Universal, MHC-E restricted killer T cell responses: Identification of a novel immune response against HIV

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Overview

With over 35 million people living with HIV today and a long history of vaccine failure, an unconventional vaccine is urgently needed. Researchers studying HIV commonly use Simian Immunodeficiency Virus (SIV) in Rhesus macaques as a reliable model for HIV infection. A new RhCMV vaccine targeting SIV showed **fifty percent efficacy and viral clearance** in vaccinated macaques. A subset of vaccine-induced, major histocompatibility complex (MHC)-class II-restricted CD8+ (killer) T cell responses **breaks a central tenet of immunology**, but the MHC-class I-restricted responses still remained uncharacterized. This research shows that **non-classical Rhesus MHC-E restricts RhCMV/Gag vaccine-induced CD8+ T cell responses.** Rhesus MHC-E is highly conserved with its human ortholog, and limited sequence diversity in **MHC-E explains the universal targeting in vaccinated macaques.** This study explains the phenomenon described as "supertope" presentation. Notably, MHC-E-restricted responses have not previously been shown for any other vaccine, and could be the explanation of the conferred immunity this vaccine provides. This data suggests that this vaccine could have efficacy in humans.

Introduction

The Human Immunodeficiency Virus (HIV) has devastated millions of lives, with 35 million infected individuals and over two million new cases each year. After HIV infection, many develop the Acquired Immunodeficiency Syndrome (AIDS), which ultimately leads to CD4+ (helper) T-cell depletion, opportunistic infections, and in many cases death.

- Since the late 1990s, anti-retroviral therapy (ART) has been the paradigm for HIV treatment and has been shown to lengthen the life expectancy of many infected individuals.
- However, significant cost barriers, availability issues, and large lifestyle changes make ART a reasonable treatment option for only a small subset of infected individuals. Additionally, ART has numerous side effects.
- Multiple vaccine trials have failed against HIV, largely due to genomic sequence diversity and glycosylation of surface proteins (see Box 3).

As a result, an unconventional, prophylactic HIV vaccine is necessary

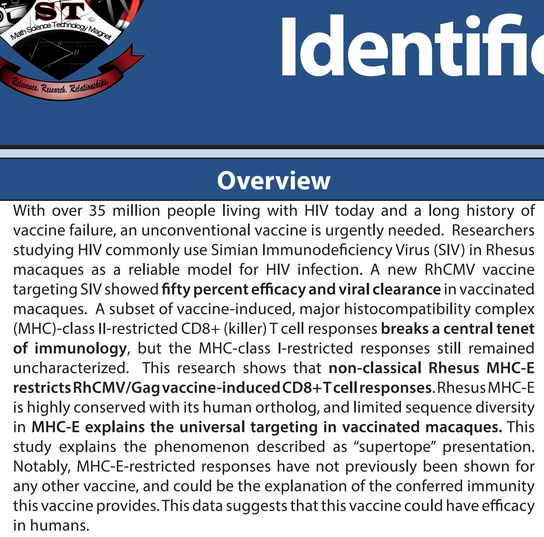


Figure 1. *Unique CD8+ T cell responses from CMV vaccination (Hansen et al, 2013).* (*1A*) RhCMV/gag-vaccinated Rhesus macaques had immune responses that targeted a breadth of epitopes much larger than conventional vaccines (3x more) and more than any other HIV vaccine and even elite controllers. (*1B*) RhCMV-vaccination induced CD8+ T cell responses restricted by both MHC class I (36%) and MHC class II (64%), covered enormous breadth, and had universally-targeted epitopes.

- In 2009, researchers developed an novel vaccine that utilizes Rhesus Cytomegalovirus (RhCMV) engineered to express Simian Immunodeficency Virus (the AIDS-causing virus for Rhesus macaques).[1]
  - The vaccine had **50% efficacy** in RhCMV vaccinated macaques.[2,3,4]
  - Vaccinees appeared **functionally cured** with **no detectable levels of viral RNA** even with the most sensitive RT-PCR assays.[3]
  - The vaccine was later shown to induce major histocompatibility complex, **MHC-class-II-restricted-CD8+ T cell responses**, which breaks a central tenet of immunology.

