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In vivo immunogenicity of Tax 11-19 epitope in HLA-A2/DTR transgenic mice: implication for dendritic cell-based anti-HTLV-1 vaccine

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Abstract

Viral oncoprotein Tax plays key roles in transformation of human T-cell leukemia virus (HTLV-1)-infected T cells leading to adult T-cell leukemia (ATL), and is the key antigen recognized during HTLV-associated myelopathy (HAM). In HLA-A2+ asymptomatic carriers as well as ATL and HAM patients, Tax(11-19) epitope exhibits immunodominance. Here, we evaluate CD8 T-cell immune response against this epitope in the presence and absence of dendritic cells (DCs) given the recent encouraging observations made with Phase 1 DC-based vaccine trial for ATL. To facilitate these studies, we first generated an HLA-A2/DTR hybrid

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Conflict of interest

The authors declare that they have no competing interests.

Authors' contributions

DS, SM, TS, PJ, and ZKK contributed to the conception and design of the paper. DS and SM performed the experimental work with the direct supervisory assistance from PJ and ZKK. JDC assisted with MILLIPLEX magnetic bead assay. DS, SM, PJ, and ZKK drafted the manuscript. TS, SJ, BW, PJ, and ZKK thoroughly read and enriched the manuscript for important intellectual content and gave final approval of the version to be published. JDC and RP gave several inputs during the revision process and thoroughly reviewed the revised manuscript.

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mouse strain carrying the HLA-A2.1 and CD11c-DTR genes. We then studied CD8 T-cell immune response against Tax(11-19) epitope delivered in the absence or presence of Freund's adjuvant and/or DCs. Overall results demonstrate that naturally presented Tax epitope could initiate an antigen-specific CD8 T cell response in vivo but failed to do so upon DC depletion. Presence of adjuvant potentiated Tax(11-19)-specific response. Elevated serum IL-6 levels coincided with depletion of DCs whereas decreased TGF- β was associated with adjuvant use. Thus, Tax(11-19) epitope is a potential candidate for the DC-based anti-HTLV-1 vaccine and the newly hybrid mouse strain could be used for investigating DC involvement in human class-I-restricted immune responses.

Keywords

HTLV-1; Tax; dendritic cells; HLA-A2.1 transgenic mice; CD11c-DTR transgenic mice

Introduction

Human T-cell leukemia virus (HTLV-1) causes a spectrum of abnormalities, the most prominent being adult T-cell leukemia (ATL) and HTLV-1-associated myelopathy (HAM) [1]. Leukemic cells are usually monoclonal with respect to the integration of HTLV-1 provirus, which is the end result of progression from polyclonality during the course of T-cell transformation with the establishment of malignant cell lineages [2, 3]. A number of epitopes of Tax, the major HTLV-1 oncogenic transactivator protein, have been recognized by HTLV-1-specific CD8⁺ CTLs in infected individuals carrying HLA-A2, -A11, or -A24 [4–10]. In HLA-A2⁺ patients, the frequency of Tax-specific CTLs can be as high as 30% of all CD8⁺ T-cells in the peripheral blood [11] and even higher in CSF [4, 12, 13], with Tax(11-19) epitope being the immunodominant epitope in both asymptomatic carriers [14], ATL [7, 15], and HAM [4, 6, 12, 16–19]. During the development of ATL, general immune suppression has been reportedly due to dysfunction of Tax-specific CTLs [7, 20]. In addition, both IFN- γ production and proliferative capacity of tetramer-binding Tax-specific CD8⁺ T-cells are severely impaired in ATL patients [21], and a Tax-low or Tax-negative phenotype is associated with clonal proliferation of ATL cells [22, 23]. Consequently, it has been established that impairment of the Tax-specific response is critically involved in ATL induction and disease progression.¹

ATL is highly aggressive and malignant with the median survival time measured in months even with standard treatment such as chemotherapy, antiviral drugs, or interferon therapy [24]. Autologous Tax-specific CTL therapy for primary ATL has shown promise leading to prolongation of survival time [25, 26]. Very recently, promising results from phase I clinical trials against ATL using Tax peptide-pulsed DC-based vaccination have been reported at the 16th International Conference on Human Retrovirology, June 26–30, 2013, Montreal, Canada (Suehiro et al., abstract no. O1-2251). A significant reduction in proviral loads and size of lymph nodes with no severe side effects were observed in patients confirming a

¹**Abbreviations:** AC, asymptomatic carrier; ATL, adult T-cell leukemia; CFSE, 5(6)-carboxyfluorescein diacetate N-succinimidyl ester; DC, dendritic cell; DT, diphtheria toxin; DTR, diphtheria toxin receptor; HAM/TSP, HTLV-1-associated myelopathy/tropical spastic paraparesis; HTLV-1, human T-cell leukemia virus type 1; IFA, incomplete freund's adjuvant; THP, tetanus helper peptide.

