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Cell Mediated Immune Responses Following Revaccination with an Influenza A/H5N1 Vaccine

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Abstract

Purpose—The study aims were to determine whether inactivated influenza A/H5N1 vaccine administration elicited cell mediated immune (CMI) responses and the impact of adjuvant, vaccine dose and subject age on these responses.

Methods—Adults who were previously primed with either adjuvanted or unadjuvanted, inactivated, A/H5N1/Vietnam/1203/2004 (Clade 1) vaccine or unprimed (received placebo) in previous vaccine studies were randomized to receive one (primed) or two (unprimed) 15- or 90-mcg doses of inactivated, A/H5N1/Indonesia/05/05 (Clade 2) vaccine. Peripheral blood

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mononuclear cells (PBMCs) were collected and analyzed from a subset of vaccinees to assess CMI responses using IFN- γ and granzyme B ELISPOT assays. Cytokine measurements were performed on PBMC supernatants after stimulation with H5N1 virus.

Results—PBMCs were available from 177 participants; 88 and 89 received 15-mcg and 90-mcg of unadjuvanted clade 2 vaccine, respectively. Following H5N1 clade 1 stimulation, IFN- γ but not granzyme B normalized spot-forming cell numbers had statistically significant increased numbers at each of the post-vaccination timepoints compared to baseline in pooled analyses of all vaccine doses and age groups. Clade 2 stimulation resulted in statistically significant increased numbers of IFN- γ cells only 180 days following the last vaccination. Responses were similar among younger and older study participants, as were responses among those primed with alum-adjuvanted or non-adjuvanted clade 1 H5N1 vaccines. The dosage of clade 2 vaccine did not impact CMI responses among primed subjects, but responses were statistically significantly greater in unprimed recipients of the 90-mcg dosage compared to unprimed recipients of the 15-mcg dosage. IFN- γ levels in the supernatants of stimulated PBMC were strongly correlated with IFN- γ ELISPOT results.

Conclusion—CMI responses occur in adults administered influenza A/H5N1 inactivated influenza vaccine.

Keywords

Inactivated influenza vaccine; H5N1; cell mediated immune response; cytokine; adjuvant

The emergence of novel influenza virus strains (H5N1, H7N9, H9N2, 2009 H1N1, H3N2v) as causes of disease in humans has led to the development of vaccines to prevent or attenuate infection [1–4]. Most studies have assessed vaccine immunogenicity using serological methods such as hemagglutination inhibition (HAI) and microneutralization (Neut) assays [5]. In particular, HAI antibodies have been identified as a correlate of protection from influenza-associated infection and illness with seasonal strains [6,7]. Other antibodies, e.g., those targeting the neuraminidase [8,9] or the hemagglutinin stalk [10,11], also provide protection against influenza virus infection and illness.

Cell mediated immunity (CMI) is another immune response involved in protection against symptomatic influenza. McMichael and colleagues observed an inverse correlation between influenza-specific cytotoxic T lymphocytes (CTL) in the peripheral blood and the duration of nasal virus shedding following experimental influenza infection in humans with undetectable influenza-specific antibodies [12], and other investigators have subsequently demonstrated a role for CMI responses in protection from influenza-associated illness [13–15]. Studies with trivalent inactivated influenza vaccines (IIV3) have demonstrated transient CTL effector responses [16,17]. More recently, interferon gamma (IFN- γ) and granzyme B (GrB) spot-forming cells (SFCs) derived from peripheral blood mononuclear cells (PBMCs) have been used to measure influenza-specific CMI responses [18,19], including those following IIV administration [20,21]. The aim of the current study was to determine whether inactivated influenza A/H5N1 vaccine administered as a booster dose would elicit CMI responses and if present, the effects of priming, adjuvant, vaccine dose and subject age on

this response. The correlation of Th1 and Th2 cytokine levels in PBMC supernatants with CMI responses was also assessed.

