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Innate-like $\gamma\delta$ T cell responses to mycobacterium *Bacille Calmette-Guerin* using the public V γ 2 repertoire in *Macaca fascicularis*

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Abstract

The V γ 2V δ 2 T cell subset responds to *Bacille Calmette-Guerin* (BCG) immunization in macaques and may be a component of protective immunity against tuberculosis. We characterized the effects of BCG on the V γ 2V δ 2 T cell receptor repertoire by comparing the starting population of V γ 2 chains in cynomolgus macaques with the repertoire found after priming or booster immunization with BCG. The starting repertoire was dominated by public V γ 2 chain sequences that were found repeatedly among unrelated animals. Primary exposure to BCG triggered expansion of cells expressing public V γ 2 chains and booster immunization was often associated with contraction of these same subsets. Thus, BCG-reactive V γ 2 chains were present at high frequency in the repertoire of mycobacteria-naïve macaques and they comprised the major response to primary or booster immunization. Normal selection processes that created the naïve V γ 2 repertoire in macaques, also encoded the capacity for rapid responses to mycobacteria. The unusual composition of a normal V γ 2 repertoire helps to explain the powerful $\gamma\delta$ T cell responses to BCG immunization.

Keywords

V γ 2V δ 2; repertoire; BCG; *M. fascicularis*

Introduction

In human and non-human primates, the major $\gamma\delta$ T cell subset in blood has the V γ 2V δ 2 T cell receptor ^{1–4}. Cells expressing this TCR receptor respond to stimulation with mycobacteria *in vitro* ^{5–9}; responses include proliferation, production of cytokines such as IFN- γ ¹⁰ or TNF- α ¹¹, and cytotoxicity against *M. tuberculosis* (Mtb) and Mtb-infected cells ¹². V γ 2V δ 2 T cells also react to purified mycobacterial components including the non-peptidic phosphoantigens called TubAg1–4 ^{8, 13}. These antigens are chemically similar to isopentenyl pyrophosphate (IPP), an intermediate in cholesterol biosynthesis that is used frequently as a model phosphoantigen for $\gamma\delta$ T cell studies ⁹. The $\gamma\delta$ T cell responses to phosphoantigens are MHC unrestricted ^{14, 15}, require expression of the V δ 2 chain ¹⁶ and depend on specific residues in the V γ 2 chain CDR3 region ^{16–18}.

Phosphoantigen responses are positively selected during normal ontogeny. Thymocytes or cord blood lymphocytes contain low frequencies of V γ 2V δ 2 T cells,^{2, 4} but within a few years after birth, the repertoire evolves because of strong selection for the V γ 2-J γ 1.2 rearrangement⁴ that is mostly paired with the V δ 2 chain^{4, 19}. It is believed that stimulation by self- or ubiquitous non-self antigens amplifies and maintains the V γ 2-J γ 1.2V δ 2+ population, thus creating the mature repertoire^{2, 4, 20–22}. In healthy human adults, the V γ 2-J γ 1.2 rearrangement accounts for more than 70% of V γ 2 chains in peripheral blood^{20, 23}. Similar results were obtained for *Macaca mulatta*^{24, 25} and *Macaca fascicularis*²⁶ monkeys although we know that monkeys have some cells expressing V γ 2 chains paired with V δ 1²⁷ (a combination that is infrequent for adult human beings) and that subset will influence the repertoire. The V γ 2-J γ 1.2+ subset responds to a variety of pathogens^{28–33} and tumor cells^{34–40}.

Bacille Calmette-Guerin (BCG) inoculation in macaques stimulates the V γ 2V δ 2 T cell subset and protects against lethal challenge with *M. tuberculosis*, arguing that antigen specific V γ 2 T cells are an important part of the protective immunity against tuberculosis⁴¹. However, the starting V γ 2 repertoire is selected during normal ontogeny, presumably in the absence of mycobacterial infection, and lymphocytes from unprimed individuals respond strongly to mycobacteria *in vitro*^{6, 42}. We hypothesize that the response to BCG is targeted toward a host molecule that is induced or modified during mycobacterium infection, and that the capacity for BCG recognition is already encoded at high levels in the starting V γ 2V δ 2 T cell receptor repertoire. In this study, we test whether BCG-naïve macaques already have the capacity for memory $\gamma\delta$ T cell responses to BCG by examining the starting V γ 2 chain repertoire and its changes after primary or booster inoculation with BCG. The nature and specificity of V γ 2V δ 2 T cell responses is important for characterizing the protective immunity against *M. tuberculosis*, and for the design and testing of new tuberculosis vaccines.

Materials and Methods

M. fascicularis immunization protocol

11 juvenile (2 years old) healthy *M. fascicularis* were housed in the Animal Core Facility at the Institute of Human Virology. Six animals were inoculated intradermally with 5×10^4 CFU/animal of BCG (Aventis-Pasteur strain) freshly reconstituted in PBS (GIBCO Grand Island, NY), five control animals received an equivalent volume of PBS alone. Five weeks later, animals that received BCG were boosted with 5×10^5 CFU/animal. For each macaque, heparinized blood specimens were collected at several time points before and after the BCG inoculations: 4 and 2 weeks before the immunization, the day of the primary immunization, and 1, 2, 3, 5, 7, 9 weeks after primary inoculation. Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Maryland Biotechnology Institute.

