# Long-Lasting T Cell Responses to Biological Warfare Vaccines in Human Vaccinees

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***Background.*** Medical countermeasures against biological warfare include the use of vaccines for anthrax and plague, which require repeated dosing and adjuvant to achieve adequate protection from threats such as inhalational anthrax and pneumonic plague. Despite the widespread use of these measures in preparation for recent military deployments, little is known about the cell-mediated immune response that is induced by these vaccines, in comparison with conventional vaccines, such as pertussis or tetanus-diphtheria vaccines.

***Methods.*** To examine this question, we used cytokine enzyme-linked immunospot assays to measure interferon-$\gamma$, interleukin (IL)–2, IL-4, and IL-13–producing cells in military service personnel vaccinated during the Gulf War of 1990–1991.

***Results.*** Our data indicate that 12–15 years after vaccination against anthrax and plague, antigen-specific T cell recall responses are present in the circulation and are comparable in magnitude to those for tetanus-diphtheria toxoids. Recall responses to anthrax were an approximately equal mixture of type 1 T helper cell (interferon-$\gamma$ and IL-2) and type 2 T helper cell (predominantly IL-13) responses, whereas plague cellular immunity was more polarized toward type 1 T helper cell responses. Responder cell frequency and type were similar to that against conventional tetanus-diphtheria (mixed type 1 and type 2 T helper cells) vaccine. When veterans were divided according to whether or not they reported multisymptom illness, there was no difference in the frequency or type of cellular response, although the number of cases in each group was small, and these data should be interpreted as preliminary.

***Conclusions.*** This study shows that, despite any putative limitations of vaccines for anthrax and plague in terms of achieving protective host immunity, long-lasting cell-mediated responses are generated with these agents.

The threat of biological and chemical warfare in recent military conflicts has led to an increasing focus on the deployment of medical countermeasures, including vaccines for category A biological warfare agents, such as anthrax *(Bacillus anthracis)* and plague *(Yersinia pestis)* [1–7]. Anthrax and plague vaccines have been in existence for several years, but they have limitations [3]. Protection against plague requires 2 doses at an interval of 1–4 weeks with boosting every 6 months [5, 7], whereas doses at 2 and 4 weeks with boosters at 6, 12, and 18 months and annually thereafter are necessary for anthrax protection [8]. Even under these circum-

stances, the protection afforded by vaccines from pneumonic plague and inhalational anthrax may not be ideal [3]. These requirements place considerable constraints on the usefulness of these agents during military deployment, when rapid protection is highly desirable.

During the 1990–1991 Persian Gulf conflict, 53,000 UK military service personnel were deployed, most of whom received medical countermeasures against anthrax and plague. In an attempt to boost the efficacy of these measures (and of anthrax protection, in particular), the whole-cell pertussis vaccine was used as an adjuvant during the immunization of UK troops. By necessity, vaccines were administered within a short period of time, and their number was extended to include protection against a range of other infectious diseases. These countermeasures came under considerable scrutiny when, on their return to the United Kingdom, many veterans sought medical advice for symptoms that they thought were related to their service in the Gulf, including fatigue, rashes, joint pain, neuropsy-

chiatric complaints, shortness of breath, sleep disturbances, and gastrointestinal problems [9]. The etiopathogenesis of these sequelae of military service, often referred to as Gulf War syndrome despite the lack of evidence for a discrete clinical entity [10], remains unclear.

Imbalanced type 1 T helper cell (Th1) and type 2 T helper cell (Th2) immunity has served as an important paradigm for the investigation of numerous inflammatory diseases, including autoimmunity (Th1) and allergy (Th2) [11]. In 1997, Rook and Zumla [12] proposed a hypothesis to explain Gulf War–related symptoms that was based on the concept that some of these symptoms (e.g., skin rashes and joint pain) could reflect the inadvertent generation of a Th2-biased immune response, and they cited multiple vaccines and the use of whole-cell pertussis as interventions that might promote such Th skewing. Subsequently, there have been several attempts to explore Th1/Th2 balance in veterans of the Gulf War by examining polyclonal (i.e., "global") T cell cytokine production [13–18]. This design is inherently flawed, because, when applied to veterans, it measures T cell immunity as a product of many years of prior exposure to vaccines, immunogens, and wild-type infections, rather than specifically examining immune responses generated in the perideployment period, when the proposed Th2-biasing conditions are present.

