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Anti-SIV Cytolytic Molecules in Pigtail Macaques

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Abstract

Release of granzymes and perforin from the cytolytic granules of SIV-specific CD8 T cells is a critically important effector mechanism facilitating the elimination of SIV-infected cells. We sequenced granzyme A, B, and K and perforin in pigtail macaques and defined polymorphisms between humans, rhesus macaques, and pigtail macaques. The pigtail macaque sequences were similar to the corresponding rhesus sequences at the mRNA and protein level and (0.4–1.1% sequence differences) but substantially different from human sequences (3.8–8.1% sequence differences). We used this sequence information to develop multiplex PCR assays to detect these genes. We also successfully studied the release of perforin and granzyme B from deregulating SIV-specific CD8 T cells by flow cytometry. These sequences and tools enable further study of the cytolytic control of SIV in pigtail macaques.

Cytotoxic CD8 T lymphocytes (CTLs) provide partial control of HIV-1 in humans and SIV in macaques. The appearance of specific CTLs coincides with control of acute infection[1] and depletion of CD8 T cells results in a brisk rise in viral load.[2] The effectiveness of CTLs is partially offset by viral mutational escape,[3] although some escape mutant viruses have impaired replication capacity (reduced "fitness").[4] CTLs mediate their effect by both the killing of virus-infected cells and through the release of bioactive cytokines and chemokines. The killing of infected cells by effector CTLs involves the release of preformed pore-forming molecules such as granzymes and perforin from within cytotoxic granules. Several forms of granzyme molecules exist (granzyme A, B, H, K, and M). Granzymes and perforin molecules are differentially expressed in single CD8 T cells in murine influenza infection models, highlighting the complexity of mammalian cytotoxic molecules.[5,6]

Several simian models of HIV-1 infection exist, with the most commonly studied models being SIV infection of rhesus (*Macaca mulatta*), and, increasingly, cynomolgus (*M. fascicularis*) and pigtail macaques (*M. nemestrina*). Most studies of cytotoxic molecules in nonhuman primates have employed only rhesus macaques. Several studies have illustrated the important roles of granzyme B and perforin during SIV infection of rhesus macaques.[7,8] Rhesus macaques have, however, limited availability in some areas and little information or tools are available in alternate macaque models. The pigtail macaque has in recent years become a robust model with a series of immunologic and virologic tools developed to study vaccines and pathogenesis issues.[9] We recently showed that release of granzyme B and perforin from tetramer-positive SIV-specific CTLs is faster following live attenuated SIV vaccination than standard viral vector vaccination of pigtail macaques.[10]

New tools are, however, required to further probe the contribution of various cytotoxic effector molecules in macaques. In particular, comparative sequence information on multiple cytotoxic molecules (including the different forms of granzyme) is required to develop useful polymerase chain reaction (PCR)-based assays to detect and analyze these molecules. Robust assays to study the functional release of these molecules in bulk SIV-specific CTLs without the requirement to gate on tetramer$^+$ cells are also needed.

We first sequenced granzyme A, B, and K and perforin RNA in pigtail macaques. Two pigtail macaques previously reported and vaccinated with viral vector vaccines were chosen (animals 5821 and 5827). Bulk mRNA was extracted from fresh EDTA-anticoagulated blood by extracting total mRNA from Ficoll-purified peripheral blood mononuclear cells (PBMCs) (Oligotex Direct mRNA kit, Qiagen) and converting the mRNA into cDNA as previously described.[11] We then designed primers to bind to the conserved regions of the human and *M. mulatta* sequences and PCR amplified and sequenced the equivalent pigtail gene section from both animals. We used this information to design pigtail-specific primers to amplify full-length cDNAs in reverse transcription reactions (SMART RACE cDNA Amplification kit, Clontech). The full-length fragments were cloned and sequenced (Zero Blunt TOPO PCR Cloning kit, Invitrogen).