Methods

Study design

This was a substudy of a parent trial to assess the safety and immunogenicity of a booster dose of inactivated influenza A/H5N1 vaccine; a full description of the parent trial is available [22]. In brief, eligible study participants included individuals who had previously participated in clinical trials of clade 1 A/Vietnam/1203/2004 (H5N1) vaccines produced by Sanofi Pasteur (subvirion) or Novartis (purified surface antigen). In previous trials, each participant received either placebo or two or more doses of vaccine ranging from 3.75–90 mcg of hemagglutinin protein (HA) with or without adjuvant (Alhydrogel® or MF59®) 1.4–3.7 years earlier [23–30]. In the parent trial, eligible subjects were adults 19 years of age or older who were in stable general health, not on immunosuppressive therapy and not pregnant. Participants were randomized to receive either 15 or 90 mcg of inactivated, monovalent, subvirion, reverse genetics-derived (rg) A/H5N1/Indonesia/05/05 (clade 2) vaccine (Sanofi Pasteur) administered intramuscularly as a single 0.5-mL dose. Placebo recipients from the earlier trials received two doses of the unadjuvanted clade 2 vaccine (15 or 90mcg) 28 days apart. Subjects were stratified by age at the time of randomization (<65 vs. 65 and older) and by previous priming dose (low dose [3.75, 7.5 or 15 mcg] vs. high dose [30, 45 or 90 mcg]). Blood for CMI studies was collected on study days 0, 8, 14, 28 and 180. For the H5N1 vaccine-naïve subjects, the 180-day blood collection was 180 days after the second vaccine dose (day 236); other blood collection days (0, 8, 14, 28) were unchanged. In this CMI study, persons in the low dose priming group were not included because it was hypothesized they may be less likely to be primed than those in the high dosing group, and a convenience sample from the remaining eligible participants was evaluated (Supplemental Figure 1). The protocol was reviewed and approved at Institutional Review Boards of each of the participating institutions and participants provided written informed consent. The trial is registered at clinicaltrials.gov (NCT00680069).

Blood collection and processing

Whole blood was collected in heparinized tubes and processed as described in the online supplement.

Viruses

Influenza A virus reassortant reference vaccine strains A/Vietnam/1203/2004(H5N1)/PR8-IBCDC-RG (clade 1) and A/Anhui/01/2005(H5N1)-PR8-IBCDC-RG5 (clade 2) were obtained from the CDC. Virus was propagated in 10-day old embryonated eggs and then used for infection of stimulator PBMCs.

ELISPOT assays

Assays were performed in quadruplicate wells for each assay condition as described previously and are described in the online supplement [20,31].

Cytokine analysis

Cell culture supernatants collected following overnight incubation of effector and stimulator cells were evaluated with the multiplex Luminex[®] assay using the MILLIPLEX MAP Multiplex Assay system (EMD Millipore). Assays were performed according to the manufacturer's instructions.

Serology

HAI and Neut assays were performed as described and reported previously [22].

Statistical analyses

The lower limits of quantification (LLOQ) for IFN- γ and GrB SFC numbers were 5 and 20 SFCs, respectively. If the number of cells detected fell below the LLOQ, a value of LLOQ/2 was imputed. Arithmetic means of quadruplicate samples were determined and used for subsequent analyses. The normalized SFCs (nSFCs) for each subject and timepoint were calculated by subtracting the negative control SFCs (medium) from the observed SFC. Due to the limited sample size, the Clade 1 placebo, age ≥ 65 stratum was excluded from the nSFC analyses. When all data were pooled (N = 158) asymptotic normality was assumed for mean nSFCs and significance of baseline difference was evaluated using an upper one-sided paired t-test. When the data were analyzed by strata, a non-parametric bootstrap method with 1,000 bootstrap replicates was applied to obtain 95% Confidence Intervals (CI) of the medians and strata were compared using a two-sided Wilcoxon Rank Sum test. In either case, no adjustment for multiple testing was carried out. An individual alpha level of 5% was applied for all two-sided tests. For one-sided tests an individual alpha level of 2.5% was used. For cytokine measurements, replicated observations were combined using the geometric mean and normalized by dividing by the negative control (medium) to obtain normalized concentrations (nC). Spearman's rank correlation coefficient was used to evaluate nSFC associations with normalized cytokine concentrations (nC) as well as antibody responses.

