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HIV-1 Tat-Based Vaccines: An Overview and Perspectives in the Field of HIV/AIDS Vaccine Development

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The HIV epidemic continues to represent one of the major problems worldwide, particularly in the Asia and Sub-Saharan regions of the world, with social and economical devastating effects. Although antiretroviral drugs have had a dramatically beneficial impact on HIV-infected individuals that have access to treatment,

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it has had a negligible impact on the global epidemic. Hence, the inexorable spreading of the HIV pandemic and the increasing deaths from AIDS, especially in developing countries, underscore the urgency for an effective vaccine against HIV/AIDS. However, the generation of such a vaccine has turned out to be extremely challenging. Here we provide an overview on the rationale for the use of non-structural HIV proteins, such as the Tat protein, alone or in combination with other HIV early and late structural HIV antigens, as novel, promising preventative and therapeutic HIV/AIDS vaccine strategies.

Keywords HIV/AIDS, vaccine, HIV-1 Tat, preclinical development, clinical trials

UNAIDS/WHO estimates that almost 33 million people are living today with human immunodeficiency virus (HIV) infection and, notably, Sub-Saharan Africa and Asia remain the regions most devastated by human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) and are characterized by continuously increasing rates of infection (http://www.unaids.org).

Even though the development of antiviral drugs has proven to be effective on HIV-infected individuals who have access to therapy, it has had little impact on the global HIV epidemic [1] and, most of all, it is unlikely to become available for routine use in poor developing countries where infrastructures and medical and economic resources are limited or absent. In addition, drugs cannot eradicate the virus and its spreading among individuals [1]. Hence, the development of a vaccine against HIV infection represents the only realistic way to control the global expansion of the HIV pandemic, especially in the developing word [2–4], and the urgency of designing an effective, safe, inexpensive, and easily administrable vaccine to protect people from HIV/AIDS is an absolute priority. However, despite an enormous effort devoted to this goal by the scientific community, the development of an effective vaccine has turned out to be an enormous challenge and, after almost three decades since the discovery of the virus, an efficacious vaccine against HIV/AIDS is still not available.

Ever since 1983, when HIV was discovered to be the cause of AIDS, HIV vaccine approaches were designed based on the use of structural proteins, which constitute the envelope (Env) of HIV, with the rationale of generating neutralizing antibodies capable of blocking virus adsorption to the target cells and protecting from infection (sterilizing immunity) [2–5]. However, few have provided significant protective immunity in non human primate models [6–13] and, most importantly, results from clinical trials, including the first phase III trial (AIDSVAX carried by VacGen) based on a monomeric gp120 Env protein, have

been largely disappointing [14–16]. These failures may be ascribed to several reasons, including the fact that i) recombinant monomeric gp120 molecules are insufficient to create the correct antigen conformation for induction of neutralizing antibodies, which indeed recognize mostly conformational epitopes on native trimeric Env proteins, and ii) neutralization sensitive conserved epitopes, mainly located in the V3 region of Env [17], are masked and not accessible to antibodies in native Env proteins because of conformation and heavy glycosylation [18, 19]. Furthermore, HIV mutates rapidly, particularly in its structural genes, and changes its glycosilation profile leading to rapid in vivo evolution of intrapatient HIV variants [20, 21] rendering the virus a hard target not only for neutralizing antibodies, but also for cellular immunity [22–24]. The diversity displayed by HIV is further complicated by the fact that infection with one subtype of the virus does not inhibit subsequent infection with another virus even the same subtype [22, 25, 26], and extensive subtype recombination may occur [27, 28]. Thereby, the generation of escape mutants early during infection and the high genetic variability among different HIV clades helps the virus to survive and to be transmitted among individuals and accounts for the inefficiency of pharmacological therapies and vaccine strategies to eradicate HIV. Despite this scenario, evidence exists that a sterilizing immunity against different HIV virus strains could be achieved. In chimpanzee HIV Env-based vaccines and in macaques live attenuated simian immune deficiency virus (SIV) vaccines protected from homologous and heterologous challenge [29–33]. In addition, reduction of viral loads following mucosal challenge with SIV was reported in rhesus macaques primed with recombinant Adenovirures expressing HIV Env and boosted with the recombinant protein [10, 13, 34]. Passive immunization with anti-Env monoclonal neutralizing antibodies was also shown to be protective against infection, and some monoclonal antibodies, alone or in combinations, were able to neutralize primary HIV-1 strains of different clades in vitro [35–46]. Thereby, the generation of a cross-clade sterilizing immunity remains a priority for the scientific community and the final challenge is to find strategies for eliciting such crucial and cryptic neutralizing antibodies, long-lasting and capable of blocking HIV infection. Notably, recent evidence indicates that HIV-1 Env trimers of gp120 fused with the ectodomain of gp41 are superior to gp120 monomers in inducing antibodies directed against conformational epitopes [47, 48] and neutralizing antibodies against R5 and X4 HIV isolates [49, 50]. However, cross-clade and broadly neutralizing antibodies, mainly directed against conserved epitopes in the V3 region of Env [51, 52], are not accessible to neutralization in native Env

proteins because of Env conformation and high glycosilation [18, 19]. Despite this scenario, novel variants of trimeric Env from HIV-1 clades B and C capable of inducing broadly neutralizing antibodies against cryptic neutralization-sensitive epitopes were recently generated by a researcher at Novartis with a deletion in the V2 region (ΔV2) of Env facilitating access to the V3 region by neutralizing antibodies. Most of all, these novel Env variants induce broader neutralizing antibodies against primary isolates of HIV than their wild-type counterparts in preclinical models [49, 50, 53–59], as well as significantly contain infection in a monkey-challenge model with the CCR5-tropic SHIVSF162P4 virus [53, 54]. Hence, trimeric ΔV2 Env antigens of both B and C HIV-1 subtypes are presently under development [53–60] and, in particular, the subtype B Env-based vaccine candidate has already advanced to phase I clinical testing [55].

As evidence indicates that T helper and cytotoxic T-lymphocytes (CTL) cellular immunity plays an important role in controlling the acute phase of infection and disease progression even in the absence of neutralizing antibodies [4, 15], efforts of several groups were also devoted to generation of strong and broad T helper and cytotoxic T cell (CTL) responses against different HIV structural targets, such as Gag/Pol antigens, which are highly immunogenic in HIV infection and more conserved than the Env proteins [61, 62]. The main goal of these approaches is generation of CTLs against epitopes that are conserved among different HIV clades capable of limiting virus replication or aborting HIV infection, providing protection from disease progression, and reducing virus transmission to healthy individuals (non-sterilizing immunity) [recently reviewed in refs. 63–67]. These strategies based on recombinant proteins, DNA plasmids, or expression vectors have proven to be effective in controlling viremia and progression to AIDS in non-human primates [68–75]. Nevertheless, evidence indicates that the targeting of T-cell responses on immunodominant epitopes can facilitate the insurgence of viral mutants that escape the vaccine-induced immune control [76, 77]. In this scenario, recent approaches have been also attempted by combining multiple HIV antigens [(i.e., HIV-1 gag/pol/nef) in a replication competent adenovirus type 5 recombinant vector (rAd5)] with the rationale of generating strong antiviral cellular immune responses capable of containing infection and progression to AIDS. However, results from the first phase IIb STOC (a screening-test-of-concept efficacy study) STEP trial based on this rationale (HIV vaccine trial based on rAd5-gag/pol/nef) developed by Merck have been largely disappointing as no protection from primary infection and no control on plasma viremia have been

achieved in volunteers who become infected during the trial [3, 78–83, http://www.nature.com/nature/journal/v449/n7161/full/449390c.html, http://www.hvtn.org/science/step_buch.html]. Hence, basic questions regarding what constitutes an effective T cell response and how such responses could be elicited by vaccination are still open.

