Ex Vivo Kinetics of Early and Long-Term Multifunctional Human Leukocyte Antigen E-Specific CD8$^+$ Cells in Volunteers Immunized with the Ty21a Typhoid Vaccine$^\nabla$

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T cells are likely to play an important role in the host defense against *Salmonella enterica* serovar Typhi, the causative agent of typhoid fever. We have shown that HLA-E can function as a restriction element for *S.* Typhi-specific CD8$^+$ T cells. Because of the potential importance of HLA-E-restricted CD8$^+$ responses in resistance to *Salmonella* infection, we characterized these responses and investigated their kinetics of appearance and persistence in volunteers immunized orally with the licensed attenuated Ty21a strain typhoid vaccine. Cells were obtained from volunteers before and at days 2, 4, 7, 10, 14, 28, 42, 56, 120, 180, 360, and 720 after immunization. An *ex vivo* multicolor staining panel including antibodies to CD107a and -b, interleukin-2, gamma interferon (IFN-γ), and tumor necrosis factor alpha (TNF-α) was used to functionally assess memory T-cell subsets by flow cytometry. Increases in cytokine-secreting CD8$^+$ cells were observed in the T effector/memory (T$_{EM}$) and CD45RA$^+$ T$_{EM}$ (T$_{EMRA}$) subsets as early as 4 days after immunization and persisted, particularly in the T$_{EMRA}$ subset, up to 2 years after immunization. The majority of HLA-E-restricted CD8$^+$ cells 28 to 56 days after immunization coexpressed CD107, IFN-γ, and TNF-α, showing characteristic features of multifunctional T cells. In summary, the multifunctionality and longevity of the HLA-E-restricted CD8 responses observed in this study highlight their significance in adaptive immunity to *S.* Typhi. Finally, this is the first demonstration, in either animals or humans, of the presence of long-term multifunctional HLA-E-restricted CD8$^+$ cells after immunization.

Salmonella enterica serovar Typhi, the causative agent of typhoid fever, is a facultative intracellular bacterial pathogen (5, 7, 37). In industrialized countries, *Salmonella* infection is rare, with most infections occurring in military personnel and in individuals traveling to areas where typhoid fever is endemic. However, it remains an important public health priority in undeveloped parts of the world. Overall, it is estimated that 16 million new cases and 600,000 deaths occur annually (5, 12, 18). Our laboratory has demonstrated that oral immunization of volunteers with attenuated typhoid vaccine strain Ty21a triggers the generation of CD8$^+$ T cells that kill *S.* Typhi-infected cells displaying human leukocyte antigen E (HLA-E) on the cell surface (27). T cells are likely to play an important role in the host defense against *S.* Typhi. They might contribute to *Salmonella* control by secreting cytokines such as gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), and interleukin-2 (IL-2) and by directly killing bacterium-infected cells (27, 28, 30–32, 38–40).

HLA-E is a protein that is part of a family of molecules known as class Ib or nonclassical HLA molecules (26). HLA-E is expressed ubiquitously on the peripheral blood mononuclear cell (PBMC; e.g., B cells, T cells, natural killer [NK] cells, and macrophages) surface at various levels, depending on the cell type (15, 34). Because HLA-E molecules are essentially monomorphic, they are likely to present a more conserved set of

bacterial peptides that could be universally recognized by CD8$^+$ T cells from most, if not all, individuals.

Given the potential significance of HLA-E-restricted CD8$^+$ responses in resistance to *Salmonella* infection, it is of great importance to study these responses in depth, including their kinetics of appearance and persistence, which currently remains largely unknown. These responses might provide key insights into the open question concerning the dual role of the HLA-E molecules in short-lived innate and long-lived adaptive immunity (35). In fact, HLA-E molecules were primarily described as ligands for CD94/NKG2A, -B, and -C receptors present on the innate NK cell surface (3). However, HLA-E-restricted T cells have recently been implicated in adaptive immunity not only to *Salmonella* but also to cytomegalovirus and *Mycobacterium* (10, 22).

Oral immunization with the licensed Ty21a typhoid vaccine is a particularly relevant model of *Salmonella* infection, which is transmitted by the ingestion of virulent *S.* Typhi. In the present studies, volunteers were immunized with the standard regimen of four spaced doses of Ty21a given every other day (8, 28). This vaccination regimen results in levels of protection ranging from 60 to 80% (9). Using cells from Ty21a vaccinees, we investigated the kinetics of appearance and longevity of HLA-E-restricted CD8$^+$ T-cell responses for up to 2 years after immunization with the Ty21a typhoid vaccine and defined in detail the various memory T-cell subsets and effector functions mediating these responses.

MATERIALS AND METHODS

Volunteers. Seven healthy volunteers 24 to 41 years old recruited from the Baltimore-Washington area and the University of Maryland at Baltimore campus

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participated in this study. Volunteers were screened for good health by medical history, physical examination, laboratory tests (including blood counts), and the absence of antibiotic treatment. The purpose of this study was explained to the volunteers, and they gave informed, signed consent. Five volunteers were immunized with *S.* Typhi strain Ty21a typhoid vaccine, and their blood was collected before and at days 2, 4, 7, 10, 14, 28, 42, 56, 120, 180, 360, and 720 after immunization. Two unvaccinated volunteers were used as negative controls, and their blood was collected at the same times as that of the vaccinees. PBMC were isolated by density gradient centrifugation and cryopreserved in liquid N_2. These PBMC were used *ex vivo* as effectors cells.

The human experimentation guidelines of the U.S. Department of Health and Human Services and those of the University of Maryland, Baltimore, were followed in the conduct of the present clinical research. All blood specimens were collected from volunteers who participated in University of Maryland Institutional Review Board-approved protocols HP-00040025 and HP-00040022, which authorized the collection of blood specimens for the studies described in this report.

Target cells. The 721.221.AEH cell line, generously provided by D. Geraghty (10, 15), were used as target cells. The 721.221.AEH cell line is an HLA class I-defective Epstein-Barr virus-transformed lymphoblastoid B-cell line which has been transfected with HLA-E fused to the HLA-A2 leader peptide and therefore expresses HLA-E*0101, but not HLA-A, -B, -C, or -G, molecules on the cell surface. The 721.221.AEH cells were cultured in RPMI 1640 (Gibco, Grand Island, NY) 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, and 10% heat-inactivated fetal bovine serum (R10) supplemented with 200 mU/ml hygromycin B (Sigma). Cells were infected as previously described (27). Briefly, cells were incubated for 3 h at 37°C in RPMI (without antibiotics) in the presence of *S.* Typhi strain ISP1820 (obtained from J. Nataro, Center for Vaccine Development) at a multiplicity of infection of 10:1 (27, 28, 31, 32). After incubation, cells were washed and incubated overnight at 37°C in medium containing gentamicin (100 μg/ml) to kill and remove extracellular bacteria. The targets were then irradiated and surface stained with antibody to CD45, a marker abundantly expressed on the hematopoietic cell surface (4). After staining, the target cells were cocultured with PBMC (effector cells rested overnight) collected before and at days 2, 4, 7, 10, 14, 28, 42, 56, 120, 180, 360, and 720 after immunization at an effector-to-target cell ratio of 5:1.