Lymphocyte sample collection

Peripheral blood mononuclear cells (PBMC) were isolated from macaque blood specimens by centrifugation over Ficoll-Hypaque (Pharmacia, Sweden) density gradient according to the manufacturer's instructions. PBMC were frozen at 5×10^6 – 10^7 /ml in fetal bovine serum (FBS) (GIBCO, Grand Island, NY) with 10% dimethyl-sulphoxyde (DMSO) (Sigma, St Louis, MO) and stored at -130°C .

Cell culture assay for V γ 2V δ 2 T cell responses

After thawing, cells were washed twice with RPMI (GIBCO, Grand Island, NY)-15% FBS (GIBCO, Grand Island, NY) and used for phenotypic characterization and cell cultures. PBMC were cultured at 10^6 cells/ml in RPMI supplemented with 10% FBS, 2mM L-Glutamine (GIBCO, Grand Island, NY), penicillin (100 U/ml) (GIBCO, Grand Island, NY), streptomycin

(100 µg/ml) (GIBCO, Grand Island, NY) and 100U/ml of human recombinant interleukin-2 (rhIL-2) (Tecin, Biological Resources Branch, NIH, Bethesda, MD). The phosphoantigen isopentenyl pyrophosphate (IPP) (Sigma, St Louis, MO) was added to a final concentration of 15µM, and in some cultures live BCG (Aventis-Pasteur) was used at 0.3 multiplicity of infection (MOI). Cells were incubated for 13 days at 37°C with 5% CO₂. Fresh medium containing IL-2 was added every 3 days.

Thirteen days after stimulation *in vitro*, cells were harvested and viable counts were determined by the trypan blue dye exclusion method. Expanded Vγ2 or freshly thawed PBMC were resuspended in PBS (GIBCO, Grand Island, NY) with 10% FBS and stained at 4°C with directly conjugated monoclonal antibodies. After 20 minutes, cells were washed twice with PBS-10%FBS and resuspended in PBS containing 2% paraformaldehyde. At least 2×10⁴ lymphocytes (gated on the basis of forward and side scatter profiles) were acquired for each sample on a FACSCalibur (BD Biosciences, San Diego, CA). Flow cytometry data were analyzed with FlowJo software (Tree Star, San Carlos, CA).

The following monoclonal antibodies cross-react with *M. fascicularis* surface molecules and were used in this study: Vγ9 (clone IMM360, Beckman Coulter, Miami, FL) (Vγ9 is an alternate nomenclature for the Vγ2 chain); CD95-PE (clone DX2, Pharmingen, BD); CD45-RA (clone F8-11-13, Cibus Biotechnology); CD8-APC (clone SK1, BD); CD27-PE (clone CLB-27/1, Caltag Laboratories, Burlingame, CA).

ELISPOT

Thawed *M. fascicularis* PBMC were used to determine the frequency of circulating cells able to produce IFN-γ in response to IPP stimulation (Cairo et al., 2005). High protein binding Immobilon-P membrane 96-well plates (Millipore, Bedford, MA) were coated overnight at 4°C with anti human IFN-γ monoclonal antibody (clone GZ4, Bender MedSystem, San Bruno, CA) diluted in PBS at 5µg/ml. PBMC were plated (10⁵ cells/well in 200µl volume) and stimulated for 24 hours with either medium alone or IPP (15µM) or PHA (10µg/ml) (Remel, Lenexa, KS); no IL-2 was added. The following day, plates were washed and the biotinylated monoclonal antibody anti-IFN-γ (clone 7-B6-1, Mabtech, DiaPharma, West Chester, OH) was added at 5µg/ml in a PBS-0.05% Tween-20–5% FBS solution. After 2 hours incubation, plates were washed and Avidin conjugated HRP (BD Biosciences, San Diego, CA) was added, diluted 1:100 in PBS-10% FBS. After 1 hour incubation, plates were washed again and 100µl of substrate solution (BD Biosciences, San Diego, CA) were added. Plates were then incubated 1 hour, rinsed with water and let air-dry over night. Plate images were resolved and recorded through a Cellular Technology Ltd ELISPOT reader; spots were counted using the instrument software (Immunospot 3.2).

RNA extraction, RT- PCR, PCR

Total RNA was extracted from 1–5×10⁶ cells using the RNeasy mini Kit (Qiagen, Valencia, CA), as described by the manufacturer. One µg of total RNA was then converted into cDNA using the reverse transcription system kit (Promega, Madison WI), as described previously²⁶. Polymerase chain reactions were performed as described²⁶ using the following primers: oligo Vγ2 (5'ATC AAC GCT GGC AGT CC 3'); oligo Cγ1 (5'GTT GCT CTT CTT TTC TTG CC 3'). PCR products were separated on 1.5% agarose/Tris-acetate-EDTA buffer (TAE) gels containing 0.5µg/ml ethidium bromide (Sigma, St Louis, MO).