Box 1: MHC restriction of Antigen-Specific T lymphocytes

- The T cell receptor (TCR) on T lymphocytes is antigen-specific
- Antigenic peptide (epitope) is presented on major histocompatibility complex (MHC) molecules to antigen-specifc T cells.
- Additionally, epitopes can only have effective T cell responses when the TCR binds to the epitope-MHC complex that it is specific to.
- Termed **"MHC restriction."**

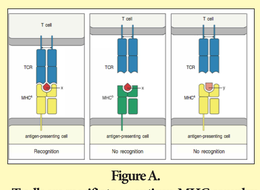


Figure A.
T cells are specific to an antigen-MHC complex.

- Responses target highly promiscuous epitopes (antigen), called "**supertopes**" (common epitopes targeted in all animals).[2]
- It is important to characterize the immune responses induced by this vaccine to determine whether this vaccine could have potential efficacy in humans.

Methods: Overview & Transfectant Design

Overview of Methodology

There were four major portions of this research.

1. MHC class I alleles on pCR-BLUNT were transferred to a mammalian cell expression vector, pCEP4 (Invitrogen).
2. The pCEP4/MHC-I constructs were transfected into either .221 or K562 (ATCC), which are MHC-class I null cell lines.
3. Then, the cells were plated with SIVgag peptide and T cell isolates in the intracellular cytokine staining protocol (ICS). Brefeldin-A and anti-CD3, -CD4, -CD8, -TNFa, and -IFNy (Abcam, BD Biosciences) antibodies were added (see top center for more information about ICS).
4. The final data were analyzed via flow cytometry on the LSR-II machine (BD Biosciences) and then on FlowJo software (Tree Star).

Transfectant Design

Designing transfectants that expressed single class I alleles was critical to successfully running restriction assays. Rhesus macaque MHC class I alleles (Mamu-A,B,E,F) were restricted and ligated into an expression vector and transfected into MHC class I null cell lines. These transfectants were able to present antigen to T cells during downstream restriction assays. See Figure 5 for the flow cytometric analysis of transfectant expression level.

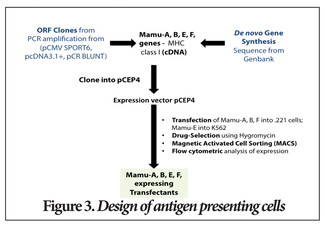


Figure 3. *Design of antigen presenting cells*

Methods: CD8+ T cell Restriction assays and Intracellular Cytokine Staining

After successfully designing MHC class I transfectants, the designed antigen presenting cells were used in intracellular cytokine staining assays to determine MHC restriction of CD8+ (killer) T cell responses. Gag69 and Gag120 epitopes were characterized first because of previous data showing supertope presentation of these peptides. Rhesus macaques that were previously RhCMV/gag-vaccinated had their PBMCs taken and used directly *ex vivo*. Peptide-pulsed transfectants were incubated with PBMCs and stained with flourescent antibodies (BD Biosciences) and analyzed through flow cytometry (BD Biosciences). Results were then gated for CD8+ T cells and cytokine production (Tree Star). See below for an ICS schematic and to the right for the ICS protocol.