MAbs for surface staining. Cells were surface stained with monoclonal antibodies (MAbs) to CD3 (clone UCHT1) (Beckman-Coulter, Miami, FL), CD4 (clone SK3), CD14 (clone M5E2) (BD Pharmingen, San Diego, CA), CD57 (clone TB01), CD107a (clone eBioH4A3), CD107b (clone eBioH4B4) (eBioscience, San Diego, CA), CD8 (clone 3B5), CD19 (clone SJ25-C1), CD45 (clone H130), CD27 (clone CLB-27/1), CD45RA (clone MEM-56), and CD62L (clone Dreg-56) (Invitrogen, Carlsbad, CA). Antibodies conjugated to the following fluorochromes were used in these studies: fluorescein isothiocyanate, phycoerythrin (PE), peridinin chlorophyll protein-Cy5.5, PE-Cy7, energy-coupled dye or PE-Texas Red conjugate, Pacific Blue, Pacific Orange, Quantum Dot (QD) 605, QD 655, QD 705, Alexa 647, allophycocyanin (APC)-Alexa 700, and APC-Cy7.

Surface and intracellular staining. PBMC from vaccinees or unvaccinated controls were stimulated with *S.* Typhi-infected 721.221.AEH target cells as described in the target cell section above, in the presence of MAbs to CD107a and CD107b (15 μl of each/1 × 10⁶ cells in 500 μl of R10 medium). The CD107a and -b antibodies were used to measure degranulation, a mechanism essential for the killing of *Salmonella*-infected targets by cytotoxic CD8⁺ cells (1). Uninfected 721.221.AEH target cells and *Staphylococcus* enterotoxin B (SEB; 10 μg/ml, Sigma, St. Louis, MO) were used as negative and positive controls, respectively. After 1 to 2 h of stimulation with *S.* Typhi antigens, the protein transport blockers monensin (1 μg/ml, Sigma) and brefeldin A (2 μg/ml; Sigma) were added to the PBMC. After overnight incubation (16 to 18 h), PBMC were harvested; stained with a dead-cell discriminator, a violet fluorescent viability dye (ViViD; Invitrogen) (14); surface stained with MAbs against surface antigens (CD3, CD4, CD8, CD14, CD19, CD27, CD45RA, CD57, and CD62L); and fixed and permeabilized with Fix & Perm cell buffers (Invitrogen, Carlsbad, CA) (Table 1). Cells were then stained intracellularly for IFN-γ (clone B27), TNF-α (clone MAb11), IL-2 (clone 5344.111) (B-D Pharmingen), and CD69 (clone TPI-55-3; Beckman-Coulter) to complete the 13-color staining panel. Cells were then resuspended in fixation buffer (1% formaldehyde) and analyzed as soon as possible by flow cytometry on an LSR-II instrument (BD Biosciences). Data were analyzed with WinList v6.0 (Verity Software House, Topsham, ME). Lymphocytes were gated based on their scatter characteristics. Single lymphocytes were gated based on forward scatter height versus forward scatter area. A "dump" channel was used to eliminate dead cells (ViViD⁺), as well as macrophages/monocytes (CD14⁺), B lymphocytes (CD19⁺), and targets (CD45⁺), from the

TABLE 1. Cell phenotypes and corresponding markers

Phenotype	Marker	Reference(s)
T-cell subsets		
Naïve	CD45RA⁺ CD62L⁺	25, 29, 33
T_{CM}	CD45RA⁻ CD62L⁺	25, 29, 33
T_{EM}	CD45RA⁻ CD62L⁻	25, 29, 33
T_{EMRA}	CD45RA⁺ CD62L⁻	25, 29, 33
Degranulation		
LAMP-1	CD107a	1
LAMP-2	CD107b	1
Dump channel		
Dead cells	ViViD	14
Monocytes	CD14	14
B cells	CD19	14
Hematopoietic cells	CD45	4

analysis. This was followed by additional gating on CD3, CD4, and CD8 to identify cytokine-producing CD8⁺ T cells. Each cytokine was gated individually. During sample acquisition, 300,000 to 500,000 events were routinely collected in the forward and side scatter lymphocyte gate. This large number of gated lymphocyte events was essential to ensure that sufficient numbers of positive cells for defined subsets would be collected for each tube analyzed.

Statistical analysis. All statistical tests were performed using Prism software (version 5.02; GraphPad Software, La Jolla, CA). Comparisons between groups were performed using Kruskal-Wallis one-way analysis of variance on ranks or Pearson product-moment correlation tests. *P* values of <0.05 were considered significant. Cubic spline curves were used for a better representation of the complex responses observed. Cubic spline curves are smooth curves through every point with the capability to be used as a standard curve or to calculate the area under the curve (AUC) (21).