Results

Study population characteristics

Samples from 177 participants were analyzed in this study (see online supplement); 88 received 15-mcg of the clade 2 vaccine dosage while 89 received 90-mcg (Table 1). Groups from the parent study that were not represented in this substudy included persons in the clade 1 low dosage groups (≤ 15 mcg) and recipients of an adjuvanted Novartis vaccine (alum or MF59).

ELISPOT Results

Statistically significant differences in the number of SFCs for the medium negative controls were observed between age groups (both IFN- γ and GrB), study days (GrB only), and treatment groups (GrB only), so data were normalized and expressed as nSFC. We first evaluated whether the administration of inactivated H5N1 vaccine elicited CMI responses. When all vaccine and age groups were pooled, nSFC for IFN- γ showed a statistically

significant increase at each of the postvaccination timepoints (days 8, 14, 28 and 180) compared to baseline for H5N1 clade 1 ($P = 0.023$, paired t-tests for each timepoint) but only day 180 for clade 2 virus stimulation ($P=0.021$; Figure 1). In contrast, statistically significant increases were not observed for GrB nSFCs at any of the timepoints. However, mean post-baseline differences in IFN- γ nSFC were low (<120 nSFC per 2×10^5 PBMCs following clade 1 and <66 nSFC per 2×10^5 PBMCs following clade 2 stimulation).

At baseline, among primed individuals in the younger age group, prior receipt of unadjuvanted Novartis vaccine compared to an unadjuvanted vaccine produced by Sanofi Pasteur was associated with a statistically significantly higher response following stimulation with the clade 1 H5N1 (both IFN- γ and GrB) and the clade 2 (IFN- γ only) strains (Table 2). The difference between the groups was not explained by gender (rates were similar between groups) or the time since priming as the median time since first H5N1 vaccination was 2.7 and 2.8 years for Sanofi Pasteur and Novartis, respectively. When pooled across Clade 2 treatments, antigen-specific responses were consistently higher in the younger age group compared to the older age group. However this trend was mainly driven by elevated baseline responses for the young primed group that had received the Novartis vaccine. A subgroup analysis restricted to the young and old age groups that received the Sanofi Pasteur priming vaccine showed opposing trends, but no statistically significant differences between the age groups for either IFN- γ (Figure 2a) or GrB (Figure 2b) were identified. On the other hand, IFN- γ and GrB nSFC levels were higher at all timepoints following polyclonal stimulation with CD2/CD2R in the older age group in both analyses (Figures 2a and 2b). Statistically significant age differences for polyclonal stimulation with CD2/CD2R were observed for Days 14 and 28 for both IFN- γ and GrB analyses and on Days 0, 8 and 180 for GrB only.

The H5-primed younger age group consistently attained higher median IFN- γ levels for both H5N1 stimulants compared to the unprimed younger age group and the primed older age group, but no statistically significant differences were observed (Figure 3a). At baseline, the H5-primed younger group had a 34% (338/252 nSFC) higher median IFN- γ level compared to the younger unprimed group when stimulated with Clade1 virus and 25% (347/278 nSFC) higher levels when stimulated with Clade 2 virus. None of the GrB responses were statistically significantly different between the two groups (Figure 3b). The primed younger age group had statistically significantly higher IFN- γ nSFC at Day 8 compared to the unprimed group after receiving 15-mcg clade 2. No effect of the clade 2 vaccine dosage level was evident in primed subjects in either age group. However, in unprimed subjects, persons who received the 90-mcg clade 2 dose developed higher IFN- γ nSFC compared to those who received the 15mcg dose on all study days for both H5N1 stimulants, and the levels were statistically significantly higher for the clade 1 H5N1 virus stimulation on Day 8 post-vaccination (median 844 vs 254 nSFCs per 2×10^5 PBMCs, respectively, $P=0.024$).