To address this limitation, we exploited the fact that, for the many thousands of military personnel receiving anthrax and plague vaccines in 1990–1991, these represented first exposures to these agents. Exposure to such neoantigens during deployment is serendipitous, providing the ideal circumstances in which to seek evidence of T helper cell bias. In the present study, therefore, we used sensitive cytokine enzyme-linked immunospot (ELISPOT) assays to examine the magnitude and Th1/Th2 balance of the recall T cell response to anthrax and plague vaccines in veterans immunized during the 1990–1991 Gulf War. Our present study, which is the first to analyze T cell cytokine responses to anthrax and plague vaccines, indicates that T cell memory against these agents is present and balanced in terms of Th1 and Th2 induction.

## MATERIALS AND METHODS

***Recruitment of participants.*** Blood samples were obtained from volunteers who had previously attended the Gulf War Illness Research Unit at King's College London School of Medicine (London, UK) and had taken part in stage I and stage II studies. Stage I was a questionnaire-based study of veterans of the Gulf conflict (1990–1991) (5046 subjects), the details of which are reported elsewhere [9], and was used to identify participants for stage II, a clinic-based evaluation. In the absence of an accepted or meaningful classification of Gulf War–related multisymptom illness, for stage II recruitment, a case definition was chosen that was based on symptomatic and func-

tional ill health as determined using the Short Form 36 Physical Functioning measure (SF-36PF), and symptomatic individuals were defined as those who scored ≤72.2. Using these criteria at stage I, a random sample of symptomatic and nonsymptomatic volunteers were invited to attend the Gulf War Illness Research Unit for stage II clinical studies. Health status was also reassessed at this point by the SF-36PF measure, and for the purposes of the present study, subjects were assigned to ill and well groups accordingly.

Eligibility for the current study required documented vaccine records. As a result, 59 subjects were eligible for our study, 29 of whom were lost to follow-up and 2 of whom refused to participate. Of the 28 subjects participating, all 28 were male (mean age ± SD, 39 ± 13 years), and 17 were classified as ill according to the SF-36PF. Blood samples were obtained from 2003 to 2005. This study was carried out with the approval of the Local Research Ethics Committee, and informed consent was obtained from all participants.

***Vaccine preparations.*** The UK human anthrax vaccine consists of a protein precipitate from the supernatant fluid of cultures of the Sterne strain of *B. anthracis*. The major immunogen is protective antigen, the nontoxic, cell-binding component of the anthrax toxin complex. The vaccine also contains lethal and edema factors. The concentration of protective antigen in the vaccine preparation is 1.3–2.2 $\mu$g/mL (whole molecule and fragments), and the concentration of lethal factor is 0.4–0.7 $\mu$g/mL. Edema factor is present in very low levels below the detection limit of the assay method. This alum-precipitated human anthrax vaccine (product licence no. PL1511/0037) was produced by the Centre for Applied Microbiology and Research (Porton Down, Salisbury, Wiltshire, UK) for the UK Department of Health. In our study, anthrax vaccine was used over a range of concentrations that resulted in 0.043–0.43 pg/mL of protective antigen (vaccine dilutions of 1:30,000 to 1:3000).

The plague vaccine (CSL Pharmaceutical) consists of a suspension of agar-grown, heat-killed organisms of *Y. pestis* in saline at $3 \times 10^9$ organisms/mL and was used in our studies in the range of $1–10 \times 10^6$ organisms/mL (i.e., vaccine dilutions of 1:3000 to 1:300). The strains used in this vaccine preparation were obtained from the Haffkine Institute, (Mumbai, India) and their virulence was confirmed by demonstration of lethal effect in rats.