RESULTS

Kinetics of *S.* Typhi-specific HLA-E-restricted CD8⁺ cells over a 2-year postvaccination follow-up. Because of the potential importance of HLA-E-restricted CD8⁺ cells in resistance to *Salmonella* infection (27), we investigated their kinetics of appearance and persistence in volunteers immunized with the *S.* Typhi Ty21a strain typhoid vaccine. Based on previous data showing that CD8⁺ responses can be detected as early as 14 days after immunization (32), we hypothesized that these responses might appear at even earlier time points. We also evaluated the longevity of these responses up to 2 years after immunization. To this end, PBMC collected before and at days 2, 4, 7, 10, 14, 28, 42, 56, 120, 180, 360, and 720 after immunization were stimulated with *S.* Typhi-infected 721.221.AEH target cells in the presence of MAbs to CD107a and -b. The 721.221.AEH targets are HLA class Ia-defective lymphoblastoid B cells which constitutively express HLA-E on their surface. The CD107a and -b antibodies were used to measure degranulation (1). Previously, we have shown that HLA-E-restricted *S.* Typhi-specific CD8⁺ T cells effectively kill *S.* Typhi-infected targets by a granule-dependent mechanism (27). In the present studies, uninfected 721.221.AEH target cells and SEB were used as negative and positive controls, respectively. After overnight incubation, PBMC were surface stained with MAbs to CD3, CD4, CD8, CD14, CD19, CD27, CD45RA, CD57, and CD62L. We selected overnight incubation to minimize bystander stimulation while maintaining a close parallel with the *in vivo* cytokine response (6). Cells were then stained intracellularly with MAbs to CD69 and IL-2,
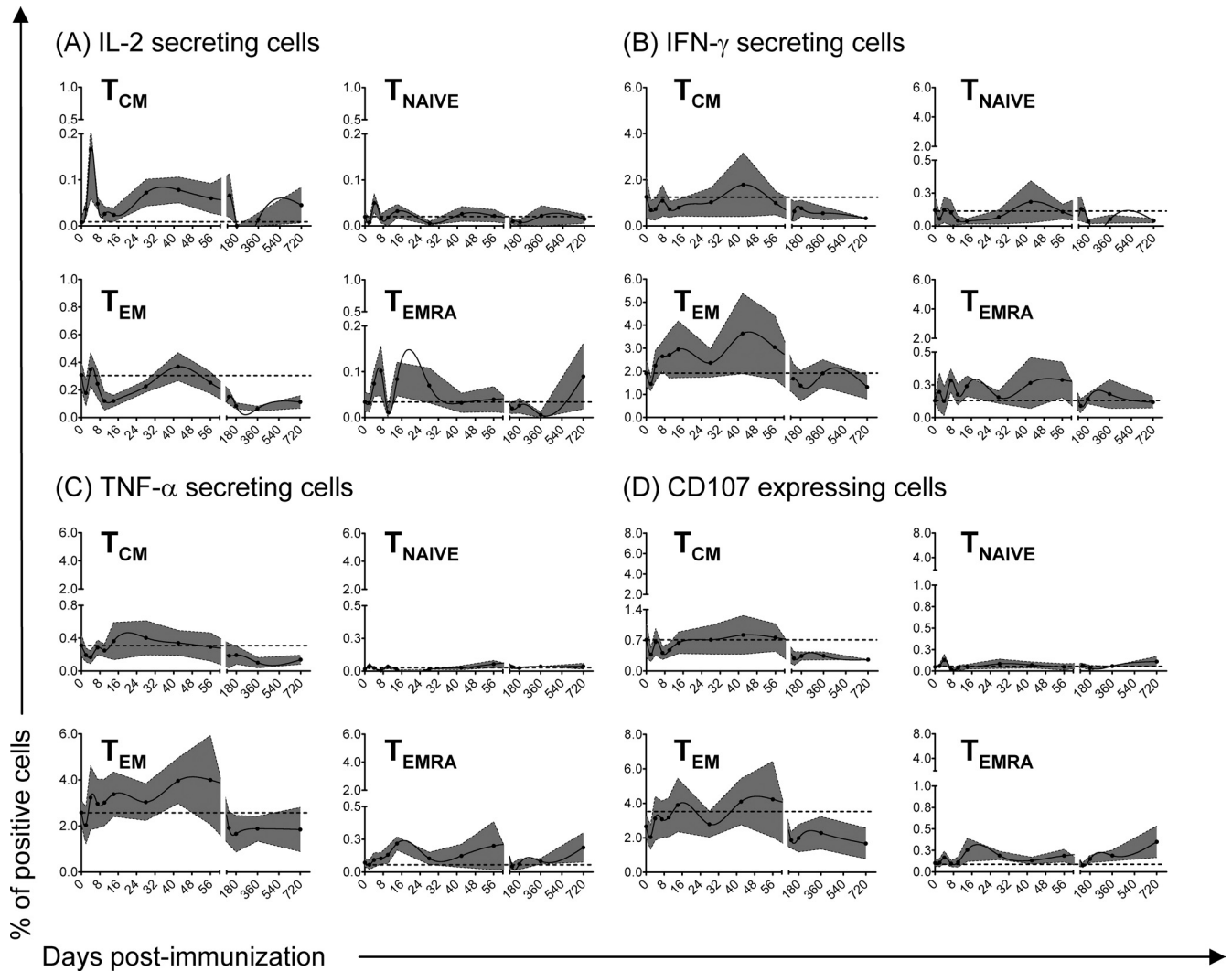
FIG. 1. Kinetics of *S.* Typhi-specific HLA-E-restricted CD8+ cells over a 2-year postvaccination follow-up. PBMC were stimulated with *S.* Typhi-infected 721.221.AEH target cells in the presence of CD107a and -b MAbs. After overnight incubation, PBMC were stained with ViViD, followed by surface staining with MAbs to CD3, CD4, CD8, CD14, CD19, CD45RA, and CD62L. After fixation and permeabilization, cells were intracellularly stained with MAbs to CD69, as well as to the cytokines IL-2, IFN-γ, and TNF-α, and analyzed by multichromatic flow cytometry. For the analysis, first a dump channel was used to eliminate dead cells (ViViD+), as well as macrophages (CD14+), B cells (CD19+), and stimulators (CD45+), from the results. This was followed by additional gating on CD3, CD4, and CD8, as well as CDR45RA versus CD62L, to analyze the three main CD8+ cell subsets: naïve T cells (CD45RA+ CD62L+), central memory T cells (T_{CM} [CD45RA− CD62L+]), and effector memory T cells (T_{EM} [CD45RA− CD62L−] or T_{EMRA} [CD45RA+ CD62L−]). Represented are cubic spline curves based on all data points to allow a better representation of the complex responses observed. The spline curves were generated by grouping the data of all five volunteers. The curves represent the mean of the results from the five different volunteers who participated in these studies. The filled area denotes the standard error bands. The dashed lines represent the baseline values (day 0).

IFN-γ, and TNF-α cytokines analyzed by multichromatic flow cytometry. We have previously demonstrated that these cytokines have the ability to serve as surrogate markers of *S.* Typhi-specific CD8+ cell responses (28, 30–32, 40). For data analysis, CD8+ cells (CD3+ CD4−) were divided into three main subsets: naïve T cells (CD45RA+ CD62L+), central memory T cells (T_{CM} [CD45RA− CD62L+]), and effector memory T cells (T_{EM} [CD45RA− CD62L−] or T_{EMRA} [CD45RA+ CD62L−]) (25, 29, 33). In agreement with previous results, cytokine-secreting (e.g., IFN-γ and TNF-α), as well as CD107-expressing, CD8+ cells were predominantly classical T_{EM} cells (41). Unexpectedly, these responses were multipha-