Alternative to these approaches, in recent years new immunization strategies capable of limiting virus replication, protecting from disease progression, and reducing virus transmission to healthy individuals have been pursued to fight HIV/AIDS. These strategies aim at targeting virus regulatory and accessory genes, in particular Tat, Rev, and Nef, which are considered ideal targets because they are the first HIV proteins expressed early after infection, even before provirus integration (Tat/Rev), are released in the extracellular environment (Tat and Nef), more conserved in their immunogenic epitopes among HIV clades and play essential and key roles in the virus life cycle and in the pathogenesis of AIDS. In addition, in HIV natural infection, humoral and cellular responses directed against Tat, Rev, and Nef correlate with the non-progression to AIDS [4, 84]. The aim of these approaches, which may be effective for both preventive and therapeutic vaccine strategies, is to provide an early control of HIV replication, leading to a containment or even abortion of early infection by the virus. Several pre-clinical and clinical studies based on HIV-1 and SIV regulatory antigens administered as recombinant proteins, DNA plasmids or expression vectors, have shown that they are safe and immunogenic in mice, monkeys, and in humans (HIV-1 negative or infected individuals) and effective in protecting from pathogenic challenge in monkeys (Tables 1 and 2). In particular, preventive and therapeutic phase I trials with a biologically active Tat protein administered by the parenteral route have been successfully completed in Italy and the data showed that the vaccine is safe and induces humoral and cellular immune responses in near all the vaccinees [84, 85, http://www.hiv1tat-vaccines.info/]. Based on the knowledge gained during these studies on HIV-1 infection and on virus-specific immune responses, consensus is growing on the idea that protective immunity, which is primarily intended as sterilizing-immunity but also as protection against disease onset in case of failure of protection against infection, likely requires the collective effect of broadly neutralizing antibodies, CD4+ and CD8+ T cell responses, and innate immunity [86–90]. Hence, current opinion is that vaccines combining early and structural viral products (combined vaccines) may be superior because they target multiple and key viral proteins [64–66, 91] and that the development of multi gene/multi antigen HIV-1 vaccines is necessary to achieve broad antiviral immunity [92–94].

TABLE I Results of Safety and Immunogenicity in Preclinical and Clinical Vaccination Studies with the HIV-1 Tat Protein or *tat* DNA

Model	Immunogen	Dose (schedule)	Adjuvant	Route	Toxic effects	Ab response	T-helper response	CTL response	References
Mice	HIV-1 Tat DNA	1–100 μg (1–3 times)	None	i.m., g.g. (i.d.), i.p., i.d.	None	+/−	+	ND	[223]
	HIV-1 Tat protein	5–50 μg (2 times)	Incomplete Freund's, RIBI	s.c.	None	+	ND	ND	[223]
	HIV-1 Tat DNA	5–10 μg (3 times)	None	i.m., i.d., i.n., intra tongue	None	+	ND	ND	[263]
	HIV-1 Tat DNA	20 μg	None	i.n.	None	+	+	ND	[264]
	HIV-1 Tat DNA (wild-type and mutants)	100 μg (6 times)	Bupivacaine	i.m.	None	+/−	+	ND	[264]
	HIV-1 Tat protein	10 μg (4 times)	LTR192G	i.n.	None	ND	ND	+	[149]
	HIV-1 Tat DNA in a self-replicating vector	1–150 μg (6 times)	None	g.g., i.m., i.d.	None	−	+	+	[129]
	HIV-1 Tat DNA	10–50 μg (twice) or 20–100 μg (twice)	None	i.m.	None	+	+	ND	[224]
	HIV-1 Tat protein	10 μg (4 times)	LTR72, Alum, Incomplete Freund's	i.n., s.c.	None	+	+	+	[148]
	HIV-1 Tat protein	10 μg (4 times)	None, MALP-2	i.n., i.d.	None	+	+	+	[184]

HIV-1 Tat protein	40 ng–10 μg (4 times)	Complete Freund's, Red blood cells	i.p.	None	+	+	+	[266]
HIV-1 Tat protein	0.5–2 μg (3 times)	None, anionic PMMA nanoparticles/microparticles, Alum	i.m.	None	+	+	+	[195, Caputo et al., submitted]
HIV-1 Tat DNA	1–30 μg (6 times)	Cationic block copolymers	i.m.	None	–	+/–	+	[200]
HIV-1 Tat DNA	1 μg (twice) + Tat protein boost in Alum	Cationic PMMA nanoparticles	i.m.	None	+	+	+	[146]
HIV-1 Tat protein	10 μg (3 times)	Alum	s.c.	None	+	+	ND	[196]
HIV-1 Tat protein	5 μg (twice)	Wax anionic nanoparticles, Alum, Lipid A	s.c.	None	+	+	IFNγ ELISA	[196]
HIV-1 Tat protein	0.2–5 μg (3 times)	Wax anionic nanoparticles, Alum, complete Freund's adjuvants, Lipid A	s.c.	None	+	ND	ND	[197]
HIV-1 Tat DNA (codon-optimized, and ubiquitin-tagged codon-optimized)	100 μg (4 times)	None	i.m.	None	ND	+	+	[151]
HIV-1 Tat protein	6–50 μg (4 times)	None, cholera toxin	Transcutaneous	None	+	+	IFNγ, IL6 ELISA	[140]

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TABLE I Results of Safety and Immunogenicity in Preclinical and Clinical Vaccination Studies with the HIV-1 Tat Protein or *tat* DNA *(Continued)*

Model	Immunogen	Dose (schedule)	Adjuvant	Route	Toxic effects	Ab response	T-helper response	CTL response	References
	HIV-1 Tat protein	10 μg (3 times)	None, cholera toxin, dsRNA motifis	i.n.	None	+	+	IFNγ, IL6 ELISA	[139]
	HIV-1 Tat protein (CyaA-E5-Tat fusion protein)	10 μg (3 times)	Adenylate cyclase (CyaA) from *Bordetella pertussis*	i.p, s.c., i.d.	None	+	ND	IFNγ, IL5 ELISA	[267]
	HIV-1 Tat DNA (wild-type, mutant)	10^8 pfu (once, twice)	Replication-competent Ad5	i.p.	None	+	+	IFNγ Elispot	[218]
	HIV-1 Tat protein	10 μg (4 times)	None, MALP-2	i.d., i.n., i.d./i.n.	None	+	+	+	[183]
	HIV-1 Tat DNA	4×10^4–4×10^6 pfu (3–4 times)	Replication-defective HSV-1 vector	s.c., i.n.	None	±	±	+	[268]
	HIV-1 Tat protein (mutant form "STLA")	10 μg (once)	Complete Freund's	s.c.	None	+	ND	ND	[137]
	HIV-1 Tat peptides (multipeptide-conjugate)	100 μg (3 times)	Complete Freund's, Incomplete Freund's	i.p.	None	+	ND	ND	[134]
	HIV-1 Tat protein (complexed with sulfate polysaccharides)	5 μg (twice)	None, Alum	s.c., i.p.	None	+	+	ND	[235]
	HIV-1 Tat DNA (center of tree sequence)	100 μg (3 times)	None	i.m.	None	ND	ND	IFNγ Elispot	[269]

	HIV-1 Tat DNA Tat-spinach transgenic plants)	1 g spinach leaves (~300 μg of Tat protein) (3 times)	None	Oral, oral/DNA boost g.g.	None	±	ND	ND	[188]
	HIV-1 Tat DNA (Tat-tomato transgenic plants)	5 μg	None	oral	None	+	ND	ND	[140]
Rabbits	HIV-1 Tat protein	100 μg (4 times)	Complete Freund's, Incomplete Freund's	i.d.	None	+	ND	ND	[152]
	HIV-1 Tat protein	100 μg (3 times)	Complete Freund's	i.d.	None	+	ND	ND	[123]
	HIV-1 Tat protein (Tat Oyi)	100 μg (4 times)	Montanide ISA 720, Aluminium hydroxide, Aluminium phosphate, calcium phospate	s.c.	None	+	ND	ND	[143]
	HIV-1 Tat protein (wild-type, mutant form "STLA")	50 μg (3 times)	PLA nanoparticles, MF59	s.c.	None	+	ND	ND	[198]
Monkeys	SIV Tat DNA in SFV and MVA viral vectors	SFV-tat (10^{8-9} $TCID_{50}$, 2 times) MVA-tat (10^{8-9} $TCID_{50}$, 2 times)	SFV, MVA	i.m.	None	ND	ND	+	[229]
	SIV Tat DNA in SFV and MVA viral vectors	SFV-tat (10^{8-9} $TCID_{50}$, 2 times) MVA-tat (10^{8-9} $TCID_{50}$, 2 times)	SFV, MVA	i.m.	None	+	+	+	[231]
	HIV-1 Tat DNA and protein	10 μg (4 times + 2 boosts with 50 μg of Tat protein)	Golden particles, RIBI	g.g.-i.d. (DNA), i.m. (protein)	None	+	+	ND	[250]

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TABLE I Results of Safety and Immunogenicity in Preclinical and Clinical Vaccination Studies with the HIV-1 Tat Protein or *tat* DNA *(Continued)*