The pertussis vaccine used in our study consists of killed whole-cell preparation of *Bordetella pertussis* W28, prepared at the Centre for Applied Microbiology and Research (Porton Down, Salisbury, Wiltshire, UK) to the original Burroughs-Wellcome procedure for the preparation of single whole-cell pertussis vaccine and provided at a strength of $4 \times 10^{10}$ organisms/mL (product licence no. 208/10/99). In our studies, the pertussis vaccine was used in the concentration range of
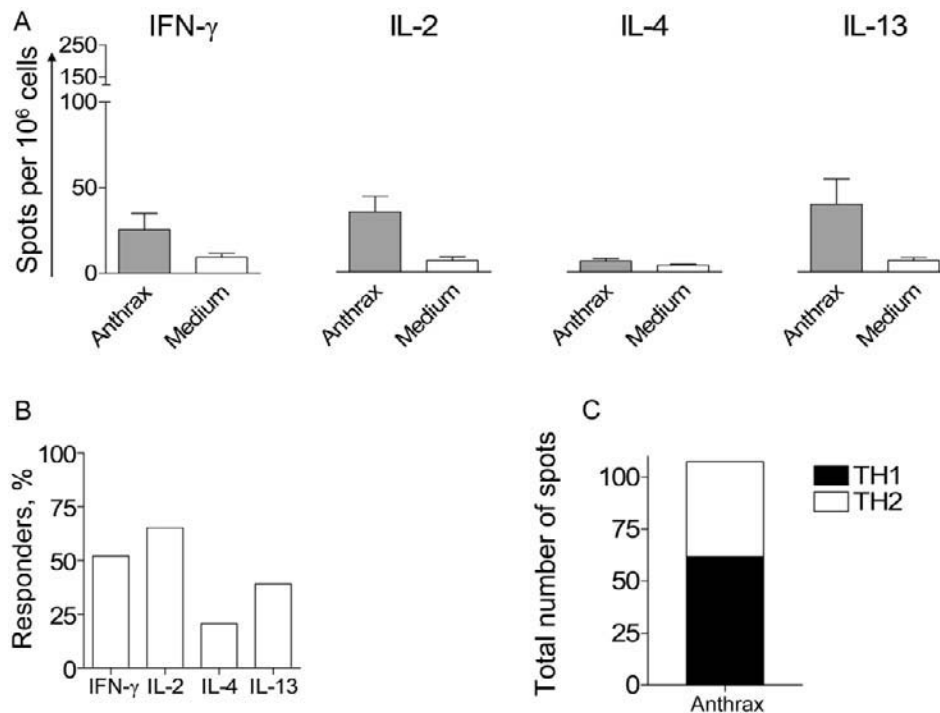
**Figure 1.** T cell immunity to anthrax vaccine. Data shown represents those obtained with the optimal anthrax vaccine dilution (1:3000, equivalent to 0.43 pg/mL of protective antigen). *A,* Cytokine (IFN-γ, IL-2, IL-4, and IL-13) enzyme-linked immunospot assay responses to anthrax vaccine in Gulf War veterans, compared with medium alone. *Bars,* mean number of spots per million cells; *error bars,* SEM. *B,* The percentage of subjects responding to anthrax vaccine with a stimulation index (ratio of vaccine response over background) >3.0. *C,* Stacked bar chart showing the summated response to anthrax vaccine in terms of type 1 T helper cell (Th1) cytokines IFN-γ and IL-2 *(filled bar)* and the type 2 T helper cell (Th2) cytokines IL-4 and IL-13 *(open bar).*

1.3–13 × 10^{7} organisms/mL (i.e., vaccine dilutions of 1:3000 to 1:300).

The tetanus-diphtheria vaccine (Diftavax; Aventis Pasteur) contained 80 IU/mL of purified tetanus toxoid and 8 IU/mL of purified diphtheria toxoid, as well as 0.9–1.7 mg/mL aluminium hydroxide in buffered saline solution. In our studies, the tetanus-diphtheria vaccine was used at dilutions of 1:3000 to 1:300 (this equals to 0.0267–0.267 IU/mL tetanus toxoid and 0.00267–0.0267 IU/mL diptheria toxoid). Vaccine concentrations used in this study were selected on the basis of previous in vitro toxicity studies and pilot ELISPOT assays [19].