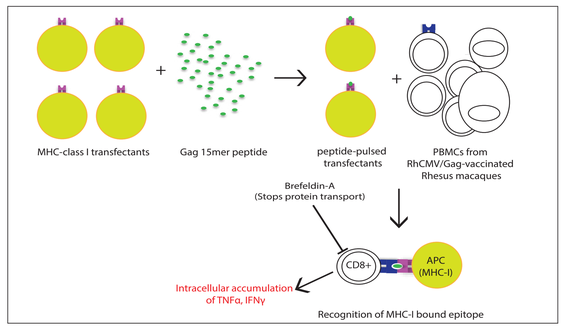


Figure 4. Schematic of Intracellular Cytokine Staining (ICS) restriction assay

Box 2: Flow Cytometry, Intracellular Cytokine Staining, and Data Analysis

- **Flow cytometry:** a laser-based biotechnology employed in biomarker detection and analysis of side scatter (granularity) and forward scatter (size). It allows for analysis of heterogenous cell populations based on surface expression of cluster of differentation (CD) molecules and other proteins, in a method referred to as *immunophenotyping.*
- **Intracellular Cytokine Staining:** a flow cytometry-based assay which detects cytokine production and accumulation.
  1. T cells are activated using antigen-pulsed transfectants
  2. Brefeldin A, which inhibits protein transport and retains cytokines within the cell, is added
  3. Antibodies for cellular markers (CD3, CD4, CD8) are added after a series of washes
  4. Cells are fixed with paraformaldehyde (PFA) and then permeabilized
  5. Anti-cytokine antibodies are added to the cells (TNF-a, IFN-y)
  6. Cell population analyzed via flow cytometry
- **Gating** used to focus on cell population of interest: CD3+; CD4-, CD8+; Quadrant Gating on TNF-a, IFN-y

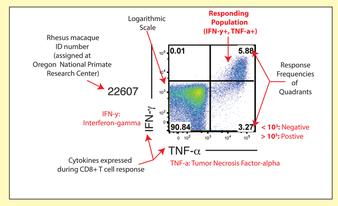


Figure B. Flow ICS Data Interpretation

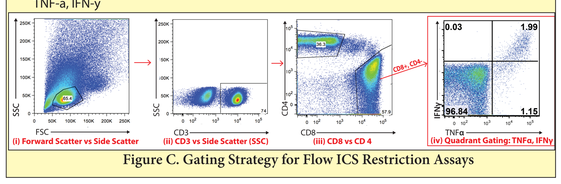


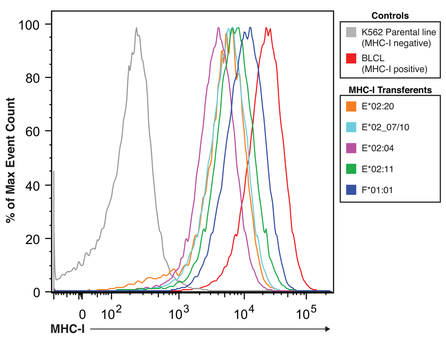
Figure C. Gating Strategy for Flow ICS Restriction Assays

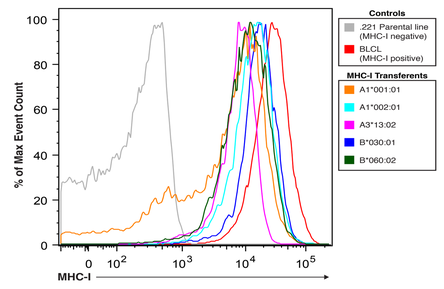
Results

Figure 5. Single MHC-class I transfetants show high levels of expression for classical and non-classical molecules (right).

Transfectants expressing single MHC class I alleles were generated when Rhesus macaque MHC alleles were transferred from pCR-BLUNT through restriction and ligation into pCEP4. The pCEP4/MHC-I constructs were transfected into 721.221 or K562 (ATCC) cell lines, which are MHC class I null cell lines. The cells were transfected using the Amaxa electroporator following the manufacturer's instructions on setting G-016. The transfectants were cultured in R10 media for a week to recover from transfection. Since pCEP4 carries mammalian antiboitic resistance for hygromycin, the cells were then cultured on media modified with hygromcin (750 ng/ul) for a month. Prior to use in any downstream immunological assays, the cells received fresh media and also underwent further selection via Magnetic-Activated Cell Sorting (MACS). Finally, cells were stained with a cross-reactive anti-MHC-class I-PE antibody (BD Biosciences) then assesed for expression using flow cytometry (BD Biosciences). All cells had mRNA isolations (Qiagen) done repeatedly to confirm the correct allele was being expressed.