sic, with a first peak response observed as early as 4 days (in two out of five volunteers) after immunization (Fig. 1 and 2), followed by a second, more pronounced and persistent, peak between days 42 and 56 (in four out of five volunteers) (Fig. 1). This second peak decreased sharply over the next few months. In three out of five volunteers, a much smaller third peak could be observed at around 180 days postimmunization. Interestingly, CD8+ T_{EMRA} and T_{CM} subsets secreting IFN-γ and TNF-α and expressing CD107 were also present, albeit at a much lower magnitude than the T_{EM} subset (Fig. 1 and 2). Like those of the T_{EM} subset, the T_{EMRA} subset responses were multiphasic, with a first peak between days 4 and 14 (day
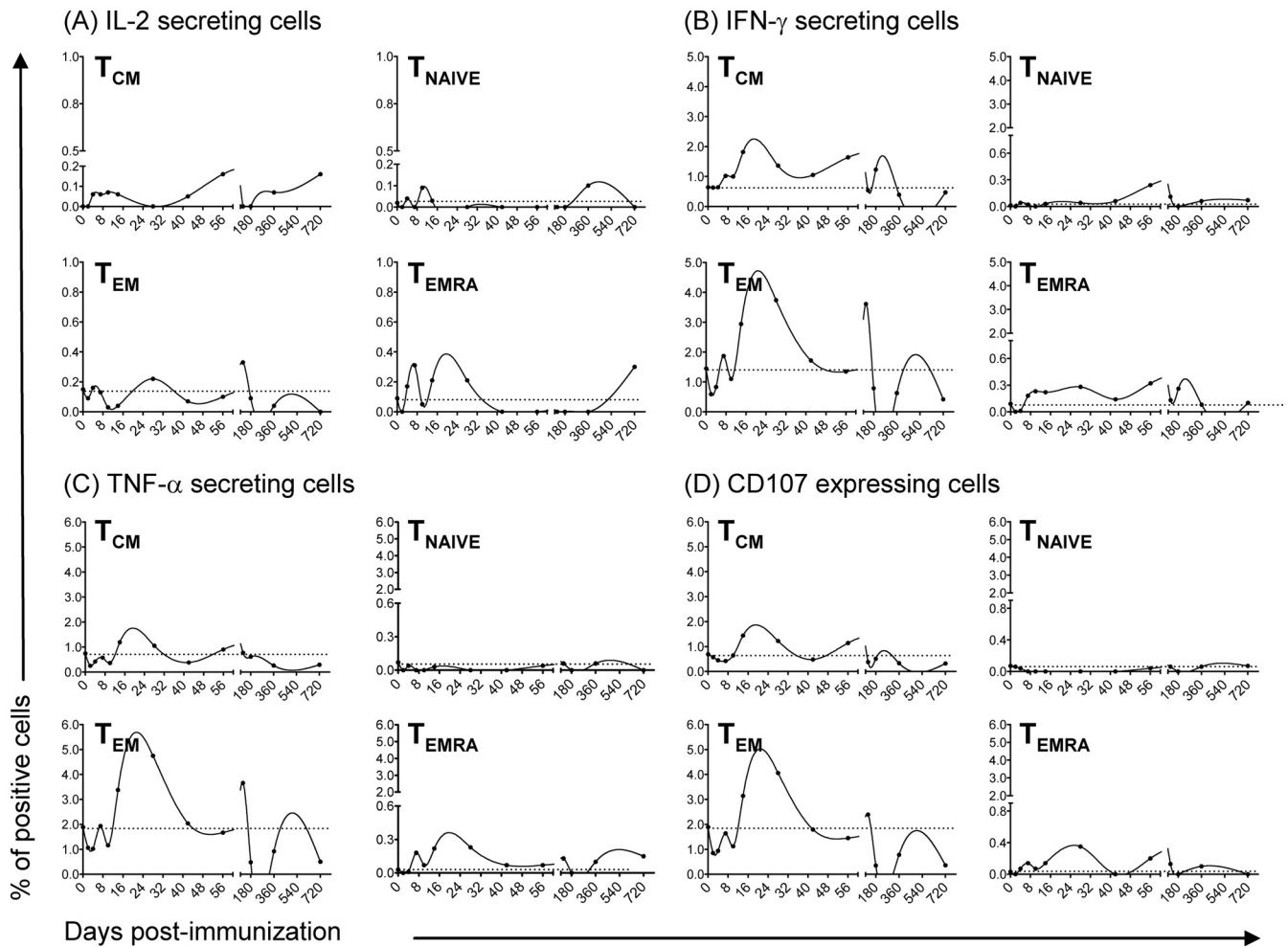
FIG. 2. Kinetics of *S.* Typhi-specific HLA-E-restricted CD8^{+} cells over a 2-year postvaccination follow-up in a Ty21a vaccinee. PBMC from one Ty21a vaccinee were stimulated with *S.* Typhi-infected 721.221.AEH target cells in the presence of CD107a and -b MAbs. After overnight incubation, PBMC were stained with ViViD, followed by surface staining with MAbs to CD3, CD4, CD8, CD14, CD19, CD45RA, and CD62L. After fixation and permeabilization, cells were stained intracellularly for CD69, as well as for the cytokines IL-2, IFN-γ, and TNF-α, and analyzed by multichromatic flow cytometry as described in the legend to Fig. 1. Represented are cubic spline curves based on all data points to allow a better representation of the complex responses observed. The curves represent the results from one vaccinee (CVD5000#04S). The dashed lines represent the baseline values (day 0).

7 in three out of five volunteers) after immunization, followed by a second peak between days 14 and 28. A third peak, if present, was observed between days 42 and 720 after immunization (day 42 in two out of three volunteers). Surprisingly, responses in the T_{EMRA} subset were more persistent than those observed in the T_{EM} and T_{CM} subsets, found to be present 720 days after immunization in two out of four volunteers. These results are in agreement with published data suggesting that T_{EMRA} subset cells are not necessarily terminally differentiated and may maintain their ability to proliferate and secrete cytokines after antigenic exposure (20). We also observed, for the first time, the presence of IL-2-secreting CD8^{+} cells. As expected, these cells were predominantly of the T_{CM} subset. However, unexpectedly, we observed IL-2 production by T_{EMRA} cells, followed, albeit at a lower levels, by T_{EM} cells. These T_{CM} subset cells appeared as early as 2 to 4 days (in four out of five volunteers) after immunization, with the fifth volunteer showing peak IL-2 production at day 7. A second peak