Effects related to adjuvant use (none vs. alum) in the priming regimen were evaluated separately by age for the Sanofi Pasteur vaccine-primed group pooled across treatments. At baseline, only GrB nSFC measured after H5N1 clade 2 virus stimulation was statistically significantly higher in older persons (> 65 years) who had been primed with a high dose clade 1 vaccine and received the alum adjuvant (median 67 vs 14 nSFCs per 2×10^5 PBMCs

with and without adjuvant, respectively, $P=0.044$). No other statistically significant effects of adjuvant on baseline responses were noted.

For the same vaccine subgroup, gender effects were evaluated separately by age group. For older persons, females had statistically significantly higher nSFC compared to males (median 482 vs. 315 nSFCs per 2×10^5 PBMCs, respectively, $P=0.023$) at Day 14 post-vaccination for clade 1 H5N1 virus stimulation as well as at Day 28 post-vaccination following H5N1 clade 2 virus stimulation (412 vs. 280 nSFCs per 2×10^5 PBMCs, respectively, $P=0.039$).

Cytokine responses

Cytokine concentrations were measured in the supernatants from a subset of samples collected from 47 persons, and results were correlated with the ELISPOT results. No increases in cytokine concentration were observed for IL-7 or IL-8 following PBMC stimulation with either H5N1 virus antigen or after CD2/CD2R stimulation. In addition, there was no increase in concentrations of IL-4, IL-5, IL-12 or IL-13 following PBMC stimulation with either H5N1 virus (although increases were observed following CD2/CD2R stimulation).

The largest increases following stimulation with either H5N1 virus or CD2/CD2R relative to the media control was for IFN- γ . nSFC levels for IFN- γ were strongly correlated ($r_s > 0.72$) with IFN- γ concentrations measured from the corresponding supernatant for all timepoints and for both H5N1 antigens (Table 3, Supplemental Figure 2). Within the primed group, the younger age group had higher correlations compared to the older age group following Clade 1 virus stimulation (Supplementary Table 3). IFN- γ concentrations also correlated with GrB nSFCs levels, but less strongly ($r_s > 0.42$). Positive correlations between nSFC levels and concentrations of other cytokines were observed, but correlation coefficients were lower or were not statistically significant at all timepoints (Table 3). IL-6 concentrations in PBMC supernatants were negatively correlated with nSFC responses at some timepoints.

Serum antibody responses

There were no statistically significant correlations between IFN- γ or GrB nSFC levels at day 0 and pre-immunization serum HAI or Neut antibody levels for persons in either age group. There was also no statistically significant correlation between IFN- γ or GrB nSFC levels and serum antibody responses at day 28 among primed study participants. However, in the unprimed young age group, IFN- γ nSFC levels at day 28 were statistically significantly correlated with serum H5N1 clade 2 virus HAI ($r_s = 0.53$ and 0.53 for H5N1 clade 1 and clade 2 stimulants, respectively, $P=0.016$) and Neut ($r_s = 0.58$ and 0.67 for H5N1 clade 1 and clade 2 stimulants, respectively, $P=0.008$ and $P<0.001$). GrB nSFC levels did not statistically significantly correlate with serum antibody responses at any timepoint.