*Cytokine ELISPOT assay.* Cytokine ELISPOT assays were performed as described [19] to examine cellular immune responses [20, 21]. Phorbol myristate acetate–ionomycin (10 ng/mL and 1 μg/mL) and medium alone constituted the positive and negative control wells, respectively. Plates were analyzed on a BioReader 3000 (BioSys), and spots of 80–150 μm were counted by researchers blinded to the health status of the participants. Triplicate spot number values were pooled and normalized to provide mean (±SEM) spots per million to indicate the frequency of responder cells. To classify subjects as vaccine responders, an arbitrary cut-off value (stimulation index, >3.0), representing the ratio of spot number in response to vaccine to spot number in the presence of medium alone, was used [19]. Mean (±SEM) responses in the presence of medium alone for all cytokines and all subjects were 1.47 ± 0.13 spots per well. Finally, total spot numbers for all cytokines in response to a vaccine were summated and expressed as either representing a Th1 response (sum of IFN-γ and IL-2 spots) or Th2 (sum of IL-4 and IL-13 spots).

*Statistical analysis.* Cytokine ELIPSOT responses between clinical groups were compared using the Mann-Whitney *U* test. *P* values <.05 were considered to be statistically significant.

## RESULTS

***Nature of recall T cell responses to anti–biological warfare vaccines.*** For IFN-γ, IL-2, and IL-13 responses to anthrax vaccine, there was clear evidence of a dose-dependent recall response, typically representing a responder frequency for each cytokine of ∼1 per 30,000 PBMCs (figure 1). IL-4+ responder cells were rare. Using an arbitrary stimulation index of >3.0 to indicate responders, the majority of subjects maintained a Th1 response to anthrax, with over one-half showing evidence of maintained IL-2+ responders indicative of central memory cells. Overall, the response to anthrax vaccine was an approx-

imately 50:50 mixture of Th1 and Th2 responses. The total responder cells approximated a frequency of 1 per 10,000 PBMCs.

Similarly, IFN-γ, IL-2, and IL-13 recall responses to plague vaccine were present, with evidence of dose dependency, typically representing a responder frequency in the range of 1 per 10,000 to 1 per 30,000 PBMCs (figure 2). IL-4+ responder cells were rare. Over 75% of subjects maintained a Th1 response to plague, and a similar proportion showed an IL-13 response. Overall, the cellular immunity to plague vaccine was dominated by a Th1-like response. The total responder cells approximated a frequency of 1 per 5000 PBMCs. Responses to plague and anthrax vaccines were not detectable in nonmilitary, healthy control subjects (data not shown).

**Nature of recall T cell responses to pertussis and tetanus-diphtheria.** Culture of PBMCs with whole-cell pertussis invoked a strong IFN-γ recall response, as well as robust IL-2 and IL-13 reactions, with evidence of dose dependency, typically representing a responder frequency in the range of 1 per 5000 PBMCs to 1 per 30,000 PBMCs for these cytokines (figure 3). IL-4+ responder cells were rare. Almost 100% of subjects maintained a Th1 response to pertussis, and over two-thirds showed an IL-13 response. Overall, the cellular immunity to pertussis

was dominated by a Th1-like response. The total responder cells approximated a frequency of 1 per 3000 PBMCs.

For all cytokine responses to tetanus-diphtheria vaccine, there was clear evidence of a dose-dependent recall response, typically representing a responder frequency for each cytokine in the range of 1 per 30,000 PBMCs to 1 per 60,000 PBMCs (figure 4). There was a greater frequency of IL-4+ responder cells than there was for the other vaccines. The highest level of response was shown by IL-2 production, indicating the presence of central memory cells. Overall, the response to tetanus-diphtheria vaccine was an approximately 50:50 balance of Th1 and Th2 responses. The total responder cells approximated a frequency of 1 per 10,000 PBMCs.