Cloning and sequencing of Vγ2 chains

PCR products were purified by gel extraction, using QIAquick gel extraction kits (Qiagen, Valencia, CA) according to the manufacturer's instructions. Purified products were denatured (1 minute at 94°C), then incubated for 30 minutes at 72°C with 2mM MgCl₂, 0.2mM dATPs,

and 2.5 units of Amplitaq gold (Applied Biosystems, Foster City, CA), then ligated into a pCR2.1 vector (TA cloning kit, Invitrogen, Carlsbad, CA). Ligated vector was transfected into TOP 10F' competent cells (TA cloning kit, Invitrogen, Carlsbad, CA), and bacterial colonies representing a library of V γ 2 chain sequences were grown overnight on agar plates containing 50 μ g/ml ampicillin (Sigma, St Louis, MO), 500 μ M IPTG (Promega, Madison WI) and 80 μ g/ml X-Gal (Promega, Madison WI). Colonies containing recombinant plasmids were cultured overnight in LB media and plasmid DNA were purified using the REAL Minipreps DNA purification kit (Qiagen, Valencia, CA). Sequencing reactions were done with a Big Dye v3.1 fluorescent sequencing kit (Applied Biosystems, Foster City, CA), with both M13F and M13R oligonucleotide primers for each sample. Sequences were loaded on an automated sequencer ABI3700 and analyzed using Sequencher (Gene Codes Corporation, Ann Arbor, MI) and MacClade (Sinauer Associates, Sunderland, MA) softwares.

Statistical analysis

Differences among groups were analyzed by one-way ANOVA test or Student *t* test. *p* values ≤ 0.05 were considered significant.

Results

Characterizing the peripheral blood V γ 2 T cell repertoire in healthy *Macaca fascicularis*. We analyzed the V γ 2 repertoire in six animals by sequencing a representative sample of V γ 2 chain cDNA clones. Macaques in this study were heterogeneous in terms of baseline J γ 1.2 segment usage (frequency of V γ 2 chains containing the J γ 1.2 segment) and V γ 2 population complexity (number of distinct V γ 2 chain sequences divided by the total number of sequences within that sample set) (Table 1). Four of the macaques had a high proportion of J γ 1.2 segments in the V γ 2 population (ranging from 50% to 88.3%). Animals 4024 and 4028 had lower J γ 1.2 segment frequencies (33% and 42%, respectively); 4028 also had high population complexity (0.8). Macaque 4024 had only a single repeated J γ 1.2+ nucleotide (referring to the nucleotide sequence of an individual V γ 2 chain), even though repeated J γ 1.2+ nucleotides were common among all other animals (not shown).

We next tested whether the V γ 2 repertoire is stable over intervals similar to what we used in the vaccination study. For macaques 4028, 4039 and 4736 we sequenced cDNA libraries from two pre-immunization time points two to four weeks apart. Then we compared the frequency for each sequence in the two sample sets (supplemental data, S1). Clones present at $\geq 6\%$ of the total V γ 2 population were found in both control specimens roughly at the same abundance, suggesting that our sample sizes were sufficient to detect these events accurately. For clones that never reached 6% abundance, we were unsure of our ability to discriminate real changes from fluctuation due to sampling error. This analysis helped establish a cut-off value of 6% abundance for sequences that could be analyzed reliably. Individual V γ 2 nucleotypes that did not exceed 6% in any specimen were not evaluated further.

Identifying *M. fascicularis* that respond to BCG immunization

We assumed that outbred macaques, like human beings⁴³, would not respond uniformly to subcutaneous BCG immunization and we developed criteria to distinguish responder and non-responder animals. We identified 4 responder macaques among 6 that were inoculated, on the basis of changing V γ 2 T cell frequencies in blood, Interferon- γ (IFN- γ) secretion after *in vitro* restimulation with IPP and proliferative responses to IPP *in vitro* (Fig. 1 and supplemental data, S2). During 3 to 7 weeks after the primary inoculation, responder animals (4028, 4039, 4087 and 4736) had significantly higher levels of V γ 2+ T cells in blood ($p < 0.05$) compared to baseline values (Fig. 1A) or to non-responders (data not shown). There were no substantial changes in the profile of memory markers CD45RA, CD95, or CD27 expression on circulating

V γ 2+ T cells (data not shown), despite the fact that IFN- γ secretion and proliferative responses to IPP were significantly higher for responder animals (Fig. 1B and 1C). Based on these characterizations, we selected 4 animals with the best V γ 2 T cell response to BCG immunization and used these macaques for our studies of repertoire and its changes following inoculation. The response rate among macaques in our study (4 of 6 or 67%) is nearly identical to the response rate among human beings vaccinated with BCG ⁴³.