The data shown in the figure is a compilation of the transfectants' expression graphs that were generated using FlowJo (Tree Star). Expression past 10^3 is considered positive compared to the negative control (parental, class I-null cell lines). B-LCL isolated from macaques served as the postive control.





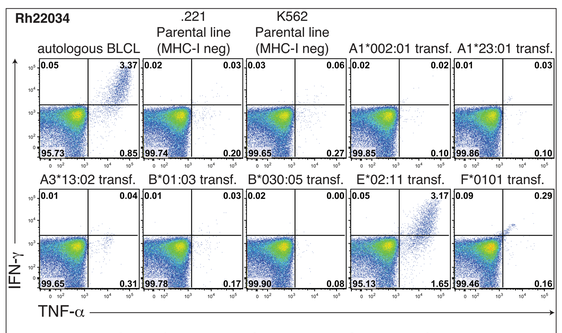


Figure 6. ***Non-classical restriction of Gag120-specific response by Mamu-E*02:11.*** Whole blood was drawn by veternarians from RhCMV/Gag-vaccinated macaque ID# 22034 (Rh22034). Peripheral Blood Mononuclear Cells (PBMCs) were isolated by using a ficoll gradient. These were incubated with SIV Gag120 peptide-pulsed transfectants with single MHC class I molecule expression Gag120 is a 15mer peptide derived from the Gag protein in SIV. After being stained with antibodies (see above), the cells were fixed and analyzed on flow cytometry. Figure 6 supports restriction by a non-classical Rhesus MHC-E molecule (Mamu-E*02:11). Additionally, this figure shows that classical MHC-I transfectants fail to present Gag69 epitope to RhCMV/gag-induced CD8+ T cells.

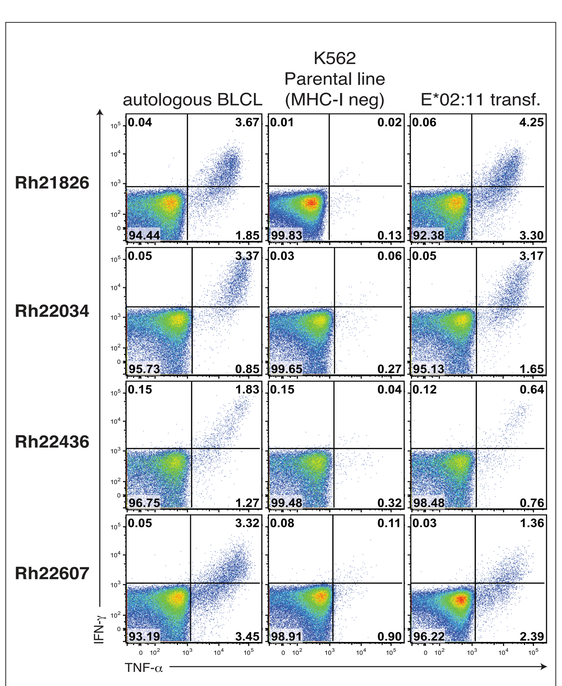


Figure 7. ***Non-classical restriction of Gag120-specific response by Mamu-E*0111.*** Whole blood was drawn by veternarians from RhCMV/Gag-vaccinated macaques. Peripheral Blood Mononuclear Cells (PBMCs) were isolated from Rh21826, Rh22034, Rh22436, and Rh22607. These were incubated with SIV Gag120 peptide-pulsed transfectants with single MHC class I molecule expression Gag120 is a 15mer peptide derived from the Gag protein in SIV. After being stained with antibodies (see above), the cells were fixed and analyzed on flow cytometry. Figure 7 supports restriction by non-classical Rhesus MHC-E molecule (Mamu-E*02:11).

