A Novel Liposome-Based Adjuvant CAF01 for Induction of CD8+ Cytotoxic T-Lymphocytes (CTL) to HIV-1 Minimal CTL Peptides in HLA-A*0201 Transgenic Mice

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Abstract

Background: Specific cellular cytotoxic immune responses (CTL) are important in combating viral diseases and a highly desirable feature in the development of targeted HIV vaccines. Adjuvants are key components in vaccines and may assist the HIV immunogens in inducing the desired CTL responses. In search for appropriate adjuvants for CD8+ T cells it is important to measure the necessary immunological features e.g. functional cell killing/lysis in addition to immunological markers that can be monitored by simple immunological laboratory methods.

Methodology/Principal Findings: We tested the ability of a novel two component adjuvant, CAF01, consisting of the immune stimulating synthetic glycolipid TDB (Trehalose-Dibehenate) incorporated into cationic DDA (Dimethyldioctadecylammonium bromide) liposomes to induce CD8+ T-cell restricted cellular immune responses towards subdominant minimal HLA-A0201-restricted CTL epitopes from HIV-1 proteins in HLA-A*0201 transgenic HHD mice. CAF01 has an acceptable safety profile and is used in preclinical development of vaccines against HIV-1, malaria and tuberculosis.

Conclusions/Significance: We found that CAF01 induced cellular immune responses against HIV-1 minimal CTL epitopes in HLA-A*0201 transgenic mice to levels comparable with that of incomplete Freund’s adjuvant. A hydrophilic positively charged dimethylammonium head–group attached to two hydrophobic 18-carbon alkyl chains (tail). In an aqueous environment, DDA self-assemble into closed vesicular bilayers [liposomes]. The adjuvant efficacy and stability of the liposomes (DDA) is increased by incorporation of the synthetic glycolipid TDB (trehalose 6,6′-dibehenate) which is a synthetic analogue to the immune stimulatory component of the mycobacterial cell wall often referred to as the cord factor or trehalosedimycolate [5,7,8].

To induce additional CTL immunity during chronic HIV-1 infection by a therapeutic vaccination we have identified infrequently targeted but conserved HLA-A0201-restricted epitopes from Gag, Pol, Env, Vpu and Vif [9,10,11]. These relatively immune silent subdominant epitopes were modified as anchor-optimized peptides to improve immunogenicity for a vaccine. During development of our CTL inducing therapeutic HIV-1 vaccine in HLA-A*0201 transgenic mice, we have used the powerful standard incomplete Freund’s adjuvant (IFA) which has, however, limited usability in humans [9,10].

Here we show that CAF01 induced also functional CD8+ T cell immune responses against HIV-1 minimal CTL epitopes in HLA-A*0201 transgenic mice to levels comparable with or better than that of IFA.
Results

Novel adjuvant CAF01 helps inducing CTL similar to incomplete Freund's adjuvant

We compared the ability of the novel adjuvant CAF01 to induce T-cell responses in HLA-A*0201 transgenic HHD mice [12] using IFN-γ ELISPOT and 51Cr-release cytolytic assay after subcutaneous (s.c.) immunization with a subdominant 9-mer epitope Vif₁₀₁ (GLADQLIHL) [10] together with the CD4⁺ T-helper epitope, PADRE [13]. The novel adjuvant CAF01 was compared with incomplete Freund's adjuvants (IFA).

We found that both the HLA-A2-restricted CD8 T-cell epitope Vif₁₀₁ and the CD4 Th epitope PADRE induced high responses in IFN-γ ELISPOT in both the IFA and CAF01 groups (Figure 1A+B). After 5 days of in vitro peptide prestimulation of splenocytes significant responses to Vif₁₀₁ stimulation and PADRE stimulation (Figure 1A+B). The Vif₁₀₁ or the PADRE responses were not significantly different between the IFA and CAF01 groups (t-test, p>0.05).

Splenocytes stimulated 5 days in vitro with Vif₁₀₁ or the PADRE peptide were analyzed for the ability to lyse peptide-loaded HHD HLA-A*0201 target cells in a 51Cr-release cytolytic assay (Figure 1C+D). We found that in the groups of animals adjuvanted with IFA only one mouse significantly lysed Vif₁₀₁-loaded target cells (Figure 1C), whereas three out of five mice adjuvanted with CAF01 significantly lysed Vif₁₀₁ loaded target cells (Figure 1D). The CTL responses correlated with the ELISPOT responses in the sense that the highest responses in the IFN-γ ELISPOT were those which gave also responses in the 51Cr-release cytolytic assay (data not shown). Thus, CAF01 adjuvants supported CD8 T cell responses as good as or better than IFA.