Public V γ 2 clonotypes among unrelated macaques

A comparison of V γ 2 sequences in the starting repertoire from each animal showed that several clonotypes (V γ 2 chain amino-acid sequences) were present in more than one animal (Table 2). Sequences found repeatedly among different animals are termed “public” in contrast to “private” sequences that are unique to individual animals. These public clonotypes accounted for a large proportion of all V γ 2-J γ 1.2+ sequences in the starting macaque repertoire being 48.6% of the J γ 1.2 sequences in macaque 4028, 62.3% in macaque 4039, 60.2% in macaque 4087, and 79.5% in macaque 4736 (the responder group). Non-responder macaques 4024 and 7646 tended to have fewer public clonotypes in their starting V γ 2-J γ 1.2 repertoire with 24.1% for 4024 and 55.9% for 7647, but they still represented a substantial portion of V γ 2 chains. By combining all V γ 2-J γ 1.2 sequences generated for every animal at all time points (approximately 1600 individual sequences), we defined 25 public clonotypes, some of which were remarkably abundant (Table 2).

The same public clonotypes could be encoded by several discrete nucleotides. This phenomenon was particularly notable for the most common clonotypes PB1 and PB2 that were encoded by 2 to 6 discrete nucleotide sequences (not shown). Additionally, one of the nucleotides (...GGGAGGTG CAACAGTTT...) encoding the PB1 clonotype was present in every animal, and it is similar to a “canonical” sequence found commonly in human beings ^{44, 45}. In many cases the same V γ 2 clonotypes were encoded by distinct nucleotides in different animals. Mechanisms that shaped the V γ 2 repertoire were selecting for T cell receptor function (related to the amino acid sequence) and not for nucleotide sequences.

Impact of primary and secondary BCG inoculation on the V γ 2 repertoire

A representative number of V γ 2 sequences were determined for each responder macaque from PBMC collected before the primary inoculation, after the primary inoculation, and following the booster inoculation. Three responder animals (4736, 4087, 4039) showed only small variations in both J γ 1.2 frequency and population complexity after the primary inoculation (Table 3). After the booster all three showed dramatic drops in J γ 1.2 clone frequency (to around half of the starting levels) that were accompanied by increases in the population complexity (Table 3).

The remaining responder macaque 4028 had an increased J γ 1.2 frequency and a decreased population complexity after both primary and booster inoculations (Table 3). In this macaque, the J γ 1.2 population expanded after both inoculations mainly due to a single clone, whose frequency increased from 4.8% (baseline) to 15.9% (after inoculation) and to 28% (after booster) of the V γ 2 population.

Pre-existing public clonotypes dominate the V γ 2+ T cell response to BCG Most of the clonotypes that were expanded after BCG inoculation were in the public group; private clonotypes represented only a minor part of the response. Consequently, the pool of J γ 1.2 sequences was even more biased to the pre-existing public sequences after BCG inoculation than in naïve animals (Table 4). Overall, these changes show a focusing of the J γ 1.2+ subset after primary BCG inoculation due to preferential expansion of T cell clones expressing public

V γ 2 sequences. As these public sequences comprise a substantial fraction of the starting repertoire, their appearance is not uniquely due to BCG immunization.

BGC responses *in vivo* and *in vitro* are not equivalent

It is common to use *in vitro* BCG stimulation for studying $\gamma\delta$ T cell responses. We compared the impact on the V γ 2 repertoire of BCG stimulation *in vitro* with what we observed in macaques. Macaque PBMC (collected before the primary inoculation) were stimulated for 13 days with live BCG (MOI 0.3). We measured V γ 2 expansion by flow cytometry (data not shown) and sequenced approximately 90 V γ 2 cDNA clones from each sample. The V γ 2-J γ 1.2 subset was expanded in every case (4736 had the lowest J γ 1.2 frequency, 71.6%, 4087 the highest, 97.8%) with a consequent decrease in the repertoire complexity (from 0.49 for 4039 to 0.43 for 4736) (Table 3).

Responses *in vivo* and *in vitro* were clearly different in terms of the variety of responding clonotypes, their frequencies, and the changes after stimulation (Table 5). In many cases the same clone was present after both *in vivo* and *in vitro* exposure, but clones that expanded the most were not always the same in the two studies. For example, a V γ 2 clone from macaque 4028 that was very frequent in the V γ 2-J γ 1.2 pool after either primary (25%) or booster inoculation (37.9%) was only 1% abundant after *in vitro* stimulation. This pattern was repeated for a number of other clones (Table 5). We also observed low frequency nucleotypes that did not react to BCG *in vivo* (e.g. 1.1% and 1.2% after primary and booster inoculation, respectively) but were expanded after stimulation *in vitro* (to 11.7%). Overall, the *in vivo* responses tended to be oligoclonal, implying a greater selection, while the *in vitro* responses tended to be polyclonal (Table 5). These data argue that *in vitro* responses to BCG are a poor model for understanding TCR recognition and the *in vivo* response to mycobacteria.

