4.

Total effector and memory CD4⁺ and CD8⁺ T cell subsets in PBMC isolated from LVS vaccinees before and after immunization. PBMC from 5 LVS vaccinees (vol. # 34, 36, 37, 40 and 42) collected before (day 0) and after vaccination (days 15, 28 and 60) were stimulated with *Coxiella* antigen (negative control). After 16–18 hours, cells were surface stained with a combination of mAb to CD3, CD4, CD8, CD14, CD19, CD45RA and CD62L, as well as ethidium monoazide (EMA) and analyzed by multichromatic flow cytometry. A “dump” channel was used to eliminate dead cells (EMA⁺), as well as CD14⁺ and CD19⁺ cells from analysis. Additional gating on CD3, CD4 and CD8, as well as CD45RA versus CD62L (to analyze the various effector and memory T cell subsets) was performed as described the legend to Fig. 3. Bar graphs show means + SE of 5 experiments using 5 different subjects.

Fig. 5.

Proliferation of T memory subsets in LVS Tularemia vaccinees. PBMC from LVS vaccinees were collected before (day 0, open bars) and after vaccination (days 15, 28 and 60) (closed bars) and stimulated with LVS 10^7 CFU/ml for 7 days. Cells were then surface stained with a combination of mAb to CD3, CD4, CD8, CD14, CD19, CD45RA and CD62L, as well as ethidium monoazide (EMA). After fixation and permeabilization, cells were stained intracellularly for Ki67 and analyzed by multichromatic flow cytometry. A “dump” channel was used to eliminate dead cells (EMA⁺), as well as CD14⁺ and CD19⁺ cells from analysis. Bar graphs show means + SE using cells from 5 different LVS vaccinees. (*, $p < 0.05$).

Fig. 6.

Specific IFN- γ producing T memory subsets in *F. tularensis* naturally-infected subjects. PBMC from naturally-infected subjects (F0715V1 and F0716V1) and a healthy unvaccinated subject (CVD4000#76) were stimulated with LVS 10^7 CFU/ml for 16–18 hours. Cells were then surface stained with a combination of mAb to CD3, CD4, CD8, CD14, CD19, CD45RA and CD62L, as well as ethidium monoazide (EMA). After fixation and permeabilization, cells were stained intracellularly for IFN- γ and analyzed by multichromatic flow cytometry. A “dump” channel was used to eliminate dead cells (EMA⁺), as well as CD14⁺ and CD19⁺ cells from analysis. Numbers correspond to the % of positive cells in the indicated quadrants in each scattergram. Major increases are bolded.

Fig. 7.

CD27 expression and homing potential of IFN- γ -producing T memory subsets in LVS tularemia vaccinees. PBMC from vaccinee #34 collected 28 days after immunization were stimulated with LVS 10^7 CFU/ml for 16–18 hours. Cells were then surface stained with a combination of mAb to CD3, CD4, CD8, CD14, CD19, CD45RA, CD62L, CD27, CD49d, integrin $\alpha_4\beta_7$, CLA and CCR4, as well as ethidium monoazide (EMA). After fixation and permeabilization, cells were stained intracellularly for IFN- γ and analyzed by multichromatic flow cytometry. A “dump” channel was used to eliminate dead cells (EMA⁺), as well as CD14⁺ and CD19⁺ cells from analysis. Numbers correspond to the % of positive cells in the indicated quadrants in each scattergram. Major increases are bolded. These results are representative of two experiments using 2 separate volunteers.

Fig. 8.

CD27 expression and homing potential of IFN- γ -producing T memory subsets in naturally-infected subjects. PBMC from a naturally-infected subject (F0715V1) were stimulated with LVS 10^7 CFU/ml for 16–18 hours. Cell staining was performed and results analyzed and shown as described in Figure 7. Major increases are bolded. These results are representative of two experiments using 2 separate volunteers.

Fig. 9.

CD27 expression and homing potential of proliferating T memory subsets in LVS tularemia vaccinees. PBMC from vaccinee #34 collected 28 days after immunization were stimulated with LVS 10^7 CFU/ml for 7 days. Cells were then surface stained as described in Figure 7. After fixation and permeabilization, cells were stained intracellularly for Ki67 and analyzed by multichromatic flow cytometry. A “dump” channel was used to eliminate dead cells (EMA⁺), as well as CD14⁺ and CD19⁺ cells from analysis. Numbers correspond to the % of positive cells in the indicated quadrants in each scattergram. Major increases are bolded. These results are representative of two experiments using 2 separate volunteers.