Including CD4⁺ T-helper epitope increases CTL responses

In order to evaluate the effect of including a CD4⁺ T-helper epitope (PADRE) with the CAF01 adjuvants for the induction of a CD8⁺ T cell response towards a minimal subdominant CD8⁺ T cell epitope (Vif₁₀₁) we immunized HLA-A*0201 transgenic HHD mice subcutaneously (s.c.) with Vif₁₀₁ with or without PADRE. The novel adjuvant CAF01 was used in both immunizations. IFN-γ ELISPOT and a 51Cr-release cytolytic assay was used to evaluate the T cell responses after five days in vitro stimulation.

We found that when immunizing with the CD8 T cell epitope Vif₁₀₁ together with PADRE Th epitope a high IFN-γ (above 1000 SFU/million cells) towards Vif₁₀₁ was seen in four out of five animals (Figure 2A) whereas when immunizing with Vif₁₀₁ alone a similar response were seen in three out of five animals (Figure 2B).

Using 51Cr-release cytolytic assay (Figure 2C+D), we found that in the animals immunized with Vif₁₀₁ together with PADRE two

Figure 1. Cellular immune responses are induced to a similar level after immunization with IFA and CAF01. IFN-γ ELISPOT responses against (A) HLA-A2-restricted CD8 T cell epitope Vif₁₀₁ and (B) PADRE Th epitope of splenocytes from HLA-A*0201 transgenic HHD mice after 10 days of s.c. immunization with Vif₁₀₁ and PADRE in either IFA (grey bars, n = 5) or CAF01 adjuvants (black bars, n = 5) and 5 days in vitro prestimulation with individual epitopes. The background of an unimmunized mouse is subtracted. Specific 51Cr-release from target cells preloaded with Vif₁₀₁ after incubation with effector splenocytes from Vif₁₀₁ and PADRE immunized mice adjuvanted with (C) IFA (grey, n = 5) or (D) CAF01 (black, n = 5). The percentage of specific lysis was calculated as 100×(experimental release-spontaneous release)/(total release-spontaneous release). Background lysis from an unimmunized mouse is shown (open circles). Significant positive levels are considered for >10% lysis at a 50:1 ratio of effector:target (E:T) cells. One representative experiment out of three. SFU, spot forming units.

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out of five significantly lysed Vif101-loaded target cells (Figure 2C), whereas one out of five mice immunized with Vif101 alone lysed Vif101-loaded target cells (Figure 1D).

Thus, CD8+ T cell responses could be induced by the CTL epitope peptides using the CAF01 adjuvants but including a CD4+ T helper epitope improved the CTL response.

Immunization with novel adjuvant CAF01 induces multiple CD8+ T cell responses with proliferative capacity

Proliferative capacity is another desirable property of vaccine-induced T cell responses. We therefore evaluated CD8+ and CD4+ T cell proliferation using CFSE labeling and 5 days in vitro stimulation with individual epitopes. Here we used multiepitope immunization with a mixture of 7 HIV-1 derived HLA-A2-restricted CTL epitopes (Gag150, Gag433, Env67, Pol606, Vpu66, Vif101 and Vif23), plus two HIV-1 specific CD4+ T-helper epitopes (Gag298 and Env570) and one universal CD4+ T-helper epitope (PADRE).

We were able to detect also proliferative CD8+ T cells against the CD4+ T-helper epitope PADRE in 10 out of 10 immunized mice (Figure 3A+B). In addition up to three minimal CTL epitopes (Gag150, Gag433, Vif101) induced CD8+ T cell proliferation in the CAF01 group and up to two epitopes (Gag150, Gag433) in the IFA group. Moreover PADRE induced CD4+ T cell proliferation in 10 out of 10 immunized mice (data not shown).

Discussion

The new lipophilic adjuvant CAF01 has been found to assist in generation antibody and CD4+ T helper responses [5]. Here we show that CAF01 also generate functional CD8+ T-lymphocyte responses towards HIV-1 derived minimal CTL epitope peptides in HLA-A*0201 transgenic mice.

Immunological features of induced T cell responses were evaluated using three different assays: IFN-γ ELISPOT, 51Cr-release cytolytic assay and proliferation using CFSE labeling. The 51Cr-release cytolytic assay is recognized as a true functional immunological activity assay where effector splenocytes from the immunized animal are allowed to interact and lyse target cells preloaded with the peptide of interest. Also proliferative capacity is a wanted feature of vaccine induced CD8+ T cell-responses. Using all three assays we were able to detect CAF01 induced cellular immune responses to levels comparable with or better than that of incomplete Freund’s adjuvant. The level of CD8+ T cell responses in the ELISPOT assay can not directly be compared to the level of CD4+ T cell responses, as this mouse model contains a very low level of CD8+ T cells compared to CD4+ T cells [12].