Discussion

Mycobacterium infection in human or nonhuman primates elicits a powerful response within the V γ 2V δ 2 T cell subset^{41, 46–48}. The magnitude and dynamics of V γ 2V δ 2 T cell responses after primary or booster BCG immunization suggested this was an antigen- specific, memory T cell response^{41, 43}. However, we showed that the response relies primarily on public V γ 2 chain sequences that exist at high frequency within the starting repertoire of BCG-naïve macaques. These same public V γ 2 chains, positively selected during normal ontogeny, encode the response to other V γ 2V δ 2 T cell antigens including alkylphosphates or tumor cells⁴⁹, and are not specific for mycobacteria. The response to BCG is either the result of cross-recognition, where the mycobacterium mimics an antigen encountered previously, or the V γ 2V δ 2 T cell receptor recognizing a host antigen that is modulated or modified during BCG infection. Interestingly, one of the common V γ 2 nucleotypes is the macaque counterpart of a “canonical” sequence that was reported previously in human beings^{44, 45}.

Our observations on the changes in V γ 2 repertoire showed that control mechanisms exist to avoid monoclonal or polyclonal responses *in vivo*. Public V γ 2 clonotypes were expanded after BCG priming, and it was reasonable to guess that booster immunization would select again on this population, expand only the most reactive clonotypes and reduce the repertoire complexity. However, booster immunization 5 weeks after the initial dose actually contracted several reactive clonotypes and tended to return the repertoire to its starting condition in 3 of 4 animals studied. This process likely reflects activation-induced cell death of reactive V γ 2V δ 2 T cell clones, because the booster was too soon after the initial immunization. In fact, to test whether the booster immunization would drive clonal depletion or responsive clones, we allowed only 5 weeks between priming and boosting compared to 3 to 5 months in another study⁴¹. For $\gamma\delta$ T cells, sensitivity to activation-induced cell death may be an important adaptation that prevents the development of autoimmunity. Since this cell subset reacts to common pathogens

and host molecules including IPP, a mechanism for limiting the expansion of individual clonotypes will guard against amplifying the response against self even during the response to infection. These data highlight practical aspects of BCG immunization showing more than 5 weeks is needed between primary and booster immunization to preserve strong V γ 2V δ 2 T cell responses to attenuated mycobacteria.

The role for a public and pre-existing V γ 2 repertoire in the response to mycobacteria raises important questions about how these cells function in human beings at risk for tuberculosis. Tuberculosis rates are linked with the epidemic spread of HIV^{50–52}, partly because dual infection worsens both diseases^{52–54}. Part of the mechanism for enhanced disease during dual infection may relate to the HIV-mediated depletion⁵⁵ of the same V γ 2-J γ 1.2V δ 2 T cells needed for the protective response to mycobacteria. Further, HIV is not the only infectious disease that alters $\gamma\delta$ T cell levels. Malaria is often prevalent in the same countries worst affected by HIV and tuberculosis. Malaria infection in endemic regions leads to expansion of V γ 2V δ 2 T cells³² while non-endemic severe malaria can deplete the V γ 2-J γ 1.2+ subset⁵⁶. These considerations are important to the success of tuberculosis immunization programs. For example, there are few data available about $\gamma\delta$ T cells in babies born to HIV+ mothers, even though many of these infants are likely to receive BCG immunization against pediatric tuberculosis. As we consider the interactions of diseases including tuberculosis, malaria and HIV, the study of $\gamma\delta$ T cell responses is likely to be of major importance.

V γ 2-J γ 1.2V δ 2 T cells are appropriately classed within acquired immunity as they represent the products of positive selection on a cell population with rearranging T cell receptor genes. Selection and peripheral amplification of public V γ 2-J γ 1.2 clonotypes during early life, creates a subset of cells that comprise between 1 in 200 and 1 in 40 of circulating T lymphocytes in adults. Their sheer abundance and memory phenotype (due to previous selection and positive amplification) ensure a rapid, innate-like response to pathogens like mycobacteria, and promote the development of protective immunity against tuberculosis.

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Figure 1. Macaques have increased V γ 2V δ 2 T cell levels and functions after BCG immunization
 A) Frequencies of V γ 2 T cells (stained with anti-V γ 2 clone IMM360) among lymphocytes during the follow-up period are shown for each BCG inoculated macaque. B) Thawed PBMC for each time point were plated in triplicate and stimulated for 24 hours with IPP (15 μ M) or control medium in ELISPOT assays for IFN- γ secretion. For each sample, background subtracted values (average number of spots for IPP stimulated PBMC)-(average number of spots for medium stimulated PBMC) were calculated. Panel B shows the background subtracted number of IFN- γ producing $\gamma\delta$ T cells $\pm$ standard deviation at different time points, for each BCG-inoculated macaque. C) PBMC were stimulated with IPP (15 μ M) in presence of IL-2 (100U/ml). Proliferation was measured by the increase in V γ 2 T cell frequency after 14 days culture (staining with anti-V γ 2 clone IMM360). The inserted legends relate symbols to individual animal numbers. The symbol * indicates $p < 0.05$.