was present in all five volunteers at days 28 to 56 and a third peak, if present (in two of five volunteers), could be observed between days 180 and 720 after immunization (Fig. 1 and 2). It is important to note that the level and timing of cytokine production varied among of the various volunteers. This is not surprising, since differences in the degree of response, as well as differences in the background level before immunization, among individuals are a common finding in human studies (32, 40, 41). Finally, as expected, only minor changes without defined patterns in cytokine secretion by *S.* Typhi-infected cells were observed in $T_{NAÏVE}$ cells or in the four subsets (T_{EM}, T_{EMRA}, T_{CM}, and T_{NAIVE}) from two healthy volunteers used as controls in these studies (Fig. 1, 2, and 3). Regardless of the subpopulation(s) showing the most pronounced responses, all five volunteers showed an increase at least one of the times examined compared to those observed before immunization. Based on these results, we conclude that HLA-E-restricted CD8^{+} cells might play a dual role, i.e., (i) at early time points
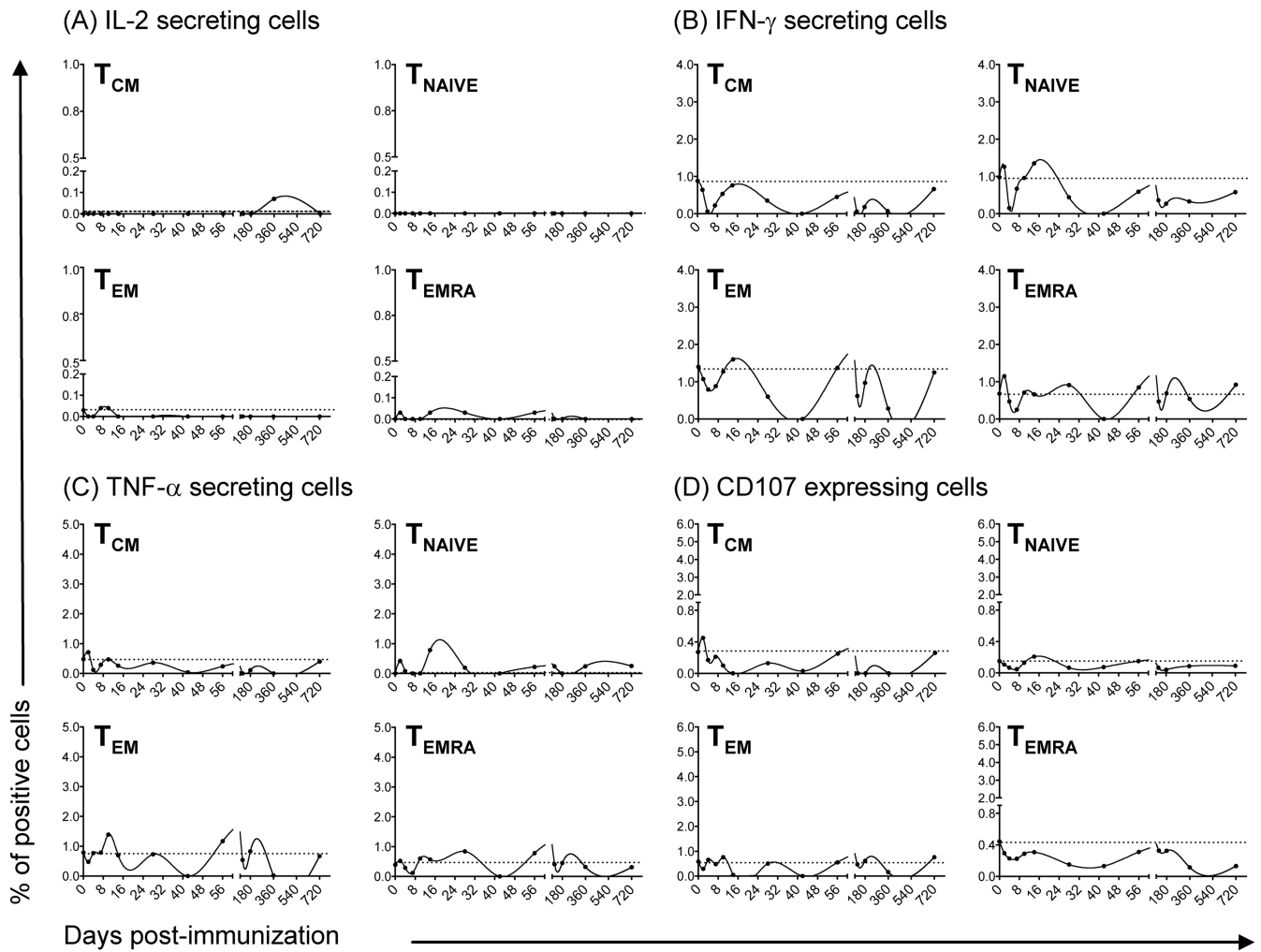
FIG. 3. Kinetics of *S*. Typhi-specific HLA-E-restricted CD8+ cells over a 2-year postvaccination follow-up in an unvaccinated volunteer. PBMC from one healthy unvaccinated volunteer were stimulated with *S*. Typhi-infected 721.221.AEH target cells in the presence of CD107a and -b MAbs. After overnight incubation, PBMC were stained with ViViD, followed by surface staining with MAbs to CD3, CD4, CD8, CD14, CD19, CD45RA, and CD62L. After fixation and permeabilization, cells were stained intracellularly for CD69, as well as for the cytokines IL-2, IFN-γ, and TNF-α, and analyzed by multichromatic flow cytometry as described in the legend to Fig. 1. Represented are cubic spline curves based on all data points to allow a better representation of the complex responses observed. The curves represent the results of one healthy unvaccinated volunteer (CVD4000#33C). The dashed lines represent the baseline values (day 0).

bridging innate and acquired immunity and (ii) at later times protecting from *Salmonella* infection by an adaptive mechanism.

Cumulative HLA-E-restricted CD8+ cell responses over the 2-year follow-up. One of the hallmarks of successful vaccination is the induction of strong and persistent memory cell responses (19, 25, 29). We studied this phenomenon by combining the level and duration of HLA-E-restricted CD8+ cell responses into a single parameter to measure differences among responses in defined cell subsets. In this case, we used AUC analysis as a cumulative measurement of the rise and persistence of HLA-E-restricted CD8+ cell responses above the baseline (day 0) over the 2-year period. The AUC was defined as the number of positive responses divided by the number of postvaccination visits. Comparisons of the various CD8+ cell subsets were performed using Kruskal-Wallis one-way analysis of variance on ranks. Nominal variables were