Discussion

The evaluation of candidate IIVs targeting avian influenza viruses has focused on serological responses following immunization. Because CMI responses have been observed in persons administered seasonal IIV3 and CMI contributes to protection against influenza

disease, we evaluated whether CMI responses could be measured in persons participating in a H5N1 IIV1 booster study. Study participants who received a clade 2 H5N1 IIV1 had modest increases in virus-specific IFN- γ nSFCs to both the clade 1 priming and clade 2 boosting antigens. The magnitude of the CMI response (up to 23% and 14% average increase over baseline for the clade 1 and clade 2 stimulants, respectively) was lower than the ~50% increase reported previously following administration of a 2009pdm IIV1 to adults [18]. The magnitude of CMI response was greater against the clade 1 virus than the clade 2 strain, raising the possibility of a priming effect. However, serological responses following the clade 2 boost were similar for both clade 1 and clade 2 strains [22].

We next sought to determine whether certain host or vaccine-specific factors affected the magnitude of the CMI response. Several investigators have reported lower CMI responses to influenza virus [32–35] and in response to influenza vaccination [36,37] in persons over 65 years of age compared to younger individuals, although Hsu et al [18] observed a greater CMI response, also measured by IFN- γ ELISPOT, in older adults before and after administration of 2009pdm H1N1 IIV1. We did not observe any statistically significant differences in the levels of IFN- γ nSFCs between these two age groups. On the other hand, we observed that the response to CD2/CD28 stimulation, which served as a mitogenic T-lymphocyte activation control [38], was statistically significantly greater in the older age group. Other investigators have reported similar responses among young and elder groups following mitogenic stimulation of PBMCs [34]. The robust response of elderly PBMC to CD2/CD28 but not to influenza antigens suggests that elderly PBMC are functional but unable to respond to specific influenza antigens. These data further suggest CD8 oligoclonal expansions in the elderly, resulting in an accumulation of dysfunctional non-specific memory cells which possibly fill the immunological space and could lead to a shrinking of the T cell repertoire for other novel antigens. Similarly, CD45RA+CD28+CD8+ T cells from elderly persons produce larger amounts of the proinflammatory cytokine IFN- γ after their stimulation with OKT3 and IL-2 than those cells of young persons [39].

IIV dosage level can affect the magnitude of the humoral response following vaccination [4,40,41]. Although persons who had received a prior H5N1 clade 1 vaccine and received the 90-mcg clade 2 booster dose were more likely to have a four-fold or greater rise and to achieve higher antibody levels than those who received the 15-mcg booster dose (parent study), no effect of the boosting vaccine dosage on the magnitude of IFN- γ nSFC response was observed among these primed subjects. In contrast, persons who received the higher clade 2 vaccine dosage as part of their initial H5N1 priming series did have a statistically significantly greater IFN- γ nSFC response compared to those receiving the lower dosage. There was also a positive correlation between serum antibody (HAI, neut) and IFN- γ nSFC levels on day 28 post-vaccination in this unprimed group. No such correlation was present among primed individuals, similar to reports from other investigators of antibody and CMI responses to other influenza vaccines [18,42,43].

Serological responses were poorer among study participants who had received seasonal influenza vaccine in the two influenza seasons prior to receipt of the booster vaccine. In contrast, no differences in IFN- γ nSFC responses were observed in persons who received seasonal influenza vaccine in the prior year. There was also no observed impact of priming

with an alum-adjuvanted vaccine. An unexpected finding was the difference in IFN- γ nSFC responses in primed adults based upon vaccine manufacturer of the clade 1 vaccine. The difference in response was not due to differences in the interval from priming to boosting since they were similar for vaccines from each manufacturer. One consideration is that there were differences in vaccines made by the two manufacturers in the content of viral proteins other than HA. Such differences in other components (e.g., neuraminidase) have been observed based upon immunological responses to individual season IIV3s made by different manufacturers [44]. The current study did not assess to which component(s) IFN- γ nSFCs were generated and cellular immune responses often target nonstructural viral proteins (e.g., matrix, nucleoprotein), so it is possible that the observed differences were due to non-HA viral proteins [45].