**Comparison of responses in Gulf War veterans with and without multisymptom illness.** As indicated, 17 of the 28 veterans participating in the study had multisymptom illness and were classified as ill with use of SF-36PF screening. These subjects were of particular interest in this study, in light of the Th2 hypothesis of multisymptom illness, the association between symptoms and multiple vaccinations, and our own study showing that this subgroup of veterans had an increased repertoire of IL-10–secreting memory cells [18]. Each of these features might impact on the quality of immunity generated
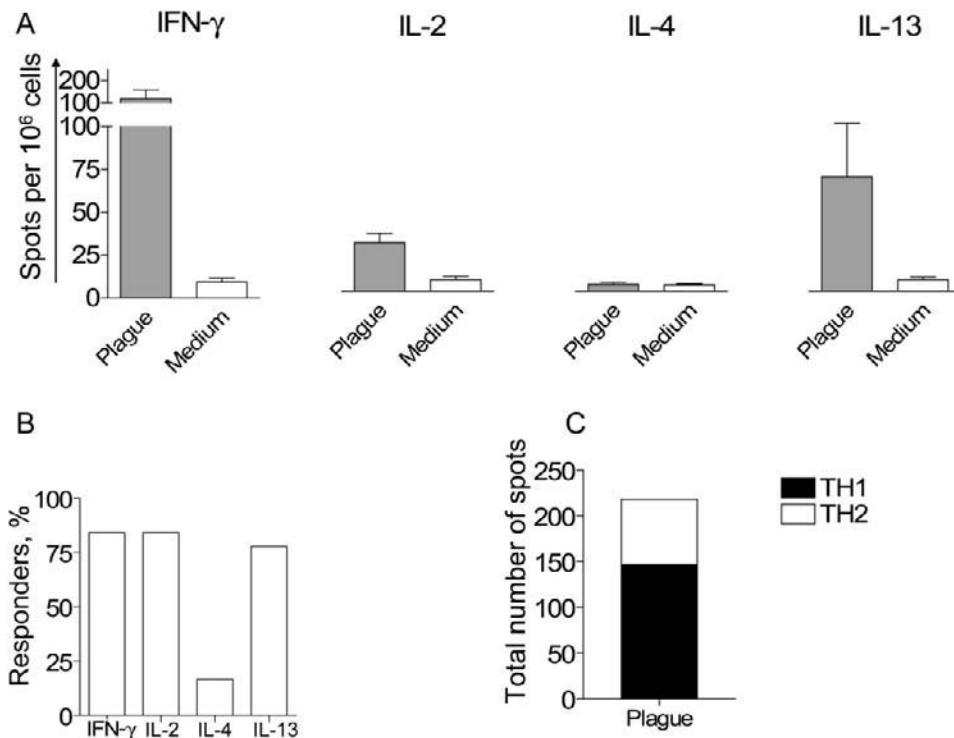


**Figure 2.** T cell immunity to plague vaccine. Data shown represents those obtained with the optimal dose-response condition (1:300, equivalent to $10 \times 10^6$ organisims/mL). *A,* Cytokine (IFN-γ, IL-2, IL-4, and IL-13) enzyme-linked immunospot assay responses to plague vaccine and medium alone in Gulf War veterans. *Bars,* mean number of spots per million cells; *error bars,* SEM. *B,* The percentage of subjects responding to plague vaccine with a stimulation index (ratio of vaccine response over background) >3.0. *C,* Stacked bar chart showing the summated response to plague vaccine in terms of type 1 T helper cell (Th1) cytokines IFN-γ and IL-2 *(filled bar)* and the type 2 T helper cell (Th2) cytokines IL-4 and IL-13 *(open bar).*