Model	Immunogen	Dose (schedule)	Adjuvant	Route	Toxic effects	Ab response	T-helper response	CTL response	References
	HIV-1 Tat DNA and protein	6–10 μg (8 times + 1 boosts with 16 μg of Tat protein with ISCOMs)	RIBI, Alum	s.c., i.d.	None	+	+	+	[169]
	HIV-1 Tat DNA and protein	0.2–1 mg (8 times + 1 boost with 16 μg of Tat protein with ISCOMs)	Methylparaben + bupivacaine	i.d., i.m.	None	+	+	+	[171]
	HIV-1 Tat protein	10–40 μg (5 times)	Incomplete Freund's, polyphospazene	i.d., i.m.	None	+	+	ND	[177]
	HIV-1 Tat DNA and protein	1 mg (7 times + 2 boosts with 25 μg of Tat protein with ISCOMs)	None	i.m.	None	+	+	+	[Ensoli et al., unpublished]
	HIV-1 Tat protein	6 μg (11 times)	None	i.d.	None	+	+	+	[Ensoli et al., unpublished]
	HIV-1 Tat DNA in SFV and MVA viral vectors	SFV-tat (10^8 $TCID_{50}$) (twice), MVA-tat (10^8 $TCID_{50}$) (twice)	SFV, MVA	s.c., i.m.	None	–	ND	ND	[232]
	HIV-1 Nef-Tat fusion protein	20 μg (3 times)	AS02A	i.m.	None	+	ND	ND	[254]
	SIV Tat	32 μg (3 times) + 1 MVA boost (5 x 10^8 pfu)	Golden particles	g.g., i.d., i.r.	None	ND	+	+	[178]

HIV-1 Tat DNA	500 μg (3 times) + 2 boost with 25 μg of Tat protein with ISCOMs)	Methylparaben+ bupivacaine	i.m.	None	+	+	+	[220]
HIV-1 Tat peptides conjugated with diphtheria toxoid	100 μg (4 times)	Complete Freund's, Incomplete Freund's	i.m.	None	+	ND	ND	[176]
HIV-1 Tat peptides	50 μg (5 times)	Montanide ISA 720	i.m., i.n.	None	+	+/–	+/–	[133]
HIV-1 Tat DNA in Ad5	1×10^{11} pfu (3 times)	Replication-defective Ad5	i.m.	None	+	ND	+ (IFNγ Elispot)	[180]
HIV-1 Tat protein (CyaA-E5-Tat fusion protein)	250 μg (3 times)	None, Alum	i.m.	None	+	ND	+/– (IFNγ Elispot)	[136]
HIV-1 Tat protein (Tat Oyi)	100 μg (4 times)	Montanide ISA 720	s.c.	None	+	ND	ND	[143]
HIV-1 Tat DNA in Ad5 and Tat protein	5×10^8 pfu (4 times)/10 μg	Replication-competent Ad5 (prime)/Alum (boost)	i.n., i.t. (prime)/s.c. (protein boost)	None	+	+	+	[181]
HIV-1 Tat DNA in Ad5 and Tat protein	5×10^8 pfu (twice times)/10 μg Tat protein (twice)	Replication-competent Ad5(prime)/Alum (boost)	i.n., i.t. (prime)/s.c. (protein boost)	None	+	+	+	[182]

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TABLE I Results of Safety and Immunogenicity in Preclinical and Clinical Vaccination Studies with the HIV-1 Tat Protein or *tat* DNA *(Continued)*

Model	Immunogen	Dose (schedule)	Adjuvant	Route	Toxic effects	Ab response	T-helper response	CTL response	References
	HIV-1 Tat protein	16 μg of Tat protein (10 μg s.c. with Alum + 6 μg i.d.) (8 times) + 16 μg i.m. of Tat protein with ISCOMs (once)	Alum, ISCOM	s.c./i.d., i.m.	None	+	+	+	[182]
Humans	HIV-1 Tat DNA	100 μg (3 times)	None	i.m.	None	+/−	+	+	[225]
	HIV-1 Nef-Tat fusion protein	20 μg (3 times)	AS02A	i.m.	None	+	−	−	[256]
	HIV-1 Tat protein	7.5, 15, 30 μg (5 times)	None, Alum	i.d., s.c.	None	+	+	IFNγ, IL4 Elispot	[84, 85, Ensoli et al., unpublished results]

TABLE II Results of Efficacy in Preclinical Vaccination Studies with the HIV-1 Tat Protein or *tat* DNA

Model	Immunogen	Dose (schedule)	Adjuvant	Route	Antibody Response	Cellular responses		Challenge			Control of infection	References
						T-helper	CTL	Virus	Dose (MID_{50})	Route		
Monkeys	SIV Tat DNA in SFV and MVA vectors	SFV-tat (10^{8-9} $TCID_{50}$ 2 times); MVA-tat ($10^{8-9}TCID_{50}$ 2 times)	SFV, MVA	i.m.	ND	ND	+	SIV_{mac32H}	50	i.v.	YES	[229]
	SIV Tat DNA in SFV and MVA vectors	SFV-tat (10^{8-9} $TCID_{50}$ 2 times); MVA-tat (10^{8-9} $TCID_{50}$ 2 times)	SFV, MVA	i.m.	+	+	+	SIV_{mac32H}	50	i.v.	YES	[231]
	HIV-1 Tat DNA and protein	10 μg (4 times) + 2 boosts with 50 μg of Tat protein	Golden particles, RIBI	g.g. (i.d.)	+	+	ND	SHIV-4	10 (MID_{100})	i.v.	NO	[250]
	HIV-1 Tat protein	6–10 μg (8 times + 1 boost with 16 μg of Tat protein with ISCOMs)	RIBI, Alum	s.c., i.d.	+	+	+	$SHIV_{89.6P}$	10	i.v.	YES	[169]
	HIV-1 Tat DNA	0.2–1 μg (8 times + 1 boost with 16 μg of Tat protein with ISCOMs)	Methylparaben + bupivacaine	i.d., i.m.	+	+	+	$SHIV_{89.6P}$	10	i.v.	YES	[171]
	HIV-1 Tat protein (toxoid)	10–40 μg (5 times)	Incomplete Freund's, polyphosp-hazene	i.d., i.m.	+	+	ND	$SHIV_{89.6P}$	2500 $TCID_{50}$	i.r.	YES	[177]

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TABLE II Results of Efficacy in Preclinical Vaccination Studies with the HIV-1 Tat Protein or *tat* DNA *(Continued)*

Model	Immunogen	Dose (schedule)	Adjuvant	Route	Antibody Response	Cellular responses		Challenge			Control of infection	References
						T-helper	CTL	Virus	Dose (MID_{50})	Route		
	SIV Tat	32 μg (3 times) + 1 MVA boost (5×10^8 pfu)	Golden particles	g.g., i.d., i.r.	ND	+	+	SIV_{mac239}	3160 $TCID_{50}$	i.r.	NO	[178]
	HIV-1 Tat DNA	500 μg (3 times) + 2 boosts with 25 μg of Tat protein with ISCOMs	Methylparaben + bupivacaine	i.m.	+	+	+	$SHIV_{89.6P}$	50	i.v.	NO	[220]
	HIV-1 Tat DNA in SFV and MVA viral vectors	SFV-tat (10^8 $TCID_{50}$) (twice), MVA-tat (10^8 $TCID_{50}$) (twice)	SFV, MVA	s.c. i.m.	–	ND	ND	$SHIV_{BX08}$	10	i.r.	YES	[232]
	HIV-1 NEF-tat fusion protein	20 μg (3 times)	AS02A	i.m.	+	ND	ND	$SHIV_{89.6P}$	5–50	i.v.	NO	[254]
	HIV-1 Tat protein (wild-type, toxoid)	100 μg (4 times)	Incompelte Freund's	i.m.	+	+	+	$SHIV_{89.6P}$	30	i.v.	YES	[179]
	HIV-1 Tat peptides conjugated with diphtheria toxoid	100 μg (4 times)	Complete Freund's, Incomplete Freund's	i.m.	+	ND	ND	$SHIV_{33}$	50	i.v.	YES	[179]
	HIV-1 Tat peptides	50 μg (5 times)	Montanide ISA 720	i.m., i.n.	+	+/–	+/–	$SHIV_{BX08}$		i.r.	YES	[176]