Administration of a booster or priming dose of clade 2 H5N1 vaccine was not associated with statistically significant changes in the levels of GrB nSFCs, in contrast to what was observed for IFN- γ nSFCs. The functionality of T cells detected by these two different ELISPOT assays differ, with GrB results more closely approximating results from cytotoxicity measured by ^{51}Cr -release assays [31]. Non-cytotoxic T cells can secrete IFN- γ , while GrB is an effector molecule involved in cytotoxicity. Thus, GrB SFC numbers did not increase after administration of a seasonal IIV3 while increases were observed following administration of a recombinant replication-deficient adenovirus type 5 vector containing an H5 HA [19,21]. The majority of IFN- γ -secreting cells induced by an IIV are expected to be CD4+ T cells [34]. One reason for the poor immunogenicity of H5 vaccines may be that the number of CD4+ T cells available to be recruited into the immune response following immunization with a novel avian antigen is less than that available for recruitment to immune response against 2009pdm H1N1 or seasonal influenza viruses [46].

Limitations of the current study include the analysis of priming effects from a heterogeneous group of persons who participated in different clade 1 H5N1 vaccine trials and analysis of only a subset of these participants in this substudy. Differences between participants in the different trials could influence the observations made in this study. As noted earlier, we did not define against which influenza antigens IFN- γ nSFCs were being generated or which lymphocytes were involved in the responses.

Conclusion

Administration of a clade 2 H5N1 IIV1 led to measurable virus-specific CMI responses, but statistically significant increases were restricted to IFN- γ nSFCs. Stimulation with H5N1 clade 1 antigen resulted in a stronger baseline increase in CMI responses compared to H5N1 clade 2 antigen stimulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- CMI responses occurred following administration of an inactivated A/H5N1 vaccine.
- High dose vaccine increased the magnitude of CMI response only in the unprimed subjects.
- IFN- γ nSFC levels correlated with IFN- γ concentrations in PBMC supernatants.

Figure 1.

Pooled mean (95% CI) of IFN- γ and GrB nSFCs per 2×10^5 PBMCs following administration of clade 2 inactivated influenza A/H5N1 vaccine. Circles represent IFN- γ levels and triangles represent GrB levels. Days are before (Day 0) or after vaccine administration, and the number of samples tested is shown below the day. The stimulating antigen is shown above each frame. *, $P < 0.025$; **, $P < 0.01$, paired t tests for IFN- γ values.

Figure 2.

nSFC per 2×10^5 PBMCs for IFN- γ (a) and GrB (b) in persons who were primed with the Sanofi Pasteur clade 1 vaccine. Individual values are shown as circles for persons 19–64 years of age and as triangles for persons over 65 years of age. Bolded values and whiskers represent median and 95% CIs. Days are before (Day 0) or after vaccine administration, and the number of samples tested is shown below the day. The stimulating antigen is shown above each frame. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, two sided Wilcoxon Rank Sum test between age groups

Figure 3.

nSFC per 2×10^5 PBMCs for IFN- γ (a) and GrB (b) in persons 19–64 years of age who were unprimed (placebo) or primed with a clade 1 H5N1 vaccine. Individual values are shown as circles for primed persons and as pluses for unprimed persons. Bolded values and whiskers represent median and 95% CIs. Days are before (Day 0) or after vaccine administration, and the number of samples tested is shown below the day. The stimulating antigen is shown above each frame. There were no statistically significant differences between groups on any of the study Days.

Table 1

Number of study participants by priming regimen (clade 1 vaccine), age, and clade 2 dosage

Age Group	Clade 1 Vaccine ^a	Adjuvant	Clade 2 Dosage (N)	
			15 mcg	90 mcg
19–64	Novartis	None	14	10
	Sanofi	None	15	22
	Sanofi	Alum	7	7
	Placebo	None	10	10
65	Novartis	None	1	0
	Sanofi	None	28	24
	Sanofi	Alum	12	14
	Placebo	None	1	2

^a All received 30, 45 or 90 mcg dosage of clade 1 vaccine; additional vaccine and demographic information provided in the online supplement