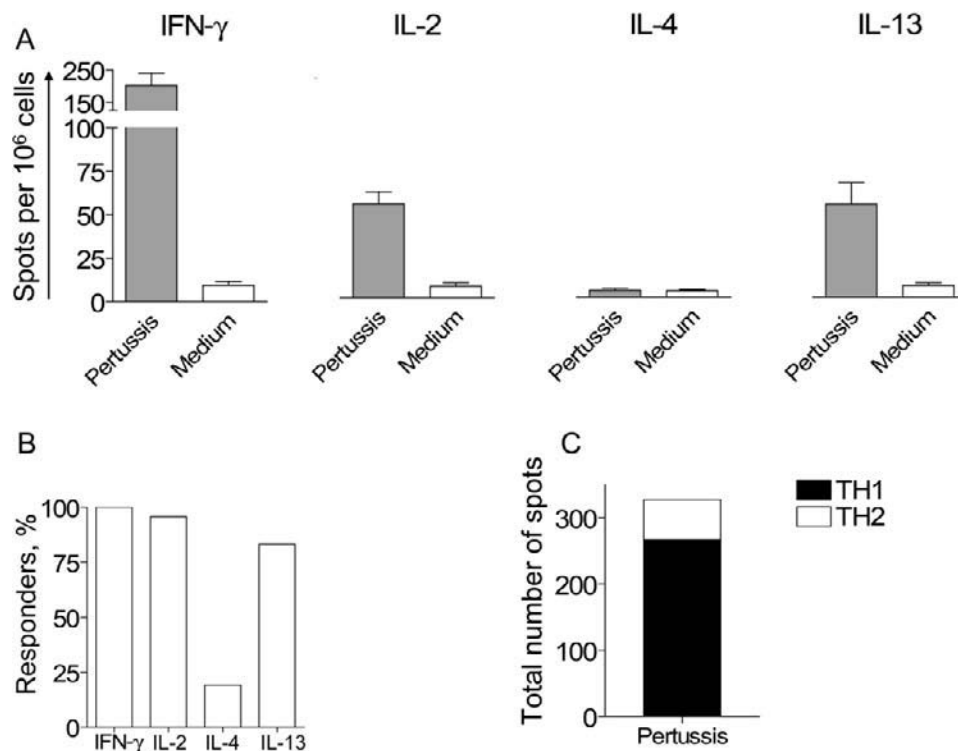4 • **CID 2006:43 (1 July)** • Allen et al.

**Figure 3.** T cell immunity to pertussis vaccine. Data shown represents those obtained with the optimal dose-response condition (1:1000, equivalent to $3.9 \times 10^7$ organisims/mL). *A,* Cytokine (IFN-$\gamma$, IL-2, IL-4, and IL-13) enzyme-linked immunospot assay responses to pertussis vaccine and medium alone in Gulf War veterans. *Bars,* mean number of spots per million cells; *error bars,* SEM. *B,* The percentage of subjects responding to pertussis vaccine with a stimulation index (ratio of vaccine response over background) >3.0. *C,* Stacked bar chart showing the summated response to pertussis vaccine in terms of type 1 T helper cell (Th1) cytokines IFN-$\gamma$ and IL-2 *(filled bar)* and the type 2 T helper cell (Th2) cytokines IL-4 and IL-13 *(open bar).*

against vaccines administered in preparation for the Gulf War. We therefore compared responder frequency for all cytokines and all vaccines between ill and healthy veterans, although this afforded us a small sample size in each group, with a consequent weakening of statistical power. As shown in table 1, there was no evidence of any difference in the magnitude or quality of antigen-specific T cell responses to any of the vaccines tested between these 2 groups.

## DISCUSSION

This is the first study to examine the magnitude and quality of the cellular immune response to anti–biological warfare vaccines. It was prompted by the need for a greater understanding of the effects of medical countermeasures, such as rapid and multiple immunization regimens, deployed in response to the threat of biological warfare. Our data indicate that 12–15 years after vaccination against biological warfare agents, such as anthrax and plague, antigen-specific T cell recall responses are present in the circulation and are comparable in magnitude to those for tetanus-diphtheria toxoids, immunogens that are typically administered with a periodicity of 5–10 years.

The hypothesis [12] that an immune system imbalance to-

wards Th2 cells might account for the multisymptom illness reported by many veterans of the first Gulf War in 1990–1991 has influenced epidemiological and mechanistic studies ever since. The major elements of the hypothesis related to Th2-biasing effects of multiple vaccines and pertussis adjuvant, administered by necessity during the rapid deployment. It has prompted several studies, using different but complementary approaches to seek evidence of Th1/Th2 imbalance in peripheral blood [13–18]. All of these studies (except one [15]) have found no evidence of Th2 skewing. However, such analyses have relied on polyclonal stimuli to elicit activation of the whole T cell memory pool [18], rather than exploring the greater subtleties of an antigen-specific immune response. So powerful has been the effect of the Th2 hypothesis that it influenced subsequent medical countermeasures for the 2003 Iraq Gulf War, in which pertussis was no longer used as an adjuvant, and both the timing of and the manner in which vaccinations were given were changed. However, as a hypothesis, it has proved difficult to examine.