HIV-1 Tat DNA in Ad5	1×10^{11} pfu (3 times)	Replication-defective Ad5	i.m.	+	ND	IFNγ Elispot	$SHIV_{89.6P}$	50	i.v.	NO	[180]
HIV-1 Tat protein (Tat Oyi)	100 μg (4 times)	Montanide ISA 720	s.c.	+	ND	ND	$SHIV_{BX08}$	337 +/- 331 AID_{50}	i.r.	YES	[143]
HIV-1 Tat DNA in Ad5 and Tat protein	5×10^{8} pfu (twice) + 2 boosts with 10 μg of Tat protein in Alum	Replication-competent Ad5, Alum	i.n., i.t. (prime)/ s.c. (protein boost)	+	+	+	$SHIV_{89.6P}$	30	i.v.	NO	[181]
HIV-1 Tat DNA in Ad5 and Tat protein,	5×10^{8} pfu (twice times)/10 μg Tat protein (twice)	Replication-competent Ad5(prime)/ Alum (boost)	i.n., i.t. (prime)/ s.c. (protein boost)	+	+	+	$SHIV_{89.6P}$	15	i.v.	YES	[182]
HIV-1 Tat protein	16 μg of Tat protein (10 μg s.c. with Alum + 6 μg i.d.) (8 times) + 16 μg i.m. of Tat protein with ISCOMs (once)	Alum, ISCOM	s.c./i.d., i.m.	+	+	+	$SHIV_{89.6P}$	15	i.v.	NO	[182]

Among the early proteins of HIV-1, the Tat protein is considered an interesting target for a combined vaccine approach as it plays a major role in HIV-1 replication and pathogenesis. Furthermore, the biologically active Tat protein displays immunomodulatory activities which can be favorably exploited for the development of novel combined subunit vaccines [95–99]. Hence, the Tat antigen is being tested in combination with other HIV structural and regulatory genes in preclinical models within the European AIDS Vaccine Integrated Project (AVIP) (http://www.avip-eu.org/) and the Mucosal Vaccines for Poverty Related Diseases (MUVAPRED) (http://www.mucosalimmunity.org/muvapred), which represent cooperative international frameworks aimed at developing an effective HIV/AIDS vaccine. In addition, the Tat antigen has been incorporated in an increasing number of combined vaccine formulations that are currently under clinical evaluation (http://www.iavi.org/trialsdb; http://www.hvtn.org/trials). These features of Tat and the advances in the development of vaccines based on Tat are reviewed below.

THE CHOICE OF TAT AS VACCINE RELEVANT ANTIGEN

The choice of Tat as an important antigen for the development of HIV/AIDS vaccine strategies essentially depends on the fact that it is a key player in HIV infection, as in the absence of Tat no infectious virus is made, and it plays an important role in AIDS pathogenesis extensively reviewed in refs. [4, 84, 100, 101]. Tat is a small polypeptide of 9–10 kDa molecular weight (and apparent molecular weight of 14 to 16 kDa in polyacrylamide-SDS gel electrophoresis), containing 86–106 amino acids (depending on the virus strain), which is encoded by 2 exons and translated from multiple-spliced 2 kb mRNAs. Tat is produced very early after infection, even before virus integration, and is essential for expression of viral genes, virus production, and cell-to-cell virus transmission. Despite its nuclear localization and transactivator function of HIV-1 gene expression, Tat is also released extracellularly by acutely infected CD4+ T lymphocytes, in the absence of cell death or cell permeability changes in vitro and in vivo. Depending on its concentration and on the target cells, extracellular Tat can be endocytosed by neighboring infected and uninfected cells where it translocates to the nucleus in an active form, enhancing replication of HIV-1, *trans* activating the replication of *tat*-defective or latent proviruses, and/or modulating the activity of cellular genes, including transcription factors, cytokines, and genes involved in cell-cycle regulation whose expression is critical for HIV replication, and thereby contributing to immune

dysregulation. Extracellular Tat also interacts with a variety of surface receptors, including integrin receptors and members of vascular endothelial growth factors family, and activates various intracellular transduction pathways. Evidence also indicates that Tat can activate the expression of a number of heterologous viral promoters, such as herpes viruses, HTLV-1 and -2, JC, and others, implying that Tat may be also involved in the development of AIDS-associated opportunistic infections. Finally, the Tat protein, both intracellular and extracellular, exerts key roles in the pathogenesis of AIDS-associated tumors [100, 102] and AIDS-associated neuropathogenesis [103]. The first exon of Tat encodes aminoacids 1–72, which include the N-terminal proline-rich (aa 1–20), the cysteine-rich (aa 21–37 containing 7 cysteines) and the core (aa 38–48) regions, representing Tat activation domain, and the basic region (aa 49–58) for nuclear localization and binding to the HIV LTR TAR RNAs. The C-terminal region of Tat, comprising the second exon, contains the arginine-glycine-aspartic acid (RGD), which is a motif present in extracellular matrix proteins [104–106]. The RGD and the basic domains are required for the interaction of extracellular Tat with cell surface molecules of the integrin family and with heparan-sulfate proteoglycans, respectively, and mediate uptake of Tat by dendritic cells (DC). Notably, the biologically active Tat protein is selectively and efficiently taken up and processed by monocyte-derived DC (MDDC), induces their maturation, and promotes their capacity of presenting antigens, eliciting immune responses of Th1-type profile, and increasing T cell responses to other antigens [95, 96]. These functions are exerted only by the monomeric biologically active Tat protein and are abolished or greatly hampered after oxidation/inactivation of the protein [95, 96]. Moreover, other experimental evidence indicates that the second exon of Tat plays a role in virus infectivity, expression, and replication in T cells and in monocyte-derived dendritic cells, mediates Tat uptake in brain cells is required for Tat-induced apoptosis and Tat and CD28 mediated immune hyper-activation of HIV-1 infected cells [107–118].

In addition to its key role in the virus life cycle and disease pathogenesis, Tat released by HIV-1 infected cells during natural infection induces Tat-specific antibody responses in approximately 20% of the infected individuals [for review see refs. 4, 63, 67, 84, 85] and 46% of anti-Tat seropositive individuals were reported when anti-Tat ELISA were carried out with Tat variants specific for the subtype infecting patients [119]. High antibody titers directed against a broad spectrum of Tat functional domains neutralize the effects of extracellular Tat on virus replication and cell functions and play an important role in

the control of the HIV infection and disease progression [105, 119–130]. Cross-sectional studies in 302 HIV-infected patients showed that anti-Tat antibodies are more frequent at the asymptomatic stage of infection compared with the symptomatic stage, whereas no differences were observed for antibodies directed against structural proteins [120]. Furthermore, a longitudinal study on the Italian cohort of 252 HIV-1 seroconverters indicates that the presence of anti-Tat antibodies strongly correlates with slower disease progression and no AIDS event occurred in persistent anti-Tat positive subjects [127]. In addition, evidence exists that natural IgM antibodies reacting with Tat may provide an early initial defense against the pathological effects of Tat after HIV infection and influence the course of AIDS progression [131, 132]. Similarly, immunization with Tat elicits antibody responses in rodents, monkeys, and humans able to block the activity of extracellular Tat on cellular entry, gene expression, and replication [4, 84, 133–142]. Moreover, strong anti-Tat antibody responses correlated with an efficient reduction in plasma viremia and long-term protection in Tat protein-vaccinated monkeys challenged intrarectally with an heterologous SHIV virus [143]. This evidence thereby suggests that the presence of anti-Tat antibodies may predict the clinical outcome and that the induction of humoral responses against Tat, either during the course of the natural infection or by vaccination, may contrast the progression to the disease. The predominant antibody responses to Tat have been mapped at amino acids 1–20 and 36–50, which are highly conserved among different HIV subtypes [120] and are key for Tat function and contain B [133, 144] and T cell [115, 145–151] epitopes. However, antibody responses against other functional domains of Tat are also detected during infection, including the cysteine, basic, and carboxy-terminal regions [120–122, 126, 129, 152, 153]. The carboxy-terminus is the most variable region of Tat but represents, however, an important epitope; indeed, recent results indicate that its high variability may account for the limitation of cross-recognition of Tat antibodies depending on Tat variants [119]. A large portion of the humoral response against Tat is against conformational epitopes whose detection requires a native, monomeric Tat protein [120, unpublished results]. This is demonstrated by the evidence that the majority of HIV-1 infected patients, or of Tat-immunized animals, seropositive for Tat have higher titers of conformational anti-Tat antibodies as compared with IgG titers directed against linear epitopes [120, Ensoli et al., unpublished results], and by data showing that non-homologous Tat proteins are cross-recognized by antibodies mainly directed against conformational epitopes [120, 143, 154].