The sequencing efforts yielded high-quality data from the pigtail macaques studied. The *M. nemestrina* nucleotide sequences and predicted amino acid sequences were

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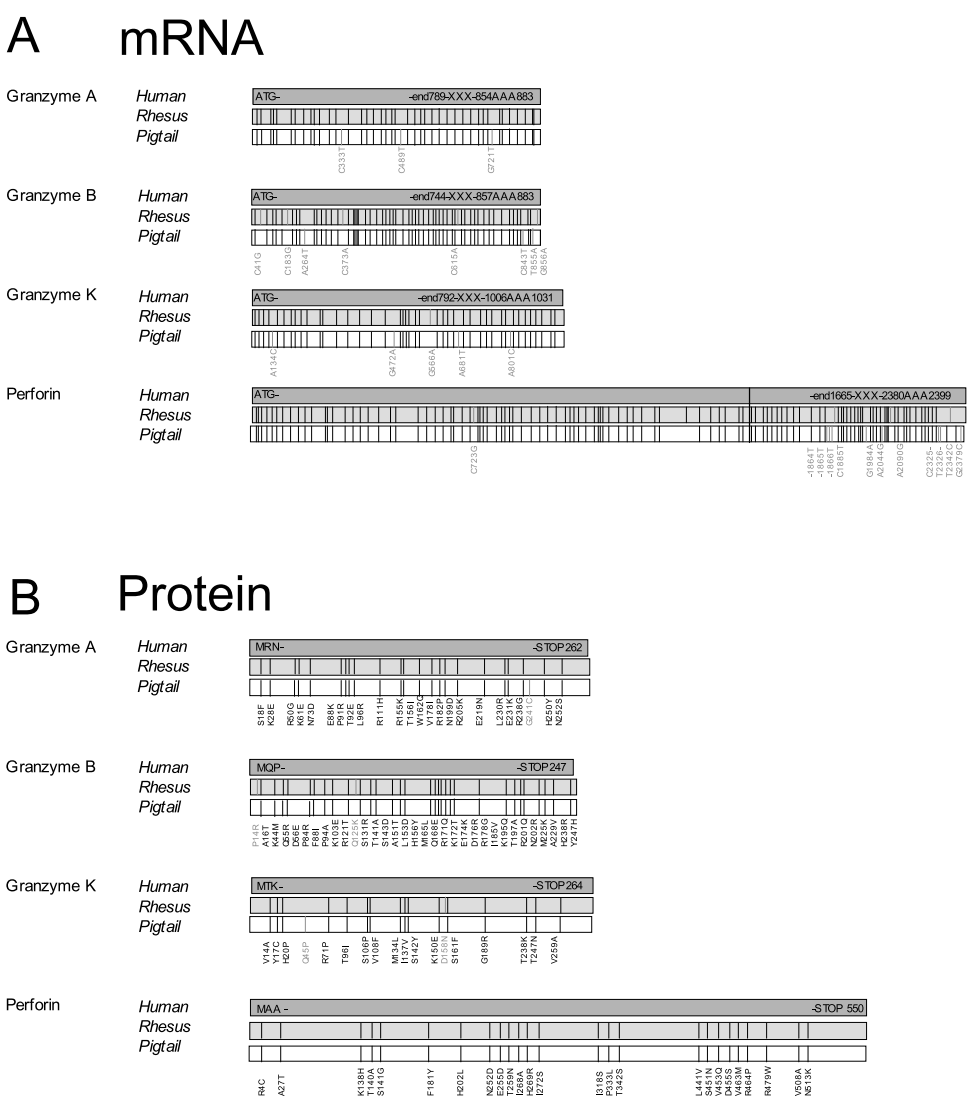