We reasoned that, if the Th2 hypothesis was correct, then one of the most sensitive approaches to unravelling it would be the examination of cellular immune responses to a neoan-
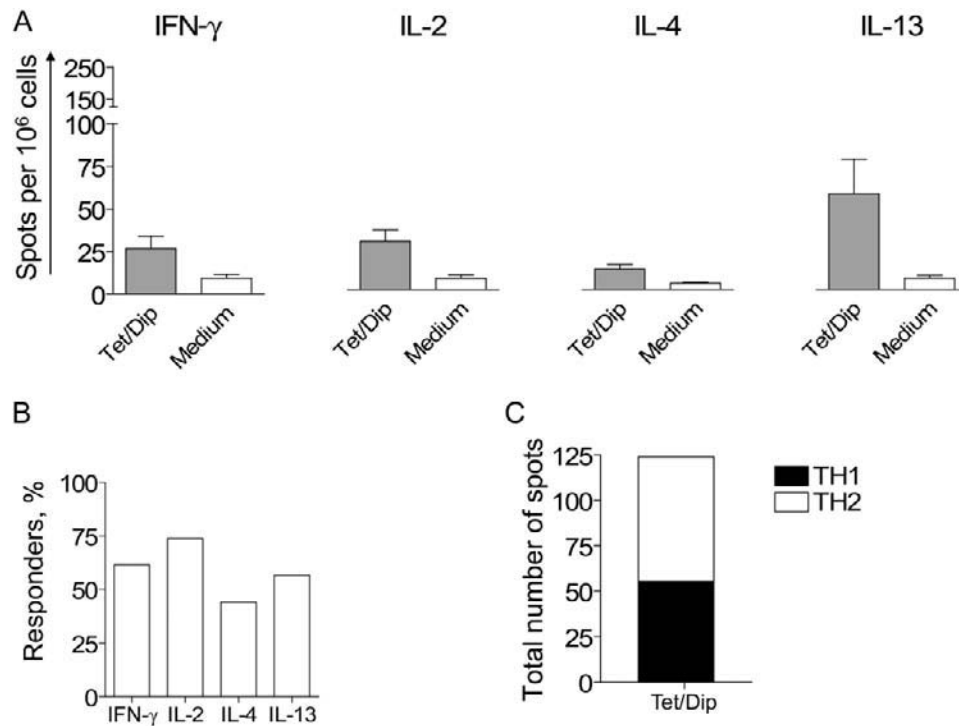
**Figure 4.** T cell immunity to tetanus-diphtheria vaccine (Tet/Dip). Data shown represents those obtained with the optimal dose-response condition (1:1000, equivalent to 100 ng/mL toxoid). *A,* Cytokine (IFN-γ, IL-2, IL-4, and IL-13) enzyme-linked immunospot assay responses to Tet/Dip vaccine and medium alone in Gulf War veterans. *Bars,* mean number of spots per million cells; *error bars,* SEM. *B,* The percentage of subjects responding to pertussis vaccine with a stimulation index (ratio of vaccine response over background) >3.0. *C,* Stacked bar chart showing the summated response to Tet/Dip vaccine in terms of type 1 T helper cell (Th1) cytokines IFN-γ and IL-2 *(filled bar)* and the type 2 T helper cell (Th2) cytokines IL-4 and IL-13 *(open bar).*