analyzed by the Student-Newman-Keuls test. The cumulative analyses of IFN-γ production demonstrated that the CD8+ T_{EM} and T_{CM} cell subsets exhibited a significantly greater AUC than the naïve and T_{EMRA} subsets. Interestingly, the AUC analyses of TNF-α production demonstrated that the CD8+ T_{EM} and T_{EMRA} subsets had a significantly greater AUC than the naïve and T_{CM} subsets (Fig. 4). Although we observed greater AUCs in IL-2-producing T_{CM} and CD107-expressing T_{EM} and T_{EMRA} cells than in the other populations, these differences did not reach statistically significance. This phenomenon might be due to the limited number of volunteers studied (Fig. 4). Taken together, these data confirm and extend the notion that CD8+ T_{EM} cells are the most effective producers of the IFN-γ and TNF-α cytokines. These data also demonstrate the relative importance of IFN-γ-secreting CD8+ T_{CM}, as well as TNF-α-secreting CD8+ T_{EMRA}, cells.
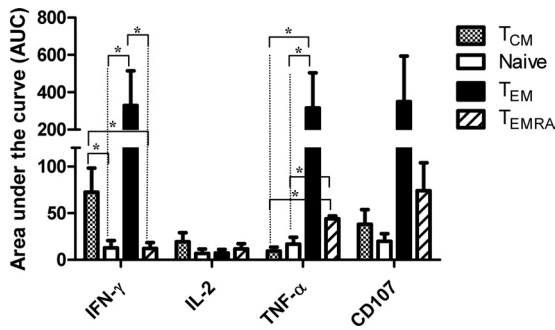
FIG. 4. Comparison of the AUCs of different CD8$^+$ subsets and their production of IL-2, TNF-α, and IFN-γ and expression of CD107 molecules. Bar graphs represent means plus standard errors of the pooled data from the five different volunteers. AUCs are expressed in arbitrary units. *, positive response (statistically significant at $P < 0.05$).

Induction of long-lasting multifunctional HLA-E-restricted CD8$^+$ memory T-cell subsets by Ty21a immunization. Because previous human studies have demonstrated a relationship between the presence of multifunctional T cells and the quality of the immune responses (2, 23), here we simultaneously measured four HLA-E-restricted CD8$^+$ T-cell functions (CD107 mobilization and IFN-γ, TNF-α, and IL-2 secretion) by multichromatic flow cytometry. Analyses of multiple cytokine patterns revealed that the majority of HLA-E-restricted CD8$^+$ T$_{EM}$ and T$_{EMRA}$ cells, particularly between days 28 and 56

after immunization, showed characteristics typical of multifunctional cells, with the specific cells exhibiting predominantly a response pattern characterized by three functions (Fig. 5). The major populations of multifunctional cells were those that concurrently secrete IFN-γ and TNF-α and express CD107. IL-2 production was also detected in combination with these three functions, but at a much lower frequency (Fig. 5). Virtually no correlations were observed between IL-2 secretion and the other three functions (Fig. 6). The only exception of a statistically significant correlation was observed between IL-2- and IFN-γ-secreting CD8$^+$ T$_{CM}$ cells at days 28 to 56 after immunization (Fig. 6). In contrast, highly significant correlations were observed between the kinetics of IFN-γ production and the expression of CD107 in all three major CD8$^+$ T-cell subsets, i.e., T$_{CM}$, T$_{EM}$, and T$_{EMRA}$ (Fig. 6). IFN-γ production was also correlated with TNF-α production in the T$_{EM}$ (at all time points) and T$_{EMRA}$ (at days 0 to 4 and 28 to 56) subsets. Of note, the vast majority of these multifunctional T cells did not express either CD27 or CD57 on their cell membrane (~90%) at any time point. Less than 10% of multifunctional T cells expressed either CD27 or CD57, but none expressed both CD27 and CD57. In conclusion, these data support the idea that multifunctional HLA-E-restricted CD8$^+$ cells are long-lived and might contribute to effective *Salmonella* immunity.

Correlation between early and late cytokine production patterns by the different CD8$^+$ memory T-cell subsets. Another important aim in vaccine development is to determine whether early responses can predict the long-term persistence of im-
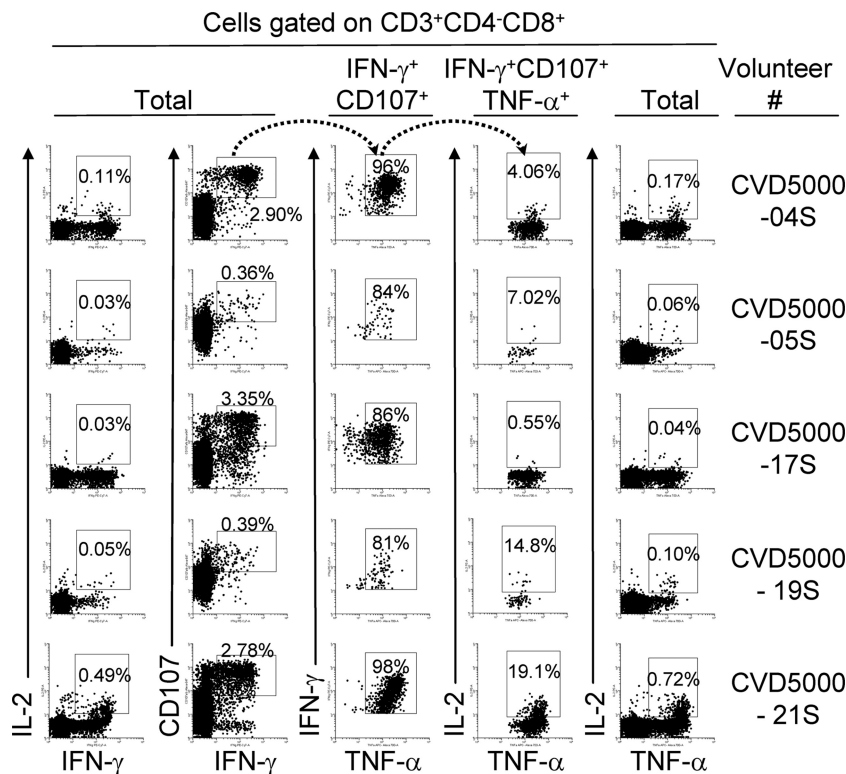

FIG. 5. Induction of multifunctional CD8$^+$ T$_{EM}$ cells by Ty21a immunization. PBMC were stimulated and analyzed as described in the legend to Fig. 1. As shown by the arrows, sequential gating was used to identify multifunctional CD8$^+$ T$_{EM}$ cells from five volunteers 42 days after vaccination. Four T-cell functions were evaluated: expression of CD107 and secretion of IL-2, IFN-γ, and TNF-α.