Table 2
Effect of unadjuvanted priming vaccine on baseline (day 0) nSFC per 2×10^5 PBMCs in persons 19–64 years of age

Stimulant	Analyte	Novartis		Sanofi Pasteur		P value ^b
		N	Median ^a	N	Median ^a	
Clade 1	IFN- γ	24	770	33	261	0.013
	GrB	24	127	33	39	0.015
Clade 2	IFN- γ	24	610	37	224	0.002
	GrB	24	116	37	58	0.069
CD2/CD2R	IFN- γ	24	1505	37	900	0.901
	GrB	24	770	37	283	0.046

^a per 2×10^5 PBMCs

^b Wilcoxon Rank Sum Test

Table 3

Significant correlations between nSFC per 2×10^5 PBMCs and normalized cytokine levels measured in PBMC supernatants following stimulation with H5N1 viruses.^a

Cytokine	Virus	IFN- γ ELISpot					GrB ELISpot				
		Day 0	Day 8	Day 14	Day 28	Day 180 Day 236	Day 0	Day 8	Day 14	Day 28	Day 180 Day 236
		r_s	r_s	r_s	r_s	r_s	r_s	r_s	r_s	r_s	r_s
IFN- γ	Clade 1	0.79	0.74	0.84	0.79	0.77	0.42	0.46	0.66	0.58	0.56
	Clade 2	0.72	0.72	0.78	0.72	0.79	0.46	0.59	0.67	0.57	0.70
IFN- γ /IL-10	Clade 1	0.74	0.55	0.64	0.74	0.68	0.36		0.43	0.51	0.32
	Clade 2	0.51	0.37	0.53	0.57	0.67				0.44	
IL-2	Clade 1	0.49	0.50	0.63	0.66	0.41	0.61	0.46	0.73	0.60	0.71
	Clade 2	0.57	0.61	0.57	0.64	0.46	0.50	0.43	0.67	0.59	0.74
IL-6	Clade 1	-0.32	-0.29	-0.31		-0.33		-0.29			
	Clade 2	-0.40		-0.31		-0.38			-0.30		
IL-12	Clade 1	0.44	0.33		0.44			0.51		0.37	0.30
	Clade 2	0.39	0.52	0.46	0.43			0.47	0.52	0.41	0.39
IL-13	Clade 1	0.40	0.35		0.41			0.50	0.52	0.37	0.67
	Clade 2		0.40	0.47	0.35			0.52	0.57	0.47	0.55
IL-1 β	Clade 1						0.42	0.41	0.35	0.36	0.45
	Clade 2						0.34	0.34			0.36
IL-4	Clade 1	0.37					0.54	0.50	0.46		0.40
	Clade 2	0.33	0.32		0.38		0.35		0.41	0.33	0.42
IL-5	Clade 1	0.33			0.34		0.34	0.51		0.42	0.36
	Clade 2	0.42	0.45					0.52	0.36		0.36
IL-10	Clade 1			0.38					0.40		0.32
	Clade 2	0.40	0.36				0.39	0.41	0.44		0.54
IL-7	Clade 1	0.30					0.33		0.31	0.36	
	Clade 2	-0.31				-0.36					
TNF- α	Clade 1	-0.39		-0.32		-0.39					
	Clade 2										

		IFN- γ ELISpot					GrB ELISpot				
		Day 0	Day 8	Day 14	Day 28	Day 180 Day 236	Day 0	Day 8	Day 14	Day 28	Day 180 Day 236
Cytokine	Virus	r_s	r_s	r_s	r_s	r_s	r_s	r_s	r_s	r_s	r_s
	IL-8						0.33				
	GM-CSF										0.36
											0.31

^aColor scheme: Dark orange, r_s 0.75; medium orange, $0.75 > r_s > 0.5$; light orange, 0.50 $r_s > 0$; light blue, -0.50 $r_s < 0$. Only significant ($P < 0.05$) correlations are shown