tigen, administered at the same time as the Th2-biasing multiple vaccines and pertussis. Serendipitously, the anti–biological warfare vaccines for anthrax and plague represented such neoantigens to the many thousands of service personnel who received them in 1990–1991. These vaccines are unavailable for general use, and wild-type infection with these pathogens is rare; the vast majority of the population is therefore immunologically naive to these agents. With use of a highly sensitive cytokine ELISPOT technique, we therefore examined recall Th1/Th2 immunity to these agents and to the non-neoantigenic tetanus-diphtheria vaccine in Gulf War veterans. We found that anthrax immunity has equal balance of Th1 and Th2 responder cells and that plague immunity is polarized towards Th1. Th2 responses to both vaccines are detected at equivalent or lower levels than responses directed against conventional tetanus-diphtheria vaccine, which is first encountered in early childhood. These data provide strong evidence against a putative pathological Th2-biasing effect of multiple vaccines, pertussis adjuvant, or, indeed, the stress of deployment, which was also invoked by Rook and Zumla [12] in their original hypothesis. The only caveat in relation to these results is that we were unable to compare our data with those obtained from veterans

receiving anthrax and plague vaccines in the absence of other multiple vaccines or pertussis vaccine, because no such cohort exists.

Our cohort allowed us to attempt to address a further question: namely, whether immune responsiveness is different in Gulf War veterans with multisymptom illness (ill veterans) than it is in those without such illness (healthy veterans). The data show no difference between these groups in terms of the magnitude and quality of the cellular immune response against anthrax and plague vaccines. However, there are important limitations to this analysis. Most notably, the small sample size does not offer adequate statistical power for us to draw definitive conclusions. In addition, there are limitations to the current case definitions for Gulf War–related illness that may impact on these data. The data should be considered preliminary and are included to indicate the feasibility of this approach to addressing the question of Th1/Th2 balance in relation to Gulf War–related illness, which may prompt other studies or facilitate future meta-analyses.

In summary, we demonstrate long-lasting and mixed Th1/Th2 cellular immunity to biological warfare vaccines for anthrax and plague in a cohort of Gulf War veterans immunized

**Table 1. Cytokine responses to anti–biological warfare vaccines in Gulf War veterans, by illness status.**

| Vaccine, cytokine response | Spot number, mean spots/$10^6$ cells ± SEM | | P |
|---|---|---|---|
| | Ill group | Healthy group | |
| **Anthrax** | | | |
| IFN-γ | 28.37 ± 15.37 | 24.00 ± 9.60 | .62 |
| IL-2 | 32.13 ± 13.83 | 38.57 ± 11.90 | .74 |
| IL-4 | 5.47 ± 1.13 | 7.67 ± 2.73 | .84 |
| IL-13 | 19.73 ± 9.57 | 63.10 ± 29.57 | .19 |
| **Plague** | | | |
| IFN-γ | 114.43 ± 63.53 | 123.77 ± 42.37 | .31 |
| IL-2 | 29.9 ± 7.27 | 26.67 ± 8.03 | .15 |
| IL-4 | 3.10 ± 1.23 | 5.27 ± 2.40 | .16 |
| IL-13 | 84.23 ± 53.83 | 45.27 ± 22.77 | .13 |
| **Pertussis** | | | |
| IFN-γ | 214.80 ± 43.77 | 168.17 ± 32.87 | .53 |
| IL-2 | 41.87 ± 7.20 | 46.57 ± 6.93 | .45 |
| IL-4 | 5.23 ± 1.43 | 4.43 ± 1.37 | .88 |
| IL-13 | 33.50 ± 8.50 | 66.87 ± 16.30 | .30 |
| **Tetanus-diphtheria** | | | |
| IFN-γ | 20.47 ± 6.03 | 20.67 ± 7.87 | .95 |
| IL-2 | 26.27 ± 4.77 | 45.77 ± 11.80 | .16 |
| IL-4 | 11.27 ± 3.30 | 16.57 ± 4.70 | .69 |
| IL-13 | 21.93 ± 9.967 | 60.20 ± 18.60 | .10 |

**NOTE.** Comparisons were made using data from the optimal dose-response condition (1:3000, 1:300, 1:1000 and 1:1000 for anthrax, plague, pertussis, and tetanus-diphtheria, respectively).

up to 15 years before analysis. Our results do not provide any evidence that Gulf War veterans have compromised immunity to these agents.

## Acknowledgments

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