Table 1

Pre-inoculation J segment usage and Vγ2 population complexity for six BCG inoculated macaques.

	4024 NR ^a	4028 R	4039 R	4087 R	4736 R	7647 NR
# Jγ1.2 ^b	29 (33%)	35 (42%)	69 (75%)	83 (88.3%)	44 (50%)	59 (67%)
# Jγ1.3/2.3	49 (56.3%)	43 (52%)	23 (25%)	10 (10.6%)	40 (45.5%)	22 (25%)
# Jγ2.1	3 (3.4%)	4 (5%)	0	0	3 (3.4%)	5 (5.7%)
# Jγ1.1	6 (6.8%)	1 (1%)	0	1 (1.1%)	1 (1.1%)	2 (2.3%)
# total	87	83	92	94	88	88
Complexity ^c	0.68	0.8	0.63	0.56	0.67	0.65

^aResponder macaques are identified by R while non-responders are identified by NR.

^bValues indicate the number and proportion of sequences within each sample set that express each known J segment. The sequences of Jγ1.3 and Jγ2.3 segments are indistinguishable at this level and were combined. The proportion is calculated on the basis of number of sequences in each set, shown in the line marked total.

^cComplexity is the proportion of unique sequences in a sample set. It is defined as the number of unique sequences divided by the total number of sequence entries in that set.

Table 2

Public Jy1.2 clonotypes present among unrelated *M. fascicularis*.

CDR3 name	Jy1.2 CDR3 sequence	# Occurrences in each animal									
		4028	4039	4087	4736	4024 ^a	7647 ^a				
PB1	CALWEV---QQFGRKVK	23	34	37	10	2	17				
PB2	CALWEV-R---QFGRKVK	12	5	8	39	2	2				
PB3	CALWE---AK---QFGRKVK	44			1						
PB4	CALWE---G---QFGRKVK		5	3	1		2				
PB5	CALWEV-H---QFGRKVK	3	4	5	24						
PB6	CALWE---A---QFGRKVK		3	5	2		6				
PB7	CALWE---P---QFGRKVK	3	2	18	2						
PB8	CALWEV-L---QFGRKVK	4	4	1	1	1	1				
PB9	CALWEV-E---QFGRKVK		1	16							
PB10	CALWEV-E---QFGRKVK	2	4	3							
PB11	CALWEV-----QFGRKVK	1	3	3		1					
PB12	CALWE---E---QFGRKVK	1	1	1	1		1				
PB13	CALWE---L---QFGRKVK	1		1	7						
PB14	CALWEV-F---QFGRKVK	2		1							
PB15	CALW---DL---QFGRKVK	2	5	1	1						
PB16	CALW---DP---QFGRKVK	1		1	1						
PB17	CALWE---T---QFGRKVK	2	2	5		1					
PB18	CALWEV-P---QFGRKVK	1		3			1				
PB19	CALWE---N---QFGRKVK	1		2			1				
PB20	CALWEV-R---FGRKVK		7	1							
PB21	CALWEV-N---QFGRKVK		1	3	3						
PB22	CALWEV-HE---FGRKVK		4		1						
PB23	CALWE---AG---QFGRKVK	1	24								
PB24	CALWE---D---QFGRKVK		1	2			2				
PB25	CALWEV-A---QFGRKVK			2	1						
Total # public sequences (Jy1.2+)		104	110	122	95	7	33				
Total # sequences (Jy1.2+)		161	164	176	114	29	59				

# Occurrences in each animal						
CDR3 name	J γ 1.2 CDR3 sequence	4028	4039	4087	4736	4024 ^a 7647 ^a
Fraction of public sequences (J γ 1.2+)						
		64.6%	67.1%	69.3%	83.3%	24.1% 55.9%

For every macaque, all J γ 1.2 sequences found in the pre-inoculation, after inoculation and after booster specimen were pooled and the fraction of sequences encoding for public clonotype was calculated as total number of public sequences (J γ 1.2+):total number of sequences (J γ 1.2+).

^aPre-inoculation time point only

Table 3Influence of BCG inoculation on J segment usage and V γ 2 population complexity