therapeutic effect. Tax oligopeptide, particularly Tax(11-19), as a dominant CTL epitope can be harnessed to induce antitumor immunity, suggesting that it could be a suitable candidate for a DC-based vaccine against HTLV-1-induced ATL [27, 28]. Previously, we have shown that following exposure to Tax, murine and human DCs undergo activation and maturation, exhibit changes in activation markers, surface phenotype, and secretion of cytokines/chemokines, leading to allogenic and Tax-specific immune responses [29–33]. We concluded that both Tax protein and its epitope (11-19) are potent enough to drive efficient antigen-specific CTL responses in naïve PBLs from normal donors as well as in HLA-A2.1 transgenic mice [34]. Hence, the promise of ATL therapy lies in reinstating a patient's anti-HTLV-1 CTL response by developing DCs pulsed with the immunodominant epitope Tax(11-19) to facilitate recognition. In this respect, we [34] and others [35] have previously reported that Tax-pulsed DCs make highly potent immunostimulators; however, in vivo evidence has not been yet reported for the immunogenicity of the naturally identified HLA-A*0201-restricted epitope 11-19 and the impact of DCs in this process.

DC priming in early stages of HTLV-1 infection has also been shown to be important for controlling disease progression [36]. To prove this, we had previously used a CD11c-diphtheria toxin receptor (DTR) transgenic mouse model [37, 38] that permits conditional transient depletion of CD11c⁺ DCs. Infection of these mice was performed with chimeric HTLV-1 virus wherein the envelope gene of HTLV-1 was replaced with that of the ecotropic Moloney murine leukemia virus (Mo-MLV) for better fusion of envelope with murine cells [39] and better induction of humoral and cellular immune responses [40]. Upon both cell-free and cell-associated infection, we witnessed an increase and decrease in proviral load respectively, in both CD4⁺ and non CD4⁺ T-cell fractions and significant reduction in IFN- γ response in the CD8⁺ T cells. These results proved the importance of DCs in controlling cell-free virus and pointed toward the involvement of DCs in cell-associated infection. Here we wish to provide direct in vivo evidence for the importance of DCs with respect to in vivo immunogenicity of the HLA-A2-restricted Tax (11-19) epitope, which is immunodominant in both carriers of HTLV-1 and patients with the disease. To this end, C57BL/6-Tg (HLA-A2.1)1Enge/J male mice that express significant quantities of the human class I MHC Ag HLA-A2.1 were crossed with B6.FVB-Tg Itgax-DTR/EGFP 57Lan/J (CD11c-DTR) mice that express the simian DTR-enhanced green fluorescent protein (EGFP) under the control of the Itgax (or CD11c) promoter. The CD8⁺ T-cell immune response in the newly hybrid HLA-A2.1/DTR transgenic mice were tested for both the naïve and a restimulation responses against Tax(11-19) antigen delivered with tetanus helper peptide (THP) in the absence or presence of adjuvant IFA (incomplete Freund's adjuvant; used as one experimental approach for immunogen delivery). In vivo depletion of CD11c⁺ DCs abrogated CD8⁺ T-cell responses in the absence and presence of adjuvant, thereby implicating the initial priming of DCs with Tax(11-19) peptide in the successful generation of an efficient antigen-specific response. Depletion of DCs coincided with a much higher level of IL-6 in sera of mice that had not received IFA. This is the first report to our knowledge wherein a transgenic hybrid mouse model carrying HLA-A2.1 and DTR transgenes together was utilized for studying the CD8⁺ T-cell immune response directed against a dominant HTLV-1 Tax(11-19) antigen. This in vivo model system could also

facilitate future studies of DC-mediated HLA-A2-restricted antigen-specific immune responses.

Results

Generation of novel transgenic hybrid mice containing HLA-A2.1/DTR transgenes

Transgenic hybrid mice generated from an intercross between HLA-A2.1 and DTR transgenic mice were all healthy and viable. The presence of the HLA-A2.1 transgene (111 bp) was observed along with a control gene fragment (MGSCv37; Mouse Genome Sequencing Consortium for The *Mus musculus* strain C57BL/6J build 37) of 200 bp and the DTR gene was detected by amplifying a 625-bp gene fragment (Fig. 1A, upper panel). The HLA-A2.1 transgene was found in 100% of the F1 hybrid progeny and the DTR transgene was shown to be present in 49% of the hybrid progeny when 66 pups of the F1 generation were analyzed (52% in females and 46% in males) (Fig. 1A, lower panel). These results were expected given the homozygous nature of HLA2.1 mice and the hemizygous nature of CD11c-DTR mice. Only double-positive mice were utilized in subsequent experiments.