FIG. 1. The pigtail macaque *(Macaca nemestrina)* mRNA and protein sequences for granzyme A, B, and K and perforin. (**A**) Alignments of granzyme A, B, and K and perforin full-length mRNA (cDNA) sequences originating from human (*Homo sapiens*), Rhesus macaque (*M. mulatta*), and pigtail macaque (*M. mulatta*). Vertical black lines within the primate schematic sequence indicate a nucleotide difference as compared to reference human sequences (GenBank accession numbers shown in Table 1). Vertical red lines indicate difference between rhesus and pigtail sequences. The pigtail-specific differences (vertical red lines) are annotated with the precise human-to-macaque nucleotide difference and its position. ATG, start codon for methionine; end, the stop codon nucleotide position; XXX, noncoding sequence downstream of the stop codon but before the poly(A) tail; AAA, poly(A) tail followed by the last nucleotide position. The reference sequences for human and rhesus were the following: granzyme A (NM006144 and XM001097639.1), granzyme B (NM004131 and CN648784), granzyme K (NM_002104 and XM001097338.1), and perforin (NM_001083116a and XM_001107967.1). (**B**) Alignments of the corresponding translated protein sequences for granzyme A, B, and K and perforin in humans and macaques. Vertical black lines indicate primate-specific amino acid differences compared to the human sequence and vertical red lines indicate differences between rhesus and pigtail sequences. The pigtail-specific differences (vertical red lines) are annotated with the precise human-to-macaque protein difference and its position. MRN, MQP, MTK, and MAA, the three initial abbreviated amino acids for each protein sequence, respectively; STOP, the stop codon followed by the terminal amino acid position.

aligned along side consensus *M. mulatta* and human reference sequences (Fig. 1 and Table 1). There were multiple conserved nucleotide changes in both *M. mulatta* and *M. nemestrina* compared to human sequences (black lines, Fig. 1A) and a smaller number of changes specific to pigtail macaques in comparison to *M. mulatta* (red in Fig. 1A). The pigtail granzyme A, B, and K sequence data showed a total of 14 specific nucleotide differences to reference rhesus macaque sequences (three for granzyme A, six for B, and five for K, Fig. 1). There were a total of three additional nucleotide changes detected in pigtail sequences that were also observed as polymorphisms among rhesus macaque sequences. Interestingly, for the much larger perforin sequence there was only a single nucleotide change in the expressed mRNA, although 11 changes within the noncoding tail.

Table 1. Comparison of Granzyme and Perforin Sequences[a]

			Comparator sequence			
			Human		*Rhesus*	
Gene	*Species*	*Size (nt/aa[b])*	*nt Δ (%, NS/S[c])*	*aa Δ (%)*	*nt Δ (%, NS/S)*	*aa Δ (%)*
Granzyme A	Human	789/262	1 (0.04, NA[d])	0 (0)	—	—
	Rhesus		41 (5.2, 59)	24 (9.2)	7 (0.9, 44)	6 (1.2)
	Pigtail		44 (5.6, 57)	25 (9.5)	3 (0.4, NA)	1 (0.4)
Granzyme B	Human	744/247	8 (0.1, 67)	4 (0.2)	—	—
	Rhesus		60 (8.1, 55)	33 (13.4)	5 (0.1, 100)	4 (0.3)
	Pigtail		57 (7.7, 55)	31 (12.6)	8 (0.8, 46)	2 (1.1)
Granzyme K	Human	792/264	0 (0, NA)	0 (0)	—	—
	Rhesus		43 (5.4, 40)	17 (6.4)	8 (0.3, 40)	1 (0.1)
	Pigtail		46 (5.8, 37)	17 (6.4)	5 (0.6, 44)	2 (0.8)
Perforin	Human	1665/550	3 (0.4, NA)	2 (0.07)	—	—
	Rhesus		64 (3.8, 40)	25 (4.6)	2 (0.04, NA)	0 (0)
	Pigtail		64 (3.8, 40)	25 (4.6)	1 (0.02, NA)	0 (0)