		Pre-inoculation	After inoculation	After booster	BCG <i>in vitro</i>
4039	# J γ 1.2	69 (75%)	64 (72%)	31 (34.1%)	91 (97%)
	# J γ 1.3	23 (25%)	25 (28%)	59 (64.8%)	3 (3%)
	# J γ 2.1	0	0	1 (1.1%)	0
	# J γ 1.1	0	0	0	0
	# total	92	89	91	94
	Complexity	0.63	0.58	0.69	0.49
4087	# J γ 1.2	83 (88.3%)	74 (84.1%)	31 (38.8%)	92 (97.8%)
	# J γ 1.3	10 (10.6%)	11 (12.5%)	47 (58.8%)	1 (1.1%)
	# J γ 2.1	0	1 (1.1%)	1 (1.2%)	0
	# J γ 1.1	1 (1.1%)	2 (2.3)	1 (1.2%)	1 (1.1%)
	# total	94	88	80	94
	Complexity	0.56	0.67	0.79	0.47
4736	# J γ 1.2	44 (50%)	45 (54.2%)	25 (29.1%)	68 (71.6%)
	# J γ 1.3	40 (45.5%)	36 (43.4%)	57 (62.3%)	26 (27.4%)
	# J γ 2.1	3 (3.4%)	2 (2.4%)	4 (4.6%)	1 (1%)
	# J γ 1.1	1 (1.1%)	0	0	0
	# total	88	83	86	95
	Complexity	0.67	0.52	0.73	0.43
4028	# J γ 1.2	35 (42%)	60 (64%)	66 (74.2%)	83 (89.2%)
	# J γ 1.3	43 (52%)	29 (31%)	19 (21.3%)	10 (10.2%)
	# J γ 2.1	4 (5%)	3 (3%)	3 (3.4%)	0
	# J γ 1.1	1 (1%)	2 (2%)	1 (1.1%)	0
	# total	83	94	89	93
	Complexity	0.8	0.63	0.5	0.48

Fraction of Jγ1.2+ sequences encoding for a public clonotype within the Jγ1.2 subset or the entire Vγ2 pool.

Table 4

	Fraction of public clonotypes within Jγ1.2 pool				Fraction of public clonotypes within Vγ2 pool			
	4028	4039	4087	4736	4028	4039	4087	4736
Pre-inoculation	48.6%	62.3%	60.2%	79.5%	20.5%	46.7%	53.2%	40.9%
After inoculation	68.3%	68.8%	66.2%	82.2%	43.6%	49.4%	55.7%	44.6%
After boosting	69.7%	74.2%	74.2%	88.0%	51.7%	25.3%	28.8%	25.6%

Table 5

Evolution of the public V γ 2-J γ 1.2 repertoire after BCG immunization in macaques and after BCG stimulation *in vitro*.

5 A	Pre-Inoculation			After Inoculation			After Booster			BCG <i>in vitro</i>		
Animal 4028	# ^b	% ^c	#	%	#	%	#	%	#	%	#	%
Unique clonotypes ^d	23	65.7%	24	40.0%	18	27.3%	24	28.9%				
CALWEVQQFGRKKVK	6	17.1%	9	15.0%	8	12.1%	12	14.5%				
CALWEAKQFGRKKVK	4	11.4%	15	25.0%	25	37.9%						
CALWEVRQFGRKKVK	2	5.7%	6	10.0%	4	6.1%	12	14.5%				
CALWEVLQFGRKKVK			4	6.7%								
CALWEVQPFGRKKVK			2	3.3%	3	4.5%	2	2.4%				
CALWEVYQFGRKKVK					2	3.0%						
CALWEVEEQFGRKKVK					2	3.0%	6	7.2%				
CALWEAREQFGRKKVK					2	3.0%						
CALWEVEQFGRKKVK					2	3.0%	4	4.8%				
CALWEVTFQFGRKKVK							7	8.4%				
CALWEAQFGRKKVK							3	3.6%				
CALWEAEQFGRKKVK							3	3.6%				
CALWEGQQFGRKKVK							2	2.4%				
CALWEEQQFGRKKVK							2	2.4%				
CALWDPQQFGRKKVK							2	2.4%				
CALWEVREQFGRKKVK							2	2.4%				
CALWEAGQQFGRKKVK							2	2.4%				
Total J γ 1.2	35	100%	60	100%	66	100%	83	100.0%				

5 B	Pre-Inoculation			After Inoculation			After Booster			BCG <i>in vitro</i>		
Animal 4039	#	%	#	%	#	%	#	%	#	%	#	%
Unique clonotypes	24	34.8%	18	28.1%	12	38.7%	26	28.6%				
CALWEVQQFGRKKVK	13	18.8%	14	21.9%	7	22.6%	26	28.6%				
CALWEAGQFGRKKVK	11	15.9%	9	14.1%	4	12.9%	3	3.3%				
CALWEVRFGRKKVK	4	5.8%	3	4.7%			3	3.3%				