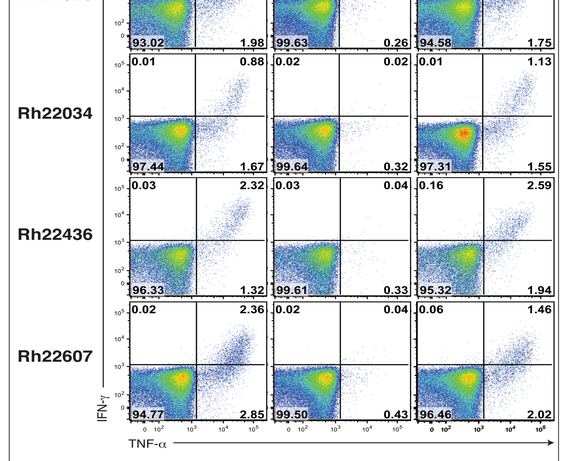


Figure 8. ***Non-classical restriction of Gag69-specific response by Mamu-E*02:04.*** Peripheral Blood Mononuclear Cells (PBMCs) were isolated from RhCMV/gag-vaccinated macaques Rh21826 Rh22034, Rh22436, and Rh22607. They were incubated with SIV Gag69 peptide-pulsed transfectants expressing single MHC class I molecules. Gag69 is a 15mer peptide derived from the Gag protein in SIV. After being stained with antibodies (see above), the cells were fixed and analyzed on flow cytometry. This figure supports universal, nonclassical presentation of Gag69 by non-classical Rhesus MHC-E (Mamu-E*02:04).

	Restricting molecule(s)	
	E*02:04	E*02:11
Gag5		X
Gag18	X	
Gag23	X	
Gag69	X	
Gag97	X	
Gag109	X	
Gag119	X	X
Gag120		X
Gag121	X	X

Figure 9. ***Summary of MHC-E restricted responses.*** PBMCs were isolated from multiple macaques and ICS assays were performed for multiple epitopes based on the data from previous blocking experiments. The epitopes listed in the tables above have repeatedly been restricted by MHC-E in multiple macaques across multiple timepoints. Additionally, the transfectants used in these assays have gone through mRNA isolation, RT-PCR, and sequence isolation to confirm MHC-E expression. As a result, these epitopes are restricted by MHC-E. E*02:04 and E*02:11 have almost complete sequence conservation.

Discussion

- The responses characterized in this study support that there are universal, classical and non-classical MHC restricted-CD8+ T cell responses induced by the RhCMV vaccine against SIV in Rhesus macaques.
- Although classical MHC class I restriction is an important arm of immunity elicited by this vaccine, the non-classical MHC restriction by *Mamu-E* is the most interesting phenomenon because it has never been seen to restrict vaccine-induced immune responses.
- Restriction by Rhesus MHC-E or any of its homologs has not been described for any other vaccine to any other pathogen, including HIV.

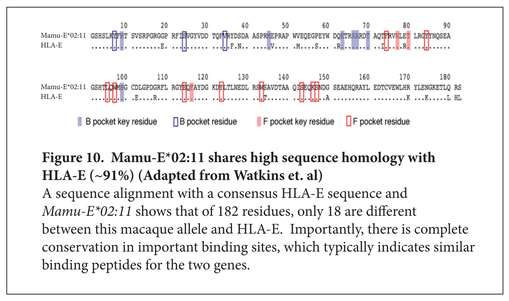


Figure 10. Mamu-E*02:11 shares high sequence homology with HLA-E (~91%) (Adapted from Watkins et. al)
A sequence alignment with a consensus HLA-E sequence and *Mamu-E*02:11* shows that of 182 residues, only 18 are different between this macaque allele and HLA-E. Importantly, there is complete conservation in important binding sites, which typically indicates similar binding peptides for the two genes.