Not many adjuvants are accessible for human use, and those, which can be used in laboratory animal experiments, are often incompatible with clinical trials in humans. CAF01 is a novel adjuvant which has an acceptable safety profile and proved successful in preclinical development of human vaccines against malaria and tuberculosis and are currently in clinical trials [4,5].
The adjuvantic properties of CAF01 for CD8+ T-cell in humans is planned to be tested in a therapeutic HIV-1 phase 1 trial using minimal CD8+ CTL and CD4+ T-cell epitope peptides, and may prove CAF01 as a very attractive adjuvant component in future therapeutic and prophylactic vaccine trials.

**Materials and Methods**

**Ethics statement**

Animal experiments were approved and done according to the Danish Animal Experimentation Act, based on the Council of Europe Convention ETS 123, on a license granted by the Ministry of Justice.

**HLA-A*0201 transgenic mice**

The HHD transgenic mice kindly provided by F.A. Lemonnier, Institut Pasteur, Paris, France, express a transgenic monochain histocompatibility class I molecule in which the C-terminus of the human β2-microglobulin (β2m) is covalently linked to the N-terminus of a chimeric heavy chain (HLA-A2.1 α1-α2, H-2D b α3-transmembrane and intracytoplasmic domains) [12]. HHD mice are homozygous for the transgene, and H-2D bα7/− and β2m−/− double knock out, respectively.

**Peptides**

Epitope peptides identified as relevant for HIV-1 vaccination [9,10,11] were synthesized by Schafer-N, Copenhagen, Denmark. The purity of the peptides was >95%. The CTL epitope peptides used in the present study were: Vif101(M9L) (GLADQLIHL), Gag150(T2L) (RLLNAWVKV), Gag433 (FLGKIWPS), Env67(V2I) (NIWATTHAC), Pol606 (T9V) (KLGKAGYVV), Vpu66(A9V) (ALVEMGGHV), and Vif23(V9) (SLVKHHMYV). As T-helper epitopes we used a Gag298 (KRWIILGLNKIVRMY) [14], Env570 (VGWIKQLOVRVLAVERYLK) [15], and the universal PADRE (aKXVAAWTLKAAa, X = cyclohexyl alanine, a = D-alanine) [13].

**HLA-A*0201 transgenic mice immunization**

Animal experiments were approved and performed in accordance with the legal requirements in Denmark. 6–7 weeks old HLA-A*0201 transgenic mice were injected subcutaneously with 100 μg of the HLA-A2-restricted minimal epitope Vif101 peptide plus 120 μg of the synthetic T-helper peptide PADRE or for multiepitope immunizations 20 μg of each of the 10 peptides. Peptides were adjuvanted either by incomplete Freund’s adjuvant (IFA) or 250 μg DDA: 50 μg TDB (CAF01). At day 10 after immunization, mice were sacrificed and the splenocytes were recovered and used for CFSE proliferation assay, or re-stimulated in vitro with the immunization-peptide, as previously described [9]. After 5 days, the cells were used for cytolitic 51Cr-release assay and IFN-γ ELISPOT assay.

**IFN-γ enzyme-linked immunospot (ELISPOT) assay**

A standard IFN-γ ELISPOT assay was used as previously described [9]. Splenocytes harvested at day 5 of re-stimulation were used in duplicates with a titration of cells ranging from 500,000 to 18,000. Restimulation was done with 10 μg of antigen/mL during 18 hours of incubation at 37°C, 5% CO₂. ConA (10 μg/mL) was included as positive control. Numbers of specific IFN-γ-secreting cells were measured in an ELISPOT reader (Autoimmun Diagnostika GmbH, Strassberg, Germany), analyzed with AiD3.1 S.R software and expressed as numbers of spot-forming units (sfu) per 10^6 input cells. The cut-off for positivity was 10 sfu (subtracted the negative background of an unimmunized mouse) per 10^6.
spleenocytes and at least twice greater than the negative control mean background activity of <3 cpm/10^6 input cells (spontaneous release) [9].

Cytotoxic T Lymphocyte assay

A standard cytotoxic 51Cr-release assay was used as previously described [9]. Briefly, HHD-EL4.S3.Rob target cells [12] were mixed with peptide loaded spleenocyte effector cells at effector:target cell ratios (E:T) of 100:1, 50:1, 25:1 and 12.5:1 in either triplicates or 6-replicates. 51Cr-release was measured using a microplate scintillation counter (Topcount, NXT, Packard, Boston, USA). Spontaneous and total 51Cr-release was measured by adding culture medium or a detergent (Triton X-100) 2% v/v, respectively. The percentage of specific lysis was calculated as

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\text{Percentage of specific lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} 
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CFSE proliferation assay