Depletion of CD11c⁺ DCs in HLA-A2.1/DTR mice by the administration of diphtheria toxin

The dose, timing, and route of DT administration were implemented as previously described [36]. In vivo depletion of conventional murine splenic DCs from hybrid mice were confirmed by assessing the frequency of CD8 α ⁺/CD11c⁺ cells before and after DT treatment (Fig. 1B). As expected, most of the splenic DC population was ablated within 24 h of DT injection and was reduced to an average of 1.3% as compared with 5.5% of total CD8⁺ splenocytes in the non-DT control group, as previously observed [36]. Similarly, the reduction in DC frequency slowly recovered by day 5 (data not shown), making it essential to complete the subsequent immunization studies within a 5-day interval. Since studies suggest the expression on CD11c on activated CD8 T cells [41, 42], we also determined the frequencies of CD8 α ⁺ T cells. It was found that DT administration did not affect either the frequency of CD8 α ⁺ T cells from which the CD11c⁺ cells were gated or CD4⁺ T cells (Supplementary Figure 1) which were also looked at.

Depletion of DCs abrogated the immunogenicity of Tax(11-19)epitope

In previous studies, we demonstrated the immunogenicity of Tax(11-19) epitope both in vitro and in vivo in line HHD II mice (expressing chimeric human and mouse HLA-A2.1 heavy chain linked to human 2-microglobulin) [34]. Here the impact of DC depletion on this process was examined in the newly hybrid strain. Levels of CFSE were first assessed on days 1 and 12 from splenocytes of control, nonimmunized mice stimulated in vitro with mitogen Con A (positive control), Tax(11-19) peptide, BMDCs, and BMDCs incubated with peptide. The twelve-day cultures were restimulated on day 5 to allow enough expansion of the anticipated low frequency of the antigen-specific cells. The average basal response of CD8⁺/CFSE^{lo} cells upon no stimulation in nonimmunized mice was 18.8%. Con A stimulation showed 34.2% proliferation whereas with Tax(11-19) it was 46.2%, with BMDCs 21%, and with BMDCs + Tax(11-19) 14% (Fig. 2A). Thereafter, in vitro recall response in non-depleted and DC-depleted mice were calculated in this manner (Fig. 2B) as indicated by percentage of division or proliferation of CD8⁺ T cells. CD8⁺ splenocytes from

non-DC-depleted immunized mice proliferated in response to Con A, as was observed with control mice, whereas those from DC-depleted mice exhibited a significantly reduced response. Stimulation with Tax peptide was also reduced significantly in the absence of DCs. Interestingly, stimulation of splenocytes with autologous BMDCs in the absence or presence of Tax peptide from non-DC-depleted mice exhibited a high degree of proliferation that was significantly hampered in cells from DC-depleted mice in both cases, which could be a combined effect of lack of splenic DCs as well as poor *in vivo* priming. There is a certain degree of proliferation that is still detected from DC-depleted mice, which could be attributed to residual DCs' presence (~1%, Figure 1B). It is noteworthy that stimulation with BMDCs + Tax(11-19) peptide showed higher proliferation of splenocytes when compared with BMDCs alone. Therefore, we sought to compare the ability of antigen-specific cells to respond in co-culture conditions in non-DC-depleted and depleted groups.

Extent of Tax(11-19)-specific CD8⁺ T-cell response among total observed responses and the impact of DC depletion

Once it was clear that immunization with Tax(11-19) peptide led to an efficient *in vivo* priming in HLA-A2.1/DTR mice, the specificity of this response was determined using PE-conjugated Tax(11-19) pentamer that has been well characterized in our previous studies [36]. In addition, pentamer staining was used on control samples to validate the specificity as well as gating for the antigen-specific T-cells. The percentage of cells positive for Tax pentamer staining was obtained for each mouse by gating on total CD8⁺ T-cells that showed low levels of CFSE at 24 h after co-culture (Fig. 3A). In non-DC-depleted mice, the frequency of pentamer-positive cells was 16%–66% out of the total CD8⁺ splenocytes. In DC-depleted mice, the frequency of antigen-specific cells was much lower (8%–42%). On day 12 of co-culture, antigen-specific CD8⁺ splenocytes were analyzed for their percentage of proliferation (Fig. 3A). In non-DC-depleted mice, 58%–100% of Tax(11-19)-specific CD8⁺ T-cells divided in co-cultures (% division) containing Tax peptide-loaded BMDCs (Fig. 3A & B). As expected, administration of DT abrogated proliferation, and the resulting CFSE dilution into daughter CD8⁺ T-cells was reduced to 13%–53% ($P < 0.05$) of Tax(11-19)-specific CD8⁺ T-cells (Fig. 3A & B).