[a]GenBank accession numbers: granzyme A-human, NM_006144, BC015739, CR456968, M18737; granzyme A-rhesus, XM_001097639.1, CN648306; granzyme A-pigtail, EU526079-80; granzyme B-human, NM_004131, AY232654.1, AY232655.1, AY232656.1, BC030195.1, J03189.1, J0407.1, M17016.1, AY372494.1; granzyme B-rhesus, CN648784, XM_001114420, XM_001114360, EE74583; granzyme B-pigtail, EU526081-2; granzyme K-human, NM_002104, U26174.1, U35237.1; granzyme K-rhesus, XM_001097338.1, EE75284, CO644825; granzyme K-pigtail, EU526083-4; perforin-human, NM_001083116, AB209604, X13224, BC047695, BC063043, NM_005041; perforin-rhesus, XM_001107967.1, XM_001107909.1, EE745301; perforin-pigtail, EU526085-6.
[b]nt, nucleotides; aa, amino acids.
[c]NS/S, % nonsynonymous over synonymous nt change.
[d]NA, too low numbers of change to give useful calculus.

Nearly half of the nucleotide changes across all four cytolytic molecules resulted in alterations at the amino acid level (Table 1). This led to differences between the macaques and human proteins of 13% for granzyme B, 9% for granzyme A, 6% for granzyme K, and 4.5% for perforin (black lines, Fig. 1B). There were much smaller numbers of amino acid differences between rhesus and pigtail macaque granzyme A, B, and K protein sequences (generally <1%, Table 1, and red lines in Fig. 1B) and none in the perforin protein sequence.

Following this sequencing effort, we were then able to design multiplex PCR reactions to sensitively detect these proteins, alongside both CD8 mRNA and LAMP-1 mRNA. The LAMP-1 gene expresses the CD107a molecule, a marker on empty vesicles, and now commonly studied in flow cytometric assays of degranulation. We chose to design a nested PCR reaction for each molecule to provide a highly sensitive means to detect these molecules in low cell sample numbers as used in murine experiments.[5,6] The multiplex primers were designed to result in bands with differing sizes readily detected on the final gel. With this assay we were able to readily detect all four cytolytic molecules from *M. nemestrina* bulk PBMCs together with CD8 and LAMP-1 (Fig. 2).

Antigen-specific release of cytolytic molecules from T cells can be studied by flow cytometry, generally by the surrogate marker of the detection of surface expression of the degranulation marker CD107a.[12,13] Fewer studies have directly studied release of cytolytic effector molecules such as granzyme B in macaques, particularly in pigtail macaque species.[10] Given the reasonable degree of sequence homology across the pigtail macaque and human protein sequences of granzyme B we identified, we were able to use available human MAb to detect intracellular granzyme B in macaque T cells.[10] We studied fresh whole blood from an SIV_{mac251}-infected pigtail macaque with the *Mane-A*10* MHC class I allele, known to present an immunodominant SIV Gag peptide KP9. The whole blood was stimulated with the KP9 peptide (1 μg/ml) for 6 h and CD8 T lymphocytes stained for the degranulation marker CD107a together with granzyme B (Fig. 3). We were able to detect a distinct CTL population from both expression surface CD107 and lower levels of granzyme B, consistent with the liberation of granzyme B upon antigen recognition.

In summary, we defined the pigtail macaque sequences of the cytolytic molecules granzyme A, B, and K and perforin, showing a reasonably high level of protein conservation across other primate species. We used this information to design nested multiplex PCRs to confirm the expression of these molecules. These multiplex assays can now be employed to probe the differential utility of these proteins in individual CTLs. Such assays in mice have revealed that single CTLs can differentially express particular cytolytic molecules during vaccination and infection scenarios.[5,6] CTL degranulation defined flow cytometrically also illustrates the cytolytic potential of SIV-specific CTLs. Future studies can now address the utility of various cytolytic molecules in control of SIV in pigtail macaques.