(A) T_{CM}

(B) T_{EM}

(C) T_{EMRA}

0-4   7-14   28-56   120-720

% of positive cells

% of IFN-γ positive cells
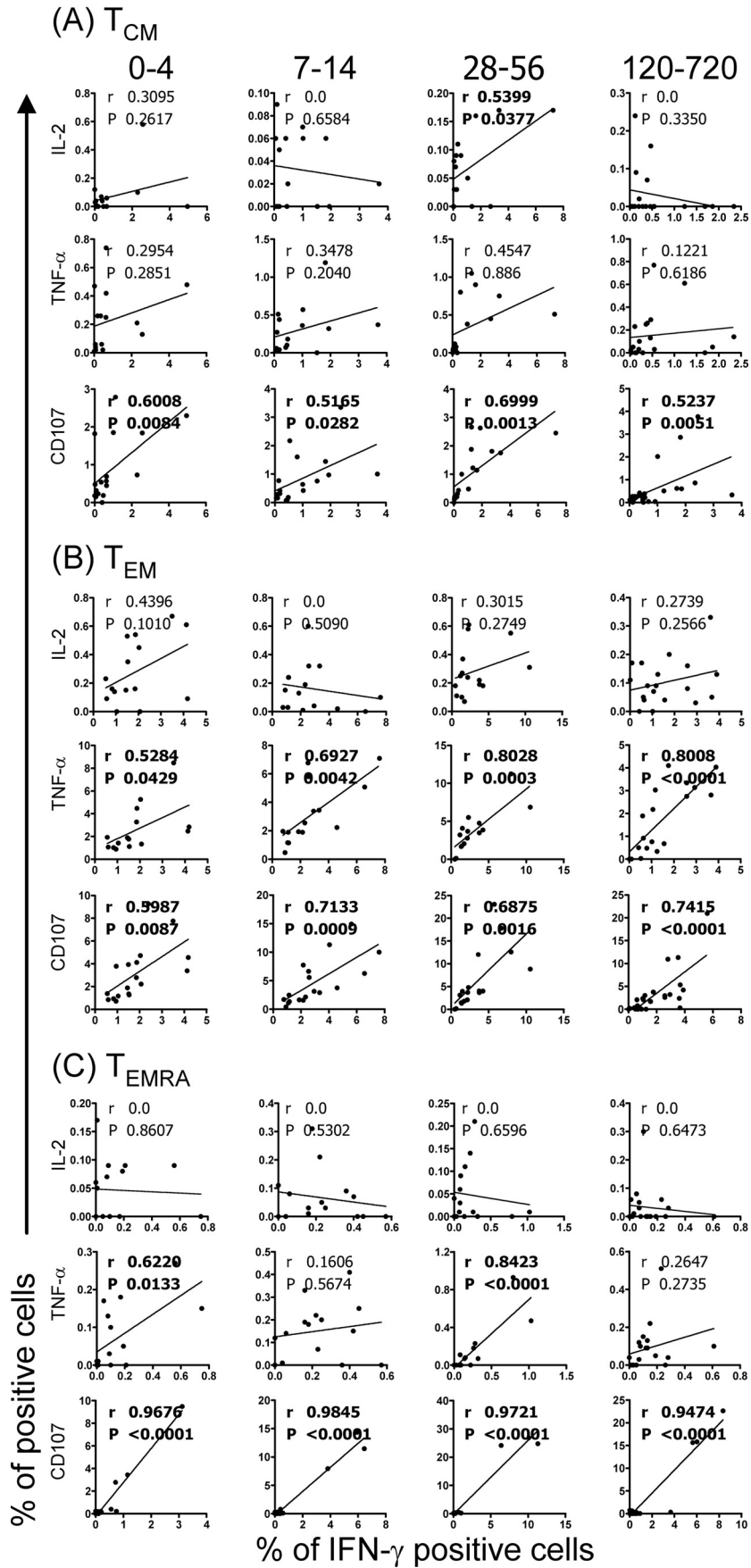
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TABLE 2. Correlations among early and long-term responses in CD8^{+} T-cell subsets

Subset and cytokine (early positive responses [<28 days])	Long-term positive responses (>42 days) (P values)[a]											
	T_{CM}				T_{EM}				T_{EMRA}			
	IL-2	IFN-γ	TNF-α	CD107	IL-2	IFN-γ	TNF-α	CD107	IL-2	IFN-γ	TNF-α	CD107
T_{CM}												
IL-2	0.4	**<0.0001**	0.472	**0.0003**	0.317	**0.0009**	**0.041**	**0.0115**	0.704	**0.0006**	**0.031**	0.784
IFN-γ	0.568	**0.002**	0.419	**0.012**	0.732	**0.036**	0.272	**0.0496**	0.918	**0.0001**	**0.049**	0.824
T_{EMRA}, IL-2	0.638	**0.001**	0.488	**0.002**	0.704	**0.047**	0.245	**0.042**	0.969	0.878	0.743	0.316

[a] Significant P values are in bold. P values of <0.05 were considered statistically significant.

munity to the organism used for immunization. This information could be important in the selection of the best early T-cell markers of systemic immune responses to evaluate future *S.* Typhi vaccine candidates. To this end, we compared the responses of the three main CD8^{+} memory T-cell subsets (i.e., T_{EM}, T_{CM}, and T_{EMRA}) to predict long-term immunity. Each of these three subsets was arranged in two groups based on the timing postvaccination. One group included early positive responses (days 2, 4, 7, 10, 14, and 28), and the other group included long-term positive responses after immunization (days 42, 56, 120, 180, 360, and 720). Comparisons among the different CD8^{+} cell subsets were performed using Pearson product-moment correlation. Interestingly, we found a striking correlation between volunteers exhibiting early IL-2- and IFN-γ-secreting CD8^{+} T_{CM} subsets and those CD8^{+} T_{CM}, T_{EM}, and T_{EMRA} subsets that later produced IFN-γ (Table 2). We also found significant correlations between the early IL-2- and IFN-γ-secreting CD8^{+} T_{CM} subsets and the CD8^{+} T_{EM} and T_{EMRA} subsets that later produced TNF-α, as well as the CD8^{+} T_{CM} and T_{EM} subsets that later expressed CD107 (Table 2). Unexpectedly, we also found a good correlation between the early IFN-γ-secreting CD8^{+} T_{EMRA} responses and the CD8^{+} T_{CM} and T_{EM} subsets that later secreted IFN-γ, as well as the T_{CM} and T_{EM} subsets that expressed CD107 (Table 2). No other early predictors of positive late responses were observed. In conclusion, these data suggest that the presence at early postvaccination of responses by CD8^{+} T_{CM} and T_{EMRA} subsets that produce sizable amounts of IL-2 and/or IFN-γ might be good indicators of long-lasting immune responses.