Adjuvant enhances immunogenicity of Tax(11-19) peptide via a DC-mediated mechanism

IFA is a well-known adjuvant for enhancing innate immunity [43], inducing cytokine expression in regional lymph nodes [44], and opening the blood-brain barrier [45]. We checked immunogenic response of splenocytes to Tax(11-19) peptide in the presence and absence of IFA via the cytotoxic activity (Granzyme B), and pro-inflammatory cytokine secretion capacity (TNF α) (Supplementary Figure 2) in BMDC + Tax peptide co-culture. Interestingly, while TNF α was increased in both IFA and non-IFA groups, Granzyme B was detected in significantly higher levels in the IFA group only. We proceeded to determine the ability of this adjuvant to increase the CD8⁺ T cell responses of Tax antigen injected in HLA-A2.1/DTR transgenic hybrid mice. The impact of IFA administration in non-DC-depleted mice was examined for the frequency of Tax(11-19) pentamer-positive CD8⁺ T-cells in the presence of IFA (49%–67%) (Fig. 4A) in the BMDC + Tax peptide co-culture. Out of these Tax(11-19) pentamer-positive cells, 36%–98% underwent proliferation as shown by the percent division (% division) of CFSE-expressing cells. The percent frequency

of Tax(11-19)-specific CD8⁺ T-cells was indeed mediated by DCs, as percent division by decreased to 2%–53% (Fig. 4A & B) in the DC-depleted group.

To compare the effect of IFA versus non-IFA, the degree of proliferation [34] of total CD8⁺ T-cells was examined. Number of generations was obtained by dividing the geometric mean fluorescence intensity (GMFI) of CFSE of the Day-0 peak by the GMFI of CFSE of each progressive peak so that the number of generations (n) = Day 0 GMFI/Day 12 GMFI. The mean number of generations on day 12 in response to BMDCs with the Tax peptide was 23.40 ± 4.297 ($N=5$, Fig. 5B) in mice that received IFA, in contrast to the high number of generations (165.1 ± 69.02) in non-IFA mice ($N=5$, Fig. 5A). This was also true for splenocytes co-cultured with BMDCs alone (13.86 ± 1.836 , $N=5$, versus 98.90 ± 33.97 , $N=5$), whereas the controls, Con A and Tax peptide, did not show many differences in the absence or presence of IFA. DC depletion primarily affected the proliferation of total CD8⁺ T-cells stimulated with BMDCs + Tax peptide in the absence and presence of IFA, but this was not significant as responses were highly variable within the non-DC-depleted groups (Fig. 5B). Furthermore, total CD8⁺ T-cells showed significantly higher proliferation in the BMDC + Tax(11-19) peptide co-culture versus the BMDC co-culture in non-DC-depleted mice in the absence or presence of IFA. Overall, although antigen specificity was higher, proliferation was reduced upon adjuvant administration.

Depletion of DCs leads to increased levels of serum IL-6

The effect of DC depletion on the serum cytokine profile was determined by multiplexed cytokine analyses performed in immunized mice in the absence and presence of IFA. Shown in Figure 6A is a summary of the cumulative levels of all cytokines from serum pooled from five mice that were PBS-injected, Tax(11-19)-immunized, and Tax(11-19) + IFA-immunized. Out of the cytokines detected (Fig. 6B), IL-6, the Th17-associated cytokine, was strikingly high in animals that were DC-depleted. It has been shown previously that IL-6 signaling prevents the conversion of conventional T-cells into Foxp3-positive Tregs *in vivo* [46]. Such high levels of serum IL-6 could be secreted to control the number of Tregs in the absence of DCs. Similarly, IL-12, the Th1 cytokine, was moderately increased in DC-depleted mice versus nondepleted-mice in both nonadjuvant (0.8 pg/ml versus undetected) and adjuvant (0.6 pg/ml versus 0.2 pg/ml) groups, supporting the possibility that the presence of DCs is important to maintain tolerance during priming of T-cells.

A decrease of about two-fold in TGF- β levels was observed in the adjuvant group as compared with the nonadjuvant group in both the absence (8.8 versus 19 pg/ml) and presence (10 versus 17 pg/ml) of DCs. TGF- β is known to be involved in maintenance of tolerance in the periphery [47–49]. The reduction in the level of this cytokine in IFA-treated mice may be associated with inducing a more robust frequency of Tax-specific T-cells, as observed in Figure 5. Another cytokine detected was IL-17A, a Th17 cytokine, which was induced (from an undetectable level to 0.8 pg/ml) upon adjuvant administration in the presence of DCs.