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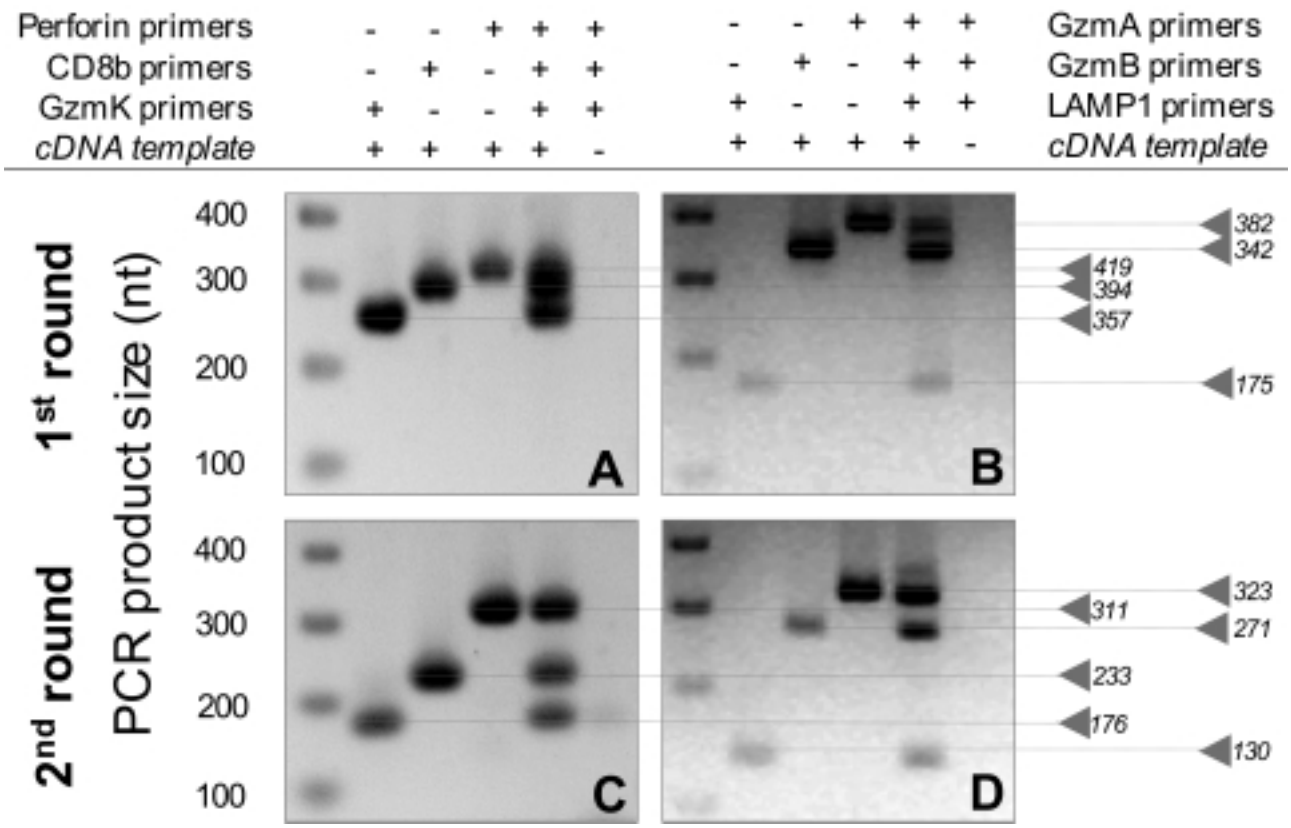

FIG. 2. A nested multiplex PCR assay to amplify pigtail macaque-specific granzymes A, B, and K, perforin, CD107, and CD8b transcripts. (**A, B**) Bulk PBMC cDNA (10 μl) is divided into two separate reactions with two different sets of outer primers [perforin/CD8b/GzmK (**A**) lane 4 versus GzmA/GzmB/CD107 (**B**) lane 9]. Single primer pair reactions (lanes 1, 2, 3, 6, 7, and 8) and no template controls (lanes 5 and 10) are also shown. (**C, D**) Of each first PCR reaction 10% is transferred into second inner primer pairs/cocktails [CD8b/GzmK/perforin (**C**) lane 14 vs. GzmA/GzmB/CD107 (**D**) lane 20]. Again single primer pair controls (lanes 11, 12, 13, 16, 1, and 18) and no template controls (lanes 15 and 20) are shown. Nt, nucleotide; +, primer pair present in the reaction; −, primer pair not included in the reaction; numbers on left y-axes indicate the even hundred nt size marker and numbers on right *y*-axes indicate the PCR expected product size (nt) for each PCR product.