About Rhesus and Human MHC-E

- Mamu-E in Rhesus macaques is orthologous to MHC-E in humans
- Mamu-E has low sequence diversity in macaques
- The Human Leukocyte Antigen (HLA) locus is one of the most highly polymorphic loci ever described, yet the HLA-E locus, which encodes MHC-E is more conserved.
- **Human CMV (HCMV) and SIV are known to upregulate MHC-E**
- Mamu-E has high sequence homology with MHC-E in humans, and there is high sequence conservation throughout all primates
- MHC-E is expressed in most tissue, including the mucosa, at low levels.
- **Only 2 MHC-E molecules exist in humans, and only differ by one amino acid.**
- MHC-E and Mamu-E have both been shown to present and bind 9mer peptides that are derived from the leader peptide of classical MHC class I alleles
- MHC-E is a ligand to NKG2 (also known as CD94), which presents to NK cells
- MHC-E is known to bind pathogen-derived peptide, and CD8+ T cells can recognize these binders from epitopes arising from *M. Tuberculosis, HCV,* and *CMV*

	MHC-E Molecules
Rhesus macaques	24
Humans	2

Figure 11. Humans have fewer MHC-E allotypes than Rhesus macaques.

Taken together, a possible explanation for presentation of supertopes by MHC class I alleles is that they bind to the non-classical, highly conserved, MHC-E molecule.

Conclusions & Outlook

- HIV is still a devestating virus today, despite numerous treatment options.
- Multiple vaccine trials have failed, so a novel vaccine modality is needed.

Box 3: HIV and a History of Vaccine Failure

- **Failed Merck STEP Trial:** This was a efficacy trial for an adenoviral vaccine (HVTN 502) but it showed no efficacy. In fact, some vaccinated individuals were at a higher risk of HIV infection.
- **RV144 Trial:** 31% efficacy shown in Thailand. However, the results have been disputed by many scientists and the prime-boost vaccine requires annual vaccination.
- **HVTN 505:** Used both an Antibody and Conventional T Cell-targeted vaccine. Research ended in 2013, and showed that the vaccine method had no efficacy.

- In 2009, a completely novel Rhesus cytomegalovirus-based SIV vaccine was shown to have 50% efficacy and protected animals appeared functionally cured.
- RhCMV-induced, SIV-specific, MHC-II-restricted CD8+ T cell responses have been characterized previously and violate classical paradigms in immunology.
- *These responses are also importantly restricted by non-classical Rhesus MHC-E molecule Mamu-E which is an ortholog to the highly conserved HLA-E in humans.*
- *MHC-E is likely responsible for promicuous presentation of viral eptiopes to CD8+ T cells in RhCMV-vaccinated macaques.*
- *Humans have essentially two major MHC-E transcripts that result in only one amino acid difference.*
- *E-restricted responses do not naturally occur in HIV-infected individuals, and this pattern of protection has never been seen for **any** vaccine.*
- **These findings suggest this vaccine could have efficacy in humans due to low polymorphism in the MHC-E locus and therefore, MHC-E-restricted responses may confer anti-viral immunity.**

Future Steps

- Further identify non-classical responses in RhCMV/SIV vaccinated Rhesus macaques.
- Develop an E-specific monoclonal antibody to show that these responses can be blocked by a high-affinity peptide.
- Develop an E-restricted, RhCMV vaccine-induced CD8+ T cell line specific to Gag peptide and show blocking with an MHC-E antibody.

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Figure Credits

- Figure 1 was adapted from *Science* (Hansen et al., 2013).
- Figures 2, 3, and 4 were designed by the student using Adobe Illustrator.
- Figures 5-8 were designed in FlowJo by the student.
- Figure 9 and 11 were designed in Adobe Illustrator by the student.
- Figure 10 was adapted from a grant application written by Watkins et al.
- Figure A was taken from Janeway's *Immunobiology* 6/e.
- Figures B and C were designed by the student using Adobe Illustrator.

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