Discussion

The role of DCs with respect to HTLV-1 pathogenesis in general and the Tax-specific CTL response in particular have been previously characterized [34]. Based on available clinical data, the majority of the class I epitopes identified has been predicted to belong to either the HLA-A2 or HLA-A24 haplotype [7][50]. Most HTLV-1-infected subjects carrying the HLA-A2 haplotype were found to be either A*0201 or A*0206, and nearly all HLA-A24 subjects were A*2402 [50, 51]. Consequently, we proceeded to determine whether immunization with Tax(11-19), the HLA-A2 restricted immunodominant epitope, was DC-dependent in vivo and whether Tax(11-19)-specific CTL expansion could be facilitated using autologous DCs upon restimulation with Tax antigen.

A DTR-based transgenic system that allowed the inducible in vivo ablation of DCs was thus used [38]. Furthermore, because the available CD11c-DTR mice are unable to present HLA-A2.1-specific antigens, they were crossbred with HLA-A2.1-transgenic mice. The homozygous mice carrying the HLA-A2.1 transgene (obtained from hemizygous mating) were crossbred with homozygous mice carrying the DTR transgene (obtained from a hemizygous $\times$ inbred mating). Hence, all F1 progeny showed HLA-A2.1 transgene expression, about half of which (49%) also contained the DTR transgene (Fig. 1A). Subsequently, a colony of mice positive for both HLA-A2.1 and DTR transgenes was maintained with depletion of splenic DCs achieved using a previously defined dose of DT [36] (Fig. 1B). A successful recall response to Tax(11-19) was observed in terms of the in vitro proliferation of CD8⁺ T-cells in control non-DC-depleted mice, whereas depletion of DCs exhibited poor or no response (Fig. 2). The observed response was specific to the antigen examined and was enhanced by the presence of adjuvant (IFA) but required the presence of DCs in vivo (Fig. 3 and 4). However, the degree of proliferation was lower in the adjuvant group as compared with the nonadjuvant group (Fig. 5). Such decreases have been reported previously under certain circumstances upon administration of IFA indicating that it induces a more controlled antigen-specific induction of disease [52–59].

These results suggest that effective DC-dependent immunotherapy can be generated by immunization with the immunodominant Tax(11-19) antigen along with adjuvant in HTLV-1-infected patients. Other epitopes including Tax [185–193], Envgp46 [247–255, 247–256, 247–257, 249–257, and 257–265] and Pol [177–185 and 177–187], restricted to HLA-B35, are also known to be recognized by HTLV-specific CTLs in vivo [60]. Epitopes associated with HLA-A3 and HLA-B14 could also be of interest while considering anti-ATL DC therapy [61]. Reports summarizing the panel of HLA-A2.1-Tax(11-19) reactive T-cell clones indicate that small allelic variations of MHC molecules could alter the functional outcome of antigen recognition [62]. In HAM/TSP, the present findings indicate that MHC class I-restricted CTLs reduce the proviral load of HTLV-1 and consequently increase the risk of HAM/TSP [63]. The DRB1*0101 HLA allele predisposes an individual to HAM/TSP in the absence of A*0201, which would usually play a protective role in the CD8⁺-mediated immune response. Therefore, the ability to engineer DC-based vaccine therapies against appropriate antigen-specific response may enable us to overcome this dysfunction thereby enhancing immunogenicity or tolerance in ATL and HAM/TSP patients, respectively.

Conclusion

Our goal for elucidating the importance of DCs in presentation of Tax to naïve CD8⁺ T-cells as the basis for induction of a Tax-specific CTL response continues as we report here that the clinically characterized epitope (11-19) of Tax can initiate a CD8⁺ T cell-mediated response in vivo but failed to do so upon DC depletion, showing the importance of Tax(11-19) in priming of DCs against HTLV-1 infection. Thus, these studies have provided data that support the development of a novel candidate vaccine directed against HTLV-1 infection and a transgenic hybrid mouse strain that may be useful in other investigations into human immune responses.