During natural infection, Tat also induces antigen-specific CD4+ [85, 147, 155] and CD8+ T cell responses [145, 150, 156–158]. CTL responses to Tat are more frequent in patients controlling viremia [145, 159] and correlate with early virus containment both in humans [156, 160] and in monkeys [161, 162].

Another important feature of Tat rendering it a relevant vaccine antigen is the conservation of its immunogenic regions among the HIV-1 M group at the sequence, functional, and conformational level [120, 142, 151, 152, 154, 163]. Indeed, the Tat protein of 86 aa from a clade B lab-adapted HIV-1 virus (HTLV-IIIB strain, clone BH-10) isolated more than 20 years ago [164] is very well recognized by sera from African individuals infected with different virus clades, including clade C, which is responsible for more than half of new HIV-1 infections worldwide [165], with similar prevalence and epitope mapping titers of anti-Tat antibodies [120]. Similarly, Tat proteins from different viral clades are cross-recognized by sera from HIV-1 seropositive individuals [166, 167], and macaques immunized with a clade B recombinant Tat protein develop anti-Tat antibodies cross-reacting with HIV-1 Tat peptides from B and C clades [142].

PRECLINICAL STUDIES WITH HIV-1 TAT VACCINES

Systemic and mucosal immunization strategies are being evaluated in animal models for safety, immunogenicity, and efficacy. Different approaches were employed including the use of biologically active or inactivated Tat proteins, wild-type or mutated *tat* DNA, or vectored Tat (protein or DNA), synthetic Tat protein, or peptides, as summarized in Tables 1 and 2. In none of these studies were local or systemic toxic effects ever reported at the biochemical, haematological, or immunological levels (Table 1). Vaccination with Tat was shown to activate specific humoral and cellular (including CTLs) immune responses in rodents and monkeys, both after systemic and mucosal administration (Table 1). However, with respect to vaccine efficacy of Tat-based vaccines, controversial results have been reported in non-human primates by different groups (Table 2). Remarkably, vaccines based on the native biologically active Tat (86 aa) protein or plasmid DNA expressing *tat*-induced long-term protective immunity (up to two years) against a pathogenic intravenous challenge with homologous $SHIV_{89.6P}$ in cynomolgus monkeys, containing virus replication in peripheral blood and tissues and preventing the development of AIDS [168–172]. In these animals, long-term protection correlated with the presence of high and stable humoral and cellular (CD4 and CD8) responses against Tat [168, 172]. Similarly,

immunization with a synthetic vaccine based on a Tat protein variant of 101 aa (Tat Oyi), isolated from a highly exposed, persistently seronegative individual in Gabon [173] and characterized by mutations never found in other Tat variants [174] and inability to transactivate HIV LTR [175], induced significant and long-term control of plasma viremia in vaccinated rhesus macaques after heterologous intrarectal challenge with $SHIV_{BX08}$, and protection correlated with strong anti-Tat antibody responses [143]. Also, vaccination of rhesus macaques with short Tat peptides containing Tat B-cell epitopes induced anti-Tat antibodies that significantly reduced the plasma viremia after intravenous challenge with $SHIV_{33}$ and prevented CD4+ T cell decline during the chronic asymptomatic phase of infection [176]. In contrast to these results, in rhesus macaques Tat toxoid or native Tat proteins (86 aa) gave partial protection [177] or did not protect [178, 179] against the $SHIV_{89.6P}$ challenge administered intrarectally or intravenously. Similarly, vaccination of rhesus macaques with long Tat peptides conferred protection against intrarectal challenge with $SHIV_{BX08}$ in one out of seven animals [133]. Also, a replication-defective adenovirus (Ad)-type 5 (Ad5)-HIV *tat* vaccine (86 aa) failed to protect rhesus macaques against homologous $SHIV_{89.6P}$ challenge administered intravenously [180]. Finally, other studies in rhesus macaques showed that priming with a replication-competent Ad5-HIV *tat* vector and boosting with the Tat protein (86 aa) did not protect against intravenous challenge with homologous $SHIV_{89.6P}$ [181]. Although the reasons for these conflicting results are not known, it is likely that the different outcomes of infection may reflect species differences with regard to immunogenicity or host resistance factors; difference in vaccine characteristics; immunization protocol; timing and route of administration; delivery systems; and/or type, dose, and route of administration of the challenge virus. To this respect, a recent comparative study of the same vaccine regimens with biologically active Tat (86 aa) in cynomolgus and rhesus monkeys showed the association of identified MHC class I and class II haplotypes with control of plasma viremia after intravenous challenge of $SHIV_{89.6P}$ and suggests that the difference in the challenge dose may also play a role in different infection outcomes [182, Cafaro et al., in preparation].

As HIV-1 infection is primarily a sexually-transmitted disease and mucosal vaccination ensures high compliance and easy administration, the safety and immunogenicity of Tat as a mucosal vaccine has been also investigated in mice after intranasal immunization with the native Tat protein alone (86 aa) or associated with mucosal adjuvants, such as MALP2, *Escherichia coli* heat-labile enterotoxin (LT) and

LT-R72, a non-toxic mutant of LT [148, 149, 183, 184]. In these studies, Tat immunization was safe and elicited Tat-specific serum and secretory antibodies as well as cellular responses. Remarkably, as several features make plants optimal systems for the development of mucosal HIV vaccines, including low-cost of production, feasibility of scaling up, increased stability, and the lack of requirement of refrigeration [185, 186], three recent studies have shown the feasibility of generating Tat-transgenic plants to deliver the vaccine orally. In one case, Tat was expressed in potatoes but no immunogenicity studies were reported [187]. In a second study, Tat was expressed in spinach plants [188], although mice fed with these plants did not develop antigen-specific antibody responses unless boosted with *tat* DNA by a gene gun. In a third study, Tat-transgenic tomato plants expressing native Tat (86 aa) were generated and mice fed orally with the tomato extracts developed mucosal anti-Tat IgA responses and serum IgG, able to neutralize Tat transactivation activity in vitro [140]. Finally, oral immunization with *tat*, *rev*, and *nef* genes was also described in humans and shown to be safe and induce antigen-specific mucosal and systemic IgA and IgG and Th1-type cellular responses [189, 190].

CLINICAL STUDIES WITH A BIOLOGICALLY ACTIVE HIV-1 TAT PROTEIN VACCINE

As safety, immunogenicity, and efficacy data in preclinical models support the development of the concept of a Tat-based vaccine, phase I preventive and therapeutic trials with the recombinant biologically active HIV-1 Tat (86 aa) protein were conducted in parallel [84, 85, http://www.hiv1tat-vaccines.info/]. These studies were directed at evaluating the safety (primary end-point) and the immunogenicity (secondary end-point) profile of the recombinant HIV-1 Tat protein vaccine in healthy, immunologically competent adult volunteers without identifiable risk of HIV-1 infection (preventive protocol), and in HIV-1 infected adult volunteers with mild immune deficiency (Clinical category A according to CDC) characterized by CD4 + $\geq$400 cells/μl and levels of plasma viremia $\leq$50,000 copies/ml (therapeutic protocol). The preventive and therapeutic studies officially started in Italy on 02/06/2004 and 01/19/2004 (first subjects enrolled), respectively, and were both closed on 11/07/2007. Both studies were randomized, placebo-controlled, and double-blinded. Volunteers were randomized to one of the two treatment arms with a different route of administration and blinded to the dosage group. In arm A, the Tat protein was given subcutaneosly in association with alum adjuvant in three dosage groups (7.5 μg, 15 μg,