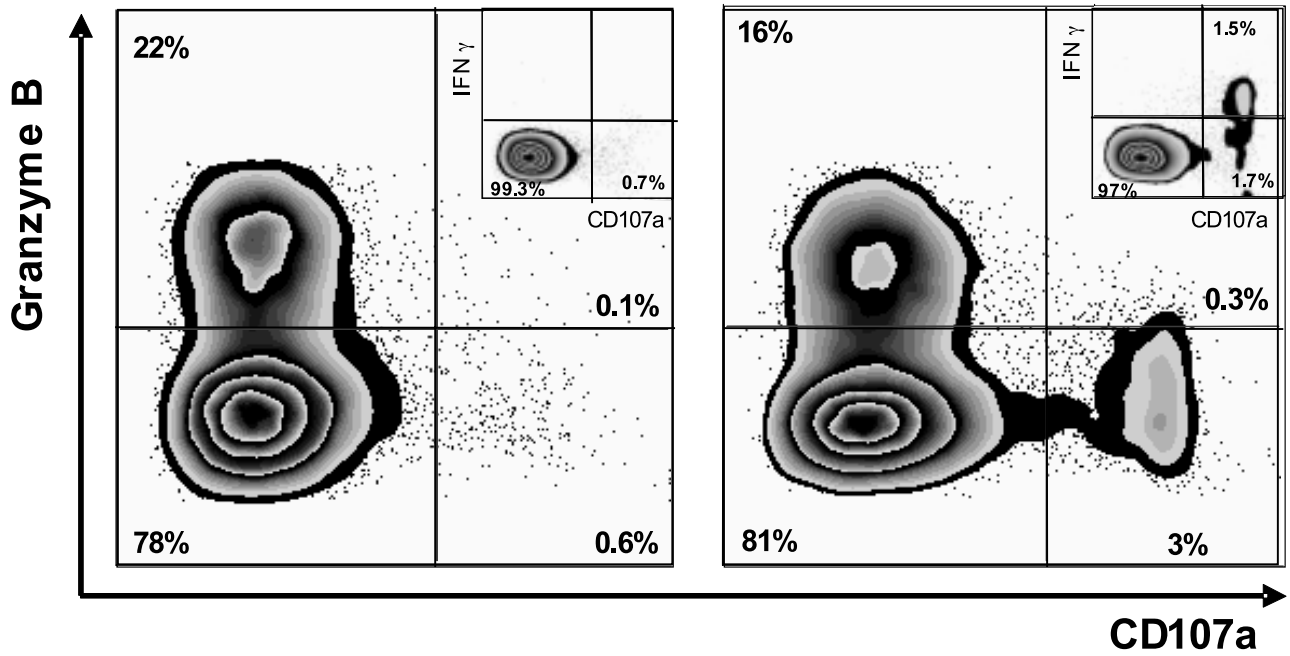

FIG. 3. SIV-specific CD8 T cell degranulation can be detected as a decrease in intracellular granzyme B levels. Pigtail macaque whole blood from animal 5612 was collected 30 weeks after inoculation with SIV_{mac239}. The blood was cultured *in vitro* for 5 h without (left panel) or with (right panel) the Mane-A*10-restricted immunodominant SIV Gag peptide KP9 (SIV $Gag_{164–172}$). The analysis shows cells gated on $CD3^+CD8^+$ lymphocytes and depicted as granzyme B vs. CD107a. The small inset panels show the corresponding $CD3^+CD8^+$ sample depicted as IFN-γ vs. CD107.

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