Materials and Methods

Generation of transgenic hybrid mice and genotyping

Male C57BL/6-Tg(HLA-A2.1)1Enge/J mice carrying the HLA-A2.1 transgene in spleen, BM, and thymus [64] and age-matched female B6.FVB-Tg Itgax-DTR/EGFP 57Lan/J (CD11c-DTR) mice carrying the DTR-EGFP transgene under the control of the CD11c promoter [38] were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred as recommended by the supplier. The animals were maintained in the housing facility of Lampire Biological Laboratories (Ottsville, PA). All animal experiments were conducted under the protocol approved by the Institutional Animal Care and Use Committee (IACUC) of both Lampire Biological Laboratories and Drexel University College of Medicine. We obtained informed consent from the supplier to cross-breed the two strains. The pups were genotyped by PCR using DNA obtained from tail clippings as previously described by The Jackson Laboratory. Specific primer sequences (Integrated Device Technology, San Jose, CA) used for genotyping included an internal control MGSCv37: F/R (5'-3') CAAATGTTGCTTGTCTGGTG/GTCAGTCGAGTGCACGTTT, HLA-A2.1: TTCTCCCTCT CCCAACCTATGTAG/CGACGACACTGATTGGCTTCT, and DTR: GGGACCATGAA GCTGCTGCG/TCAGTGGGAATTAGTCATGCC. For all experiments double-positive mice carrying both the HLA-A2.1 and DTR genes were utilized.

Confirmation of DC depletion in HLA-A2.1/DTR transgenic mice

DT was purchased from Sigma-Aldrich (St. Louis, MO) and DC depletion was confirmed in newly hybrid mice as previously described [36]. Six- to 10-week-old mice (M:F ratio 2:3) were given (i.p.) either PBS as control or a uniform dose of DT (a total of 100 ng per animal or ~5 ng/g body weight of mouse). Spleens were harvested after 24 h from control (–DT) and DT-administered (+DT) groups and stained with FITC-conjugated anti-CD4 marker, PE-conjugated mouse anti-CD11c and APC-conjugated mouse anti-CD8α. A minimum of 50,000 events were acquired using a FACSCalibur (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (Tree Star, Ashland, OR). Live cells were gated to include conventional murine splenic DCs (CD11c⁺/CD8α⁺) and plotted as double-positive cells for five mice from each group. The same number of mice was used in all subsequent studies.

Immunization of HLA-A2.1/DTR transgenic mice

Twenty-four hours prior to immunization, animals were injected with either PBS or DT as described above. In vivo priming was performed by immunizing mice with 100 µg of synthetic 11-19 peptide (LLFGYPVYV, Alpha Diagnostics, San Antonio, TX) in conjunction with 50 µg THP (LQTMVKLFNRIKNNVA) in a total volume of 100 µl through both i.d. (50 µl) and s.c. (50 µl) routes. In adjuvant studies, Tax peptide was emulsified in IFA (Sigma). Within 5 days, both spleen and bones were harvested and processed as previously described [36] from each mouse. Cells were cultured for 8 days with 20% murine Fms-like tyrosine kinase 3 ligand-containing supernatant obtained from an SP2/0 transfected cell line [65]. BMDCs were harvested and examined for purity using a PE-conjugated anti-CD11c antibody (eBioscience, San Diego, CA) using flow cytometry.

Effect of DC depletion on the in vivo immunogenicity of Tax(11-19)epitope

BMDCs were used alone or stimulated with Tax peptide (100 nM) for 1 h at 37°C and co-cultured (1:1) with corresponding splenocytes labeled with CFSE (Sigma). On day 5, the co-culture was supplied with restimulated autologous BMDCs. Controls were set up in parallel by directly stimulating labeled splenocytes with Con A, 1 µg (Sigma), or Tax peptide alone. All cultures were harvested on days 0, 1, and 12 and stained with APC-conjugated anti-mouse CD8 antibody (eBioscience). Supernatant was collected from co-cultures at Day 12 and cytokine secretion was measured using the MILLIPLEX magnetic bead assay according to manufacturer's instructions (Millipore, Billerica, MA). Proliferation of total CD8⁺ T-cells was determined by the level of CFSE dilution. Percentage of division (% Division) was obtained by using the "Proliferation" function using the FlowJo 8.8.6 software. A baseline of undivided cell population for each experimental sample was set to calculate this proliferation function. This baseline is indicated in the histograms as gates for the relevant figures. To examine an antigen-specific immune response, cells were stained with PE-conjugated Tax(11-19) pentamer (ProImmune, Sarasota, FL) and analyzed using flow cytometry as described above. Where necessary, the number of generations was calculated [34] on the basis of reduction in the CFSE fluorescence by using the formula: number of generation (n) = geometric mean fluorescence intensity at day 0/geometric mean fluorescence intensity at day 12.