DISCUSSION

In spite of the putative importance of HLA-E-restricted responses in protection from infectious agents, very little information is available about either mice or humans (11, 35, 36). In this report, we present the first detailed *ex vivo* kinetic analysis and characterization of the induction and persistence of HLA-E-restricted CD8^{+} memory T (T_M) cells up to 2 years after immunization of volunteers with the licensed Ty21a typhoid vaccine. Interestingly, we observed two or three waves of elevated levels of HLA-E-restricted CD8^{+} T_M cells over time in the absence of additional exposure to the organism beyond

day 7 (the last day of vaccination). This phenomenon is remarkably different than the typical one-wave (expansion-contraction) responses observed in classical class Ia HLA-A-, B-, and C-restricted T_M responses (24). The first wave occurred as early as 2 to 4 days after immunization with *S.* Typhi, with an increase in the levels of all three major HLA-E-restricted CD8^{+} T_M subsets (T_{EM}, T_{EMRA}, and T_{CM}). This phenomenon further supports the contention advanced by us and others (27, 35) that these HLA-E-restricted responses mediated by CD8^{+} cells might bridge the innate and adaptive immune responses. The first wave was followed by a decline in the levels of HLA-E-restricted CD8^{+} T_M cells in circulation. We speculate that this might be the result of T-cell recirculation, with some T-cell effectors homing to the gut. This assumption is based on our previous work showing the presence of *S.* Typhi-specific CD8^{+} T_M cells coexpressing gut-homing molecules (high levels of integrin α_4/β_7, intermediate levels of CCR9, and low levels of CD103) in the circulation (31, 41). The second wave, the one with the most pronounced levels of HLA-E-restricted CD8^{+} T_M cells, occurred between days 28 and 56. Interestingly, we observed that these HLA-E-restricted CD8^{+} memory cells consisted of heterogeneous populations that exhibited a distinct kinetic behavior depending on the memory T-cell subset. For example, while high levels of T_{EM} responses were observed 56 days after immunization, they declined sharply afterward. In contrast, low levels of T_{EMRA} responses persisted for up to 2 years after immunization. These results are in agreement with our previous studies showing the presence of specific CD8^{+} T-cell responses even 3 years after immunization, although these responses could only be evidenced following *in vitro* expansion in the presence of specific antigens (28, 31). It is widely accepted that strong immune responses are determined not only by their magnitude but also by their quality and persistence. Consequently, it is reasonable to speculate that T_{EM} responses of high magnitude but short duration and T_{EMRA} responses of lower magnitude but long duration might have synergistic and/or complementary roles in the control of *Salmonella* infection. In this scenario, some antigen-specific T-cell clones may remain for long periods of time as memory T cells that, upon subsequent exposure to antigen, provide a stronger, rapid, and sometimes qualitatively different specific

FIG. 6. Correlations among different CD8^{+} subsets that produce IFN-γ and those that produce IL-2 or TNF-α or express CD107 molecules. PBMC were stimulated and analyzed as described in the legend to Fig. 1. Results were divided into four groups: 1, responses at days 2 to 4; 2, responses at days 7 to 14; 3, responses at days 28 to 56; 4, responses at days 120 to 720. Coefficients of determination (r values) and P values are shown. Significant P values are in bold. P values of <0.05 were considered statistically significant.

immune response (13, 24). The third wave was present in three of the five volunteers between days 42 and 720, depending on the T$_M$ subset evaluated. These long-term cell-mediated immune responses might help explain the observations in field trials showing that three doses of the enteric-coated formulations of Ty21a given within 1 week provided 67% efficacy during the first 3 years of follow-up (17) and 62% protection over 7 years of follow-up (16) and that four doses of Ty21a in enteric-coated capsules given within 8 days are significantly more protective than two or three doses (8).

We also observed that Ty21a immunization elicited multifunctional HLA-E-restricted CD8$^+$ cells that can degranulate (CD107$^+$), a mechanism essential for cytotoxic responses against S. Typhi-infected targets, and produce IFN-γ, TNF-α, and IL-2, which are critical components of a robust inflammatory response. However, the observations regarding IL-2 appear to be in contrast to our previous results obtained with volunteers immunized with the attenuated S. Typhi strain CVD 909, which resulted in significant increases in IFN-γ, TNF-α, and IL-10, but not IL-2, production (40). It is likely that this discrepancy is the result of the use of different methodologies to analyze CD8$^+$ T-cell responses. Here, CD8$^+$ T cells from Ty21a vaccinees were stimulated with S. Typhi-infected targets whereas CD8$^+$ T cells from CVD 909 vaccinees were stimulated with S. Typhi flagella or particulate S. Typhi antigen. Interestingly, we also observed that S. Typhi-specific multifunctional HLA-E-restricted T$_M$ cells do not express either CD27 or CD57. These results are in agreement with those obtained with CD8$^+$ T cells of volunteers immunized with vaccinia virus (23) but in contrast to the observations of CD8$^+$ cells in volunteers immunized with cytomegalovirus (42). These observations strongly suggest that different pathogens elicit different T$_M$ and effector populations. Multifunctional T cells are widely believed to be of great importance in determining the quality of immune responses to vaccination or exposure to wild-type organisms (2, 23). The fact that the multifunctional HLA-E-restricted T$_M$ cells described herein appear to dominate early responses and persist for long periods of time adds further support to the contention that this might be an important mechanism of the host immune response to S. Typhi.

Finally, we observed a striking correlation among volunteers who showed strong CD8$^+$ T$_{CM}$ subsets which secreted IL-2 and IFN-γ at early times and the presence in these volunteers of long-term immune responses. We speculate that this phenomenon might be due to the fact that IL-2- and/or IFN-γ-secreting CD8$^+$ T$_{CM}$ subsets at early times after vaccination results in the development of a larger pool of long-lived specific CD8$^+$ T$_M$ cell subsets (CD8$^+$ T$_{CM}$, T$_{EM}$, and T$_{EMRA}$), which might lead to an improved ability of the host to control reinfection. In this scenario, following the contraction phase, only low levels of CD8$^+$ T$_{EMRA}$ cells remain detectable in circulation. These CD8$^+$ T$_{EMRA}$ cells might play an important role in the expansion of the other CD8$^+$ T$_M$ subsets upon reexposure to the pathogen.

In sum, this is, to our knowledge, the first demonstration in humans of the presence of persistent multifunctional HLA-E-restricted CD8$^+$ cells up to 2 year after immunization with attenuated S. Typhi. These findings argue that HLA-E-specific CD8$^+$ cells play an integral role in the innate/adaptive immune response against Salmonella infection. These observations are particularly important because they could guide the selection of appropriate systemic immune responses to evaluate in the development of future vaccine candidates, not only for S. Typhi, but also for other infectious enteric pathogens.

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