or 30 μg), at weeks 0, 4, 8, 12, 16, and one group of volunteers received alum plus saline solution as a placebo. In arm B, the Tat protein was administered intradermally without adjuvant in three dosage groups (7.5 μg, 15 μg, or 30 μg) at weeks 0, 4, 8, 12, 16, and one group of volunteers received saline solution as a placebo. Twenty volunteers were enrolled for the preventive protocol (10 for each arm), while 27 volunteers were enrolled for the therapeutic protocol (15 in arm A and 12 in arm B). Safety was evaluated by monitoring local and systemic adverse reactions during the course of the trial. Clinical evaluation of safety also included monitoring of hematological, biochemical, immunological, and virological parameters. Assessment of safety was performed at baseline and at several time points during the study [85]. The data collected during the treatment (24 weeks) and the follow-up (48 weeks) periods show that in both preventive and therapeutic protocols no significant alterations were observed clinically or at the laboratory analyses for all the haematological, biochemical, and immunological parameters tested. Local and systemic alterations were transient, below severity grade 3, and were not associated with the vaccine dosage. Thereby, the data indicate that the Tat protein vaccine is safe and well tolerated both locally and systematically. In addition, the Tat protein vaccine was immunogenic in both studies, preventive [Ensoli et al., submitted] and therapeutic [85]. In particular, in the therapeutic protocol, anti-Tat IgM were present in 17% of the subjects at baseline as a response to the natural infection. After vaccination, anti-Tat IgM were induced in 83% of the immunized individuals. Anti-Tat IgG was present in 11% of the subjects at baseline as a response to the natural infection and, after vaccination, were induced in all immunized individuals. Anti-Tat IgA, which were absent in all subjects at baseline, were induced in 61% of the immunized individuals. In the placebo groups, one subject out of seven (14%) was positive for IgM at baseline, and a transient induction (at 1 time point) of anti-Tat IgM was reported in one volunteer randomized in the placebo group. One subject out of seven (14%) was positive for IgG at baseline, and one subject out of seven (14%) was positive for IgA at baseline. However, no induction of anti-Tat IgG nor IgA was reported in the placebo groups. A cellular immune response against Tat (γIFN production and/or IL4 production and/or proliferation) was present at baseline as response to the natural infection in 83% of the individuals randomized in the groups to be treated with the Tat vaccine, and in 86% of the individuals randomized in the placebo groups. After immunization, anti-Tat cellular responses were observed in 100% of vaccinated individuals, while a decrease was observed in the placebo groups (57%). Remarkably, the analysis of the CD4+ T cell counts and

viral load (primary safety parameter) indicated the preservation of the levels of circulating CD4+ T cells and the absence of significant plasma viremia rebounds. A significant correlation was observed between the CD4+ T cell counts and the titers of anti-Tat IgA in both arms or IgG in arm B, whereas no significant correlation was reported between CD4+ T cell numbers and anti-Tat IgM. Based on these results showing full achievement of both primary and secondary endopoints in either protocols, the Tat vaccine has already advanced further clinical testing in a phase II therapeutic trial officially started in Italy in July 2008 (http://www.hiv1tat-vaccines.info/).

PRECLINICAL STUDIES OF HIV-1 TAT VACCINE FORMULATIONS INCLUDING NANO- AND MICROPARTICLES

A peculiar feature of the HIV-1 Tat protein is that it oxidizes very easily with air and light and is labile at room temperature due to the presence of seven cysteines in its sequence. Oxidation leads to protein multimerization, aggregation, and loss of the biological activity, which requires the native monomeric Tat protein [84, 95, 96, 191]. In addition, a fully biologically active Tat protein is required for the immunomodulatory effects of Tat, as illustrated in the following section, and for control of replication and block of disease progression in monkeys vaccinated with Tat and challenged with a pathogenic simian immunodeficiency virus [133, 143, 168, 169, 172, 177]. Hence, in a fashion similar to different vaccine antigens, the use of a biologically active Tat protein as a vaccine antigen represents a critical issue since the preservation of the native conformation permits the induction of an effective Th1-type cellular immune response, the induction of antibodies directed against conformational epitopes, and allows to retain its adjuvant properties. As Tat contains a basic domain rich in arginine and lysine, novel anionic biocompatible core-shell nano- and micro-spheres were developed consisting of a poly(methylmethacrylate) (PMMA) core and an expanded hydrophilic shell made of a functional poly(methacrylic acid-*st*-ethyl acrylate) copolymer, whose commercial name is Eudragit L100-55 [63, 192]. These particles are able to bind, deliver, and release the HIV-1 Tat protein, both in vitro and in vivo, while protecting it from oxidation and preserving its biological activity. As a result, prevention of oxidation and increased stability of the vaccine formulation was obtained. The stabilization effect of surface adsorption of Tat on these microspheres is likely due to the highly hydrophilic shell, which may accomodate native protein molecules, i.e., HIV-1 Tat monomers in the Eudragit chains, thus preventing protein

multimerization and loss of biological activity. Notably, Tat protein vaccine formulations, including these core-shell nano- and microparticles, were well-tolerated and not reactogenic in mice after multiple injections by systemic and mucosal routes and enhanced the anti-Tat humoral and cellular responses, including CTLs [192–195, Caputo et al., submitted]. In addition, one microsphere sample loaded with the HIV-1 Tat protein was further characterized in cynomolgus macaques, confirming the safety and vaccine potency of this delivery system and new vaccine formulation [Titti et al., manuscript in preparation]. Notably, the synthesis on a large scale of these microspheres is feasible, reproducible, easy, and inexpensive [Sparnacci et al., manuscript in preparation], and the synthetic and purification procedures allow the preparation of endotoxin-free nano- and micro-spheres to be used in vivo in agreement with the EMEA guidelines (ICH topic Q3C) hence, achieving an important task in view of future clinical development of this technology. Recently, other groups have also shown that the delivery of Tat protein by means of anionic wax nanoparticles enhances the cellular [196] and humoral responses against Tat in mice as compared to the use of alum adjuvant [197], while adsorption of a mutated Tat protein on poly(D,L-lactide) nanoparticles impacts the breath of serum and fecal antibody responses in rabbits [198]. Alongside these studies, novel cationic block copolymers [199, 200] and novel cationic PMMA core-shell nanoparticles [146] have also been produced to develop safe, efficacious, and easily administrable second generation *tat* DNA-based vaccines with increased shelf-life and stability, capable of triggering broad, efficacious, and long-lasting immune responses with a reduced amount of antigen and number of boosts in mice [194].

IMMUNOMODULATORY ACTIVITY OF HIV-1 TAT

The biologically active Tat protein is known to have several immunostimulatory effects, only partially characterized at molecular level, rendering it an attractive adjuvant for other antigens. Tat presents an RGD domain that through integrin receptors binding allows the protein to enter different types of cells, particularly moncyte-derived dendritic cells (MDDCs). The RGD-mediated entry in MDDCs occurs in a time-, dose-, and maturation-dependent fashion and is hampered by oxidation of the protein and loss of biological activity and by low temperatures. Moreover, the biologically active (but not oxidized) Tat protein induces maturation of MDDCs, as indicated by the dose-dependent increase of surface expression of MHC, CD40, CD80, and CD86 molecules, and of IL-12, TNFα, and β-chemokines (MIP-1α, MIP-1β, and RANTES)

production driving Th-1 type immune responses. Consistently, Tat increases the capability of MDDCs to present both allogeneic and recall antigens, hence potentiating T cell responses against heterologous antigens [95, 96]. In addition to the RGD domain, Tat contains in its sequence a short and highly basic region (aa 47–59) termed "protein transduction domain" (PTD), a feature shared with very few other proteins called "penetratins" [201, 202], which enables Tat- or PTD-conjugated proteins to efficiently enter the cells in an energy- and receptor-independent fashion [203, 204]. Thereby, the PTD peptide or the Tat protein have been largely used to deliver conjugated antigens to the MHC class I restricted-antigen presentation pathway, and to increase CTL responses to the conjugated antigen [187, 205–210]. It has also been suggested that the PTD domain itself, in addition to its antigen-delivery capability, exerts an adjuvant effect on the conjugated antigen, in a fashion similar to poly-L-arginine peptides [211], leading to increased expression of specific MHC-I/peptide complexes and activation of T cell responses [202]. In this context, it was demonstrated that a biologically active Tat protein (86 aa), intracellularly expressed or given extracellularly, modifies the catalytic subunit composition of the immunoproteasome in B and T cell cultures [98, 212]. In particular, Tat up-regulates the LMP7 and MECL1 subunits and down-modulates the LMP2 subunit. Down-regulation of the LMP2 subunit occurs at transcriptional level as Tat interferes with the formation of the STAT1-IRF-1 complex, inhibiting IRF-1 binding to the LMP2 promoter [212, 213]. The Tat-induced changes on the expression of the catalytic subunits correlate with the increase of all three major proteolytic activities of the proteasome and results in a more efficient generation and presentation of subdominant MHC-I binding CTL epitopes of heterologous antigens. Hence, Tat modifies the antigen processing and modulates the generation of CTL epitopes. This in vitro evidence suggested that Tat may function in vivo as both an antigen and a novel adjuvant with the capacity to increase T cell responses, particularly those that are subdominant. Indeed, it was shown that co-immunization of mice with ovalbumin (OVA) and Tat proteins induces CTL responses against subdominant and cryptic OVA-derived epitopes, which were not detected in mice vaccinated with OVA alone [97]. Similarly, mice vaccinated with the HIV-1 Gag, Env, or V2-deleted Env antigens in combination with the biologically active Tat showed Th-1 type and CTL responses directed to a larger number of T cell epitopes, as compared to mice vaccinated with Gag, Env, or V2-deleted Env proteins alone. Specifically, mice immunized with the Gag protein alone responded to 6 different Gag-derived T cell epitopes while mice immunized with Gag and Tat proteins responded to 11 different T cell epitopes, 5 more than those detected in