Detection of serum cytokines

Serum samples from each group were analyzed for cytokines using the mouse Multi-Analyte ELISA Array procedure as described by the manufacturer (Qiagen, Germantown, MD). This assay included Th1 cytokines IL-2, IL-10, IL-12, IFN-γ, and TNF-α; Th2 cytokines IL-2, IL-4, IL-5, IL-10, and IL-13; and Th17 cytokines IL-6, IL-17A, IL-23, TNF-α, and TGF-β. Undiluted serum (50 µl) from each sample or standard was added and incubated for 2 h at room temperature. Plates were washed and incubated with detection antibody followed by avidin-HRP solution, and absorbance was read at 450 nm using the Rainbow Microplate reader (Tecan Group, Ltd., Männedorf, Switzerland). Cytokine concentrations were determined from the respective standard curve and represented as pg/ml.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- HLA-A2/DTR transgenic mouse strain was generated to study immunogenicity of Tax 11-19 epitope.
- A reduced proliferation of CD8⁺ splenocytes from Tax 11-19 immunized DC-depleted mice is observed.
- Adjuvant usage demonstrated higher frequency of Tax 11-19-specific cells.
- Tax 11-19 epitope can be a potential candidate for a DC-based anti-HTLV-1 vaccine.

Figure 1. Genotyping mice to confirm presence of HLA-A2.1 and DTR transgenes and verification of splenic DC-depletion

Male C57BL/6-Tg (HLA-A2.1)1Enge/J mice carrying human MHC class I antigen HLA-A2.1 and age-matched female B6.FVB-Tg Itgax-DTR/EGFP 57Lan/J (DTR) mice were bred as described by standard guidelines provided by the supplier. The genotype of the pups was determined by PCR using tail DNA and specific primers for a control gene fragment, MGSCv37 (Mouse Genome Sequencing Consortium version 37, the most recent version of the C57BL/6J mouse genome assembly), HLA-A2.1, and DTR genes. A) Upper panel, representative data from 14 pups. M represents the DNA molecular weight marker, 1–14 indicates number of pups, NC denotes negative primer control, and PC denotes positive control DNA provided by the supplier. Lower panel, percentage and distribution of transgenes in females (F) and males (M) derived from 66 pups of F1 generation. Mice were given (i.p.) either PBS as the control or a uniform dose of 100 ng DT. B) Splenocytes were harvested and stained for CD11c and CD8α markers, gated for CD8α⁺ T-cells and further

plotted as CD11c⁺/CD8α⁺ murine splenic DCs. Data shown are for –DT and +DT groups 24 h after DT administration. Number 1–5 represents mice from each group.

Figure 2. DC depletion in HLA-A2.1/DTR transgenic mice abrogates CD8 T-cell response to Tax(11-19) antigen

Mice were immunized with Tax(11-19) peptide along with tetanus helper peptide (THP) in the absence or presence of diphtheria toxin (DT) treatment as described in Materials and Methods. At the end of the experiment (day 5 after DT injection), both spleen and bones were harvested from each mouse. Bones were processed to obtain BMDCs as detailed in Materials and Methods. Splenocytes were CFSE labeled and co-cultured with Con A (1 μ g), Tax(11-19) peptide (100 nM), BMDCs alone, or BMDCs prepulsed with Tax(11-19) antigen in vitro. Cells from co-cultures were harvested at days 0 and 12 and stained with APC-conjugated anti-mouse CD8 antibody and analyzed by flow cytometry. A total of 50,000

events collected for each sample were gated to include the CD8⁺/CFSE⁺ population. Proliferation of CD8⁺ T-cells at different conditions was analyzed by determining CFSE dilution into the daughter T-cells. Data presented indicate A) proliferation histograms from days 1 and 12 showing percent division at day 12 from nonimmunized mice; and B) percentage of dividing CD8⁺ T-cells (% Division) under all culture conditions from mice immunized with Tax(11-19) + THP in the absence or presence of DT administration. Percentage of division was obtained by calculating proliferation using the FlowJo 8.8.6 software and plotted as a graph showing the median value. Data are presented from five mice per treatment per injection group. The horizontal line in the graphs represents median values. P values for statistical comparisons within a group were calculated using two-tailed, nonparametric paired *t* test (Wilcoxon); for comparisons between groups, values were calculated using two-tailed nonparametric unpaired *t* test (Mann-Whitney). *P • 0.05. **P • 0.01.

Figure 3. Antigen specificity of the observed responses based on the detection of Tax(11-19) pentamer-positive cells

Mice were immunized with Tax(11-19) peptide along with tetanus helper peptide in the absence or presence of diphtheria toxin (DT) treatment as described in Materials and Methods. At the end of the experiment (day 5 after DT injection), both spleen and bones were harvested from each mouse. Bones were processed to obtain BMDCs as detailed in Materials and Methods. Cells from co-cultures between splenocytes and BMDCs or BMDCs preprimed with Tax(11-19) peptide were harvested at days 1 and 12 and stained with APC-conjugated antihuman CD8 Ab and PE-conjugated Tax(11-19) pentamer and analyzed by flow cytometry. A) CD8⁺ T-cells labeled cells were used on day 1 for obtaining CD8⁺ T-cells/Tax(11-19) pentamer-positive cells from co-culture of splenocytes with BMDCs preprimed with Tax(11-19) antigen from non-DC-depleted and DC-depleted mice immunized with Tax(11-19) + THP. Proliferation of Tax pentamer-positive cells from these groups was then analyzed on day 12 by determining CFSE dilution as incorporated into the daughter T cells. B) Percent division (% Division) comparison between non-DC-depleted and DC-depleted mice. Percentage of division was obtained by calculating proliferation using the FlowJo 8.8.6 software and plotted as a graph showing the median value. Numbers