For measuring specific proliferation of mouse spleenocytes we used a standard CFSE proliferation assay in which spleenocytes were labeled with CFSE (5 μM) and stimulated with target peptide (20 μg/ml) and cultured in vitro for 6 days before labeling with anti-CD3-APC (BD Biosciences), anti-CD4-PE (BD Biosciences) and anti-CD8-PerCP (BD Biosciences). PHA (10 μg/ml) was included as positive control and media alone as negative control. Data were acquired on a BD LSRII instrument using FACSDiva software (BD Biosciences) and analyzed with FlowJo software (TreeStar). The percentage of proliferating cells (having reduced CFSE dye) was analyzed within CD3/CD4+ or CD3/CD8+ lymphocytes.

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Author Contributions

Conceived and designed the experiments: AF. Performed the experiments: GJG. Analyzed the data: GJG IK. Contributed reagents/materials/analysis tools: EMA PA. Wrote the paper: GJG IK AF.

References

1. Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MB (1994) Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. J Virol 68: 6103–6110.
2. Kougu RA, Safrit JT, Cao Y, Andrews CA, McLeod G, et al. (1994) Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J Virol 68: 4650–4653.
3. Jin X, Bauer DE, Tuttleton SE, Lewin S, Gettie A, et al. (1999) Dramatic rise in cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. J Virol 68: 6103–6110.
4. Davidsen J, Rosenkrands I, Agger EM, Andersen P, et al. (2005) A comparative study of cationic liposome and niosome-based adjuvant systems for protein subunit vaccines: characterisation, environmental scanning electron microscopy and immunisation studies in mice. J Pharm Pharmacol 58: 787–799.
5. Davidsen J, Rosenkrands I, Christensen D, Vangala A, Kirby D, et al. (2005) Characterization of cationic liposomes based on dimethylidioctadecylammonium and synthetic cord factor from M. tuberculosis (trehalose 6,6′-dibehenate)-a novel adjuvant inducing both strong CMI and antibody responses. Biochim Biophys Acta 1718: 22–31.
6. Lindenstrom T, Agger EM, Korsholm KS, Darrah PA, Aagaard C, et al. (2009) Tuberculosis subunit vaccination provides long-term protective immunity characterized by multifunctional CD4 memory T cells. J Immunol 182: 8047–8055.
7. Korsholm KS, Agger EM, Foged C, Christensen D, Dietrich J, et al. (2007) The adjuvant mechanism of cationic dimethylidioctadecylammonium liposomes. Immunology 121: 216–226.
8. Christensen D, Korsholm KS, Rosenkrands I, Lindenstrom T, Andersen P, et al. (2007) Cationic liposomes as vaccine adjuvants. Expert Rev Vaccines 6: 785–786.
9. Thorn M, Tang S, Therrien D, Kloverpris H, Vinner L, et al. (2007) Sequence conservation of subdominant HLA-A2-binding CTL epitopes in HIV-1 clinical isolates and CD8+ T-lymphocyte cross-recognition may explain the immune reaction in infected individuals. APMIS 115: 757–768.
10. Corbet S, Nielsen HV, Vinner L, Lauemoller S, Therrien D, et al. (2003) Optimization and immune recognition of multiple novel conserved HLA-A2, human immunodeficiency virus type 1-specific CTL epitopes. J Gen Virol 84: 2409–2421.
11. Kloverpris HN, Karlsson I, Bonde J, Thorn M, Vinner L, et al. (2009) Induction of Novel CD8+ T Cell Responses During Chronic Untreated HIV-1 Infection by Immunization with Subdominant CTL Epitopes. AIDs 23: 1329–1340.
12. Pascolo S, Bievra N, Ure JM, Smith AG, Lemonnier FA, et al. (1997) HLA-A2.1-restricted education and cytolytic activity of CD8(+) T lymphocytes from beta2 microglobulin (beta2m) HLA-A2.1 monochain transgenic H-2Db beta2m double knock out mice. J Exp Med 185: 2043–2051.
13. Alexander J, Sidney J, Southwood S, Ruppert J, Oseroff C, et al. (1994) Development of high potency universal DR-restricted helper epitopes by modification of high affinity DR-blocking peptides. Immunol 1: 751–761.
14. Wilson CC, Palmer B, Southwood S, Sidney J, Hargisimoto Y, et al. (2001) Characterization and antigenicity of broadly cross-reactive and conserved human immunodeficiency virus type 1-derived helper T-lymphocyte epitopes. J Virol 75: 4195–4207.
15. Malkota U, Holte S, Zhu T, Delpe E, Hantsberry C, et al. (2003) Early induction and maintenance of Env-specific T-helper cells following human immunodeficiency virus type 1 infection. J Virol 77: 2663–2674.