mice vaccinated with Gag alone. Similarly, mice vaccinated with Env or with V2-deleted Env in combination with Tat responded to 17 peptides, 12 more than mice vaccinated with the Env proteins alone. The effect was specific as Tat did not affect Th2-type responses to these structural HIV proteins [97, 214]. In contrast with these results, using a DNA vaccination approach Agwale and co-worker reported that vaccination with a bi-cistronic *gp120-tat* DNA diminished IFN-γ CD8+ T cell responses against the immunodominant HIV-1 gp120 Env peptide in mice compared to those induced by vaccination with DNA encoding gp120 alone and that the effect of Tat was abolished by a sequence deletion (aa 31–50) in the cys-core domain of Tat (2). Morevoer, using a DNA vaccination approach, Gupta and co-workers [215] reported that vaccination with *tat* DNA in combination with *env* DNA inhibited T cell responses against Env through Tat-mediated induction of IL10. At present, the reasons for these different results are not completely understood and require further investigation. One possible explanation for these different results may depend on the fact that in the studies [215, 216] T cell responses directed against Env were searched using only a few Env-derived peptides, which are not representative of the whole Env antigen, as opposite to our studies where sets of peptides representing the full-length Gag, Env, or with V2-deleted Env proteins were employed. Alternatively, differences in the vaccine formulation and immunization protocol may possibly account for these diverse results.

Also, Tat has adjuvant properties on the induction of humoral immune responses; this property has been mapped to the core region and does not depend on the presence of cysteins required for the trans-activating capacity of Tat. Indeed, a synthetic dimeric Tat constructs obtained by oxidation of two cysteine residues at position 34 demonstrated adjuvant properties comparable to that of the wild-type protein [99]. Hence, altogether this evidence suggests that Tat, and in particular the biologically active protein, may represent a useful tool to drive and increase Th-1 and CTL responses as well as antibody responses against heterologous antigens, and could therefore be considered an optimal co-antigen for the development of novel vaccination strategies against HIV/AIDS.

PRECLINICAL STUDIES WITH COMBINED VACCINES BASED ON HIV-1 TAT AND OTHER HIV ANTIGENS

Attractive strategies against HIV-1 are subunit-based vaccines targeting several viral components including stuctural and regulatory proteins. These vaccines are designed to broaden the antibody, CD4+ T

helper, and CTL responses and possibly target a greater number of HIV-1 strains. They may be suitable either for therapeutic and prophylactic use and capable even of inducing a sterilizing immunity that, until now, has not been possible to achieve.

Tat in Combination With HIV-1 Env and/or Gag Structural Antigens

Novel approaches with Tat combined with Gag and Env are under preclinical and clinical evaluation within the AVIP and MUVAPRED projects in Europe to develop vaccine strategies in which Tat-adjuvant and immunomodulatory activities are also exploited. These combined vaccines should be more efficacious because they may attack the virus on early and late gene products. In these studies, mice vaccinated subcutaneously with the HIV-1 Gag, Env, or V2-deleted Env protein antigens in combination with the biologically active Tat protein developed strong humoral and cellular (Th1- and Th2-type) immune responses against Tat and the co-administered antigen and broadened the Th1-type and CTL responses against Gag or Env antigens [97, 214]. In addition, these strategies were safe, immunogenic, and effective in containing $SHIV_{89.6P}$ replication in cynomolgus macaques [Ensoli et al., manuscript in preparation], and based on these preclinical results, a Tat/V2-deleted Env protein vaccine will soon undergo phase I clinical trial testing in Italy (http://www.hiv1tat-vaccines.info/).

Long lasting local and systemic humoral and cellular immune responses were also detected in mice immunized intranasally with the combination of HIV-1 Tat and Env, V2-deleted Env, or SIV Gag in the presence of the detoxified LTK63 mucosal adjuvant [217]. It was also reported that mice immunized intraperitoneally with replication-competent Ad5 recombinants expressing HIV-1 Tat together with recombinants expressing SIV Gag developed modest but significant enhancement of Gag-specific IFN-γ secreting cells and lymphoproliferative responses as compared to mice vaccinated with Gag alone [218]. Complete protection against a challenge with HIV-1/MuLV-infected murine cells was also described in mice immunized with plasmids encoding Env, Gag, and Tat with or without boosting with the corresponding proteins formulated in the MF59 adjuvant [219].

The combination of Tat with Gag and Env was also evaluated in non-human primates using a DNA prime/protein boost vaccine regimen. Vaccinated rhesus macaques developed strong antibody and cellular responses and were protected from CD4+ T cells decline and disease progression up to 62 weeks after challenge with $SHIV_{89.6P}$ [220].

Similarly, rhesus macaques primed intranasally and intratracheally with a replication-competent Ad5 virus expressing Tat and Env and boosted with the Tat protein subcutaneously and with the Env protein intramuscularly mounted strong antibody and cellular responses against Tat and Env, which correlated with better protection from intravenous $SHIV_{89.6P}$ challenge as compared to control monkeys [181]. Also, a DNA immunization regimen with SIV *gag/tat* in conjunction with antiretroviral therapy in SIV-infected macaques resulted in better viral containment and stable CD4+ T cell counts in 17 out of 20 animals. This effect was associated with vaccine-induced T cell responses which were greater in magnitude and broader in epitope specificity than those induced by viral infection alone [221]. Different findings were, however, reported when the effect of a Tat protein boost was evaluated in rhesus macaques vaccinated with ALVAC-based formulations expressing *gag-pol* or *gag-pol-env*, as in this setting the boost with the Tat protein did not further contribute to containment of viral replication and preservation of CD4+ T cells [222]. This result suggests that the timing of Tat inoculation with respect to the co-administered antigen is likely relevant for Tat to exert its immunomodulatory activity on the co-antigen, as at a later time from priming the development of an immunity against Tat that may become inefficient because of the Env and/or Gag dominancy.

These findings, together with the capability of Tat being presented in association with MHC class I molecules and to induce Th-1 type immune responses and CTLs, which correlated with protection in vaccinated monkeys, its immunomodulatory activity on dendritic cells and on other antigens, and the evidence that Th-1 responses and CTLs correlate with reduced viral loads and containment of infection also with Env-based vaccines, suggest that these Tat-based combined vaccines could support an effective and long-lasting antiviral immunity capable of controlling virus replication and blocking disease onset, and may be even capable of inducing a sterilizing immunity. The goal of prophylactic and therapeutic immunization may be closer based on these recent advancements in vaccine development and in the comprehension of Tat-immune system interactions.

Tat in Combination With HIV-1 Regulatory Antigens

The regulatory HIV antigens are abundantly expressed early in the viral life cycle and CTL recognition may bring about early killing of infected cells. The feasibility of a vaccine approach based on early HIV antigens was first demonstrated by the Wharen's group, which showed

that HIV-1 *tat*, *rev*, and *nef* genes (individually or combined) induce broad antibody and cellular immunity in mice [223, 224]. Furthermore, studies in HIV-infected patients have confirmed that DNA vaccination with either *tat*, *rev*, or *nef* is safe (no evidence of increased viral load) and induces B- and T-cell responses (including CD8+ CTLs) that were absent or low prior to immunization [225, 226], demonstrating the feasibility of genetic immunization to induce new immune responses in HIV-infected patients. More recently, vaccination with DNA encoding consensus Tat, Rev, and Nef antigens, derived from a clade C HIV-1 virus and devoided of undesired functional activities, was shown to induce strong CD8+ T cell responses in mice, and it was suggested that these genes hold great promise for novel HIV/AIDS vaccine strategies [227]. Notably, the combination of Tat together with Rev and Nef delivered by MVA vectors in a therapeutic approach of SIV-infected rhesus monkeys demonstrated a little benefit in a subset of vaccinated animals [228]. In this regard, co-immunization with Tat and Rev together had been already reported to protect macaques in a preventative approach [229]. In this study, cynomolgus monkeys were primed with recombinant Semiliki Forest virus (SFV) and boosted with recombinant modified virus Ankara (MVA), producing SIV_{mac32H} Rev and Tat. After the challenge with SIV_{mac32H} (pJ5) plasma, viremia could be detected transiently only during the first 4 weeks after infection, and no cell-associated viremia was found, whereas in control monkeys high levels of plasma viremia and productively infected cells were easily detectable. Control of virus infection correlated with the presence of antigen specific CTL responses in the vaccinated animals [230, 231]. Subsequently, the efficacy of an HIV-1 Tat or Rev/Tat DNA or SFV prime/MVA boost regimen was evaluated in rhesus macaques using the $SHIV_{BX08}$ model [232]. Plasma viremia was reduced more than 200-fold in 30% of vaccinated macaques, whatever the vaccine regimen they received, as compared to controls. Taken together these studies not only confirm the safety, immunogenicity, and efficacy of Tat-based vaccine approaches but also underscore the importance of the immune targeting of early proteins in controlling the primary infection.