5 B	Pre-Inoculation		After Inoculation		After Booster		BCG <i>in vitro</i>	
	#	%	#	%	#	%	#	%
Animal 4039								
CALWEVREFGRKKVK	4	5.8%						
CALWEVHEFGRKKVK	3	4.3%						
CALWEVRQFGRKKVK	3	4.3%						
CALWEVEQFGRKKVK	3	4.3%					3	3.3%
CALWQQFGRKKVK	2	2.9%					3	3.3%
CALWDLQQFGRKKVK	2	2.9%	2	3.1%				
CALWEGQQFGRKKVK			4	6.3%				
CALWEAQQFGRKKVK			2	3.1%			5	5.5%
CALWEPQQFGRKKVK			2	3.1%				
CALWEVPQQFGRKKVK			2	3.1%				
CALWEVLQFGRKKVK			2	3.1%				
CALWDSQQFGRKKVK			2	3.1%	2	6.5%		
CALWEQFGRKKVK			2	3.1%				
CALWETPAGRKVK			2	3.1%				
CALWEVQFGRKKVK					3	9.7%		
CALWEVHQFGRKKVK					3	9.7%		
CALWEVEKFGRKKVK							6	6.6%
CALWEVFQFGRKKVK							5	5.5%
CALWEVEQQFGRKKVK							3	3.3%
CALWEDQQFGRKKVK							2	2.2%
CALWEVGQFGRKKVK							2	2.2%
CALWEVDQFGRKKVK							2	2.2%
CALWDSEQFGRKKVK							2	2.2%
Total Jy1.2	69	100%	64	100%	31	100%	91	100%

5 C	Pre-Inoculation		After Inoculation		After Booster		BCG <i>in vitro</i>	
	#	%	#	%	#	%	#	%
Animal 4087								
Unique clonotypes	25	30.1%	32	43.2%	10	32.3%	22	23.9%
CALWEVQFGRKKVK	14	16.9%	19	25.7%	4	12.9%	13	14.1%
CALWEPQFGRKKVK	9	10.8%	6	8.1%	3	9.7%	3	3.3%
CALWEVEQFGRKKVK	5	6.0%	3	4.1%	8	25.8%	3	3.3%

5 C	Pre-Inoculation			After Inoculation			After Booster			BCG <i>in vitro</i>		
	#	%		#	%		#	%		#	%	
Animal 4087												
CALWEVHQFGRKVK	4	4.8%								4	4.3%	
CALWEESQFGRKVK	4	4.8%		3	4.1%		2	6.5%		9	9.8%	
CALWEVRQFGRKVK	3	3.6%		3	4.1%		2	6.5%		2	2.2%	
CALWETQQFGRKVK	3	3.6%										
CALWEGQQFGRKVK	2	2.4%										
CALWEVAQFGRKVK	2	2.4%								2	2.2%	
CALWEVHSQFGRKVK	2	2.4%										
CALWEAHDQFGRKVK	2	2.4%										
CALWEVPVWQKSQ	2	2.4%										
CALWETQQQFGRKVK	2	2.4%		3	4.1%							
CALWEVQFGRKVK	2	2.4%										
CALWEAQFGRKVK	2	2.4%		2	2.7%					2	2.2%	
CALWEVNFGRKVK				3	4.1%							
CALWEVPQFGRKVK							2	6.5%		2	2.2%	
CALWEVEQFGRKVKX										11	12.0%	
CALWEVRKQFGRKVK										6	6.5%	
CALWEVLQFGRKVK										6	6.5%	
CALWEDEEFGRKVK										3	3.3%	
CALWEEQQFGRKVK										2	2.2%	
CALWGNQQFGRKVK										2	2.2%	
Total Jy1.2	83	100.0%		74	100.0%		31	100.0%		92	100.0%	
5 D												
Animal 4736	Pre-Inoculation			After Inoculation			After Booster			BCG <i>in vitro</i>		
	#	%		#	%		#	%		#	%	
Unique clonotypes												
CALWEVRQFGRKVK	13	29.5%		5	11.1%		4	16.0%		8	11.8%	
CALWEVHQFGRKVK	13	29.5%		20	44.4%		6	24.0%		4	5.9%	
CALWEVHSQFGRKVK	10	22.7%		6	13.3%		9	36.0%		25	36.8%	
CALWEVQFGRKVK	4	9.1%		3	6.7%		3	12.0%		8	11.8%	
CALWEVLVGRKVK	2	4.5%		4	8.9%					18	26.5%	
CALWEVKTQFGRKVK	2	4.5%										
CALWELOQFGRKVK				3	6.7%		3	12.0%				

5 D		Pre-Inoculation		After Inoculation		After Booster		BCG <i>in vitro</i>	
		#	%	#	%	#	%	#	%
	Animal 4736								
	CALWEVNQFGRKKVK			2	4.4%				
	CALWEVQRFGRKVK			2	4.4%				
	CALWDQLIQFGRKKVK							3	4.4%
	CALWEVQRQFGRKKVK							2	2.9%
	Total J _γ 1.2	44	100.0%	45	100.0%	25	100.0%	68	100.0%

^aThe amino acid sequence covering the V_γ2 chain CDR3 regions are shown. Bold type indicates public sequences. Unique clonotypes incorporate all sequences appearing a single time in that sample set.

^bThe number of appearances for each clonotype (or the sum of all unique sequences) in each sample set. When no value is listed, the sequence was not detected or was detected only once (and included in the unique clonotypes pool).

^cThe percentage of all V_γ2-J_γ1.2 sequences represent by each clonotype or by the pool of unique sequences.