Tat in Combination With Multiple HIV-1 Early and Late Antigens: Multiantigens and Multiclade Vaccines

Alternative attractive vaccine strategies against HIV-1 are represented by vaccine formulations containing multiple HIV antigens, including full-length structural and regulatory proteins as well as viral enzymes and auxiliary genes, and/or HIV multi-epitopes. The induction of

immune responses against multiple targets of the HIV proteome may indeed be more adequate for the generation of protective T cell responses in outbred populations with diverse MHC genes and may likely restrict opportunities for the selection of viral escape mutations. Within this frame several multi-component vaccines containing Tat have been tested in mice and in non-human primates [6, 93, 233–255 and http://hiv-web.lanl.gov/content/vaccine/home.html] using recombinant proteins, DNA, viral vectors, different adjuvants, vaccine doses, vaccination schedules, and routes of administration and are shown to be safe, highly immunogenic, and effective in control of SIV or SHIV infection in macaques as recently reviewed [4]. However, it is often difficult to evaluate the effect of Tat and/or of each single antigen included in these vaccine formulations as, in most of the reported studies, a comparative analysis of the vaccine's immunogenicity and efficacy, with and without Tat or each individual antigen, is not described. Notably, some of the approaches that have shown promising safety, immunogenicity, and efficacy results in preclinical models have advanced further clinical evaluation (http://www.iavireport.org/trialsdb). For instance, the efficacy of HIV-1 Tat in combination with Nef (as TatNef fusion protein) and gp120 (clade B) proteins was tested by researchers at GlaxoSmithKline Biologicals in genetically unselected rhesus macaques and shown to be important for gp120 vaccine efficacy against a challenge with a partially heterologous SHIV, as the proteins formulated in AS02A adjuvant conferred solid protection against the development of AIDS [254]. Similarly, a DNA prime with *tat*, *nef*, and *env* genes followed by protein boost conferred protection in mice against challenge with an HIV-1/MuLV challenge [251]. Also, more recently, the multi-component Tat/Nef/Env protein vaccine was shown to be safe and highly immunogenic in healthy HIV seronegative individuals [256]. Other studies have shown the safety, immunogenicity, and efficacy of a DNA prime with a *gag, pol, env, vpu tat,* and *rev* multi-gene vaccine followed by recombinant *gag* / *pol* fowlpox boost regimen in animal models, in particular for the generation of effective CD4+ and CD8+ T cell responses, as lower viral loads were observed after SIV_{mn229} challenge in macaques [257–259]. These vaccines administered via the same route and schedule of administration resulted, however, poorly immunogenic in humans [260]. In a fashion similar to other DNA vaccines boosted with other poxviruses [261], the researchers postulated that the discrepancy between the responses in animal models and humans likely depend on different dose-response curves in macaques and in humans, and thus, further trials with these constructs are required to determine the effect of higher dose of the vaccine in inducing strong immunity.

Alternative and promising strategies aimed at generating immune responses against multiple viral early and late antigens of different HIV clades (multigene and multiclade vaccines) have also been developed, such as the multi-HIV DNA vaccine (GTU®-MultiHIV vector) approach expressing non-structural and structural genes of consensus sequence of multiple HIV clades (A-C subtypes and F-H sybtypes) in the form of multi-HIV fusion protein. The GTU-multiHIV, multiclade strategy has proven to be safe, immunogenic, and effective after gene gun administration in a murine challenge model with subtype A and B HIV-1/MLV [245, 248]. Although safety and immunogenicity of GTU®-based vaccines was also demonstrated in phase I/II studies conducted both in seronegative and seropositive individuals vaccinated intradermally or intramuscularly, however, the results of these studies again indicate poor immunogenicity of these DNA vaccines in humans. In these studies, it is postulated that the discrepancy between the results in animal models and in humans may mainly depend on the less effective routes of vaccine delivery used in the clinical studies as compared to gene gun. Hence, new experiments are evaluating the performance of intradermal delivery combined to electroporation [245]. Another promising multigene, multiclade HIV-1 DNA vaccine approach based on intramuscular or intradermal co-injection of several DNA plasmids encoding either early and late HIV-1 genes of different clades, with or without recombinant granulocyte colony stimulating factor, has been described by other researchers [91, 93, 262]. These strategies have been shown to be safe, immunogenic, and effective in animal models [93, 247] and safe and highly immunogenic in humans especially when using a DNA prime/MVA boost regimen [262]. Altogether, the experimental evidence indicates that multigene and multiclade HIV strategies are promising and feasible in terms of safety and immunogenicity without significant antigen interference, and deserve further attention to improve the dose, route, and vaccination protocols for best effectiveness.

CONCLUSIONS AND PERSPECTIVES

Novel strategies have been developed that are aimed at blocking virus replication and disease onset in the absence of sterilizing immunity by including HIV regulatory genes, such as Tat, which is expressed very early after infection before provirus integration and is essential for virus replication and infectivity and provides also adjuvanticity for other antigens. A Tat vaccine based on the biologically active Tat protein alone is safe and highly immunogenic in seropositive and

seronegative individuals and may reduce HIV-1 replication and disease progression in seropositive patients, as suggested by the results of different cross-sectional and longitudinal studies. Based on these promising clinical data, the Tat-vaccine has recently entered a phase II clinical trial on HAART-treated subjects in Italy. In this scenario, novel modified Env immunogens have been also developed by researchers at Novartis that can induce cross-clade neutralizing antibodies. Since Tat targets and induces maturation of dendritic cells, has immunomodulatory activities, and drives Th-1 and CTL responses, immunization with Tat may drive or increase these immune responses also against other HIV antigens to support an effective, long-lasting and, hopefully, even sterilizing antiviral immunity as suggested by the results in non-human primates. At the present time, preclinical testing of Tat/ΔV2Env combined vaccines in rodents and non-human primates holds great promise as it indicates that the inclusion of both proteins may lead to a vaccine formulation with superior efficacy and which might even have the chance to fulfill the primary goal of the HIV/AIDS global vaccine strategy. Meanwhile, preparation for clinical trials is being actively pursued both in developed and developing countries and clinical testing of Tat and ΔV2Env as single antigens administered parentally has been completed. Hence, the Tat/ΔV2-Env combination will undergo phase I trials soon. Alternative and novel multi-antigen, multi-clade HIV vaccine strategies, including Tat and multiple regulatory and/or structural viral gene products, are also being successfully pursued and are now also in an advanced clinical stage of development. These strategies aim at generating broad and cross-clade immunity against multiple HIV antigens expressed during the early and late phase of infection, possibly restricting opportunities for the selection of viral escape mutations and targeting a greater number of HIV-1 strains. Mucosal delivery of Tat-based protein or DNA vaccine formulations has also proven to be safe, facilitates vaccine administration, and prime potent and persistent immune responses systemically and at mucosal sites. Moreover, the inclusion of appropriate delivery systems and immunomodulating factors in the vaccine formulation has proven to contribute to stability of the antigen(s), ease of transportation and administration, and increase the immunogenicity of the vaccine without adverse effects.

Nevertheless, efforts are further required to optimize the combination of vaccine, delivery, and immunization protocol in order to obtain optimal safety, immunogenicity, and efficacy profiles. The goal of prophylactic and therapeutic immunization may be closer based on these recent advancements in vaccine development.

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Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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