1–5 represent mice from each group. P values for statistical comparisons within a group were calculated using two-tailed, nonparametric paired t test (Wilcoxon); for between groups comparisons, values were calculated using two-tailed nonparametric unpaired t test (Mann-Whitney). *P • 0.05. **P • 0.01.

Figure 4. Detection of Tax(11-19) pentamer-positive cells in HLA-A2.1/DTR transgenic mice immunized along with the adjuvant

Mice were immunized with Tax(11-19) peptide along with tetanus helper peptide (THP) and incomplete Freund's adjuvant (IFA). Two additional groups of mice were given a uniform dose of diphtheria toxin (DT) (100 ng) prior to immunization. At the end of the experiment (day 5 after DT injection), both spleen and bones were harvested from each mouse. Bones were processed to obtain BMDCs as detailed in Materials and Methods. Cells from co-cultures between splenocytes and BMDCs or BMDCs preprimed with Tax(11-19) peptide were harvested at day 12 and stained with APC-conjugated antihuman CD8 Ab and PE-conjugated Tax(11-19) pentamer and analyzed by flow cytometry. A) Labeled CD8⁺ T-cells were used for obtaining CD8⁺ T-cells/Tax(11-19) pentamer-positive cells from co-culture of splenocytes with BMDCs preprimed with Tax peptide from Tax(11-19) in conjunction with THP and IFA without (upper panel) and with (lower panel) DT administration. CFSE dilution histograms were obtained from the Tax(11-19) pentamer-positive population. B) Percentages of dividing CD8⁺ T-cells/Tax pentamer-positive cells (% Division) on day 12 of BMDC plus Tax(11-19) co-culture were plotted to compare DC-depleted and nondepleted groups of mice showing the median value. Numbers 1–5 represent mice from each group. P values for statistical comparisons within a group were calculated using two-tailed, nonparametric paired *t* test (Wilcoxon); for comparisons between groups, values were calculated using two-tailed nonparametric unpaired *t* test (Mann-Whitney). **P* • 0.05. ***P* • 0.01.

Figure 5. CD8⁺ T-cell proliferation in mice immunized with Tax(11-19) peptide in presence or absence of adjuvant

Mice were immunized with Tax(11-19) peptide along with tetanus helper peptide in the absence or presence of incomplete Freund's adjuvant (IFA) as described in Materials and Methods. Two additional groups of mice were given a uniform dose of diphtheria toxin (DT) (100 ng) prior to immunization. At the end of the experiment (day 5 after DT injection), both spleen and bones were harvested from each mouse. Bones were processed to obtain BMDCs as detailed in Materials and Methods. Splenocytes were CFSE labeled and co-cultured with Con A (1 μ g), Tax(11-19) peptide or BMDCs alone or BMDCs prepulsed with Tax(11-19) antigen in vitro. Cells from co-cultures were harvested at days 0 and 12 and stained with APC-conjugated anti-mouse CD8 antibody and analyzed for the CD8⁺ T-cell/CFSE⁺ population by flow cytometry. Proliferation of CD8⁺ T-cells at different conditions was analyzed by determining CFSE dilution into the daughter T-cells. The number of generations (n) was calculated on the basis of reduction in the CFSE fluorescence in comparison to basal CFSE labeling ($n = \text{geometric mean fluorescence intensity at day 0} / \text{geometric mean fluorescence intensity at day 12}$) and plotted as a graph showing the median value. Data indicate proliferation in mice immunized A) without and B) with IFA for both -DT and +DT groups. Data are presented from five mice per treatment per injection group. P values for statistical comparisons within a group were calculated using two-tailed, nonparametric paired *t* test (Wilcoxon); for comparisons between groups, values were

calculated using two-tailed nonparametric unpaired t test (Mann-Whitney). * $P < 0.05$ and ** $P < 0.01$.

Figure 6. Serum cytokine profiles of DC-depleted and non-depleted transgenic mice immunized with or without adjuvant

Serum samples from mice were analyzed for cytokines using a Multi-Analyte ELISA Array (Qiagen, Germantown, MD) as described in Materials and Methods. Th1-related cytokines such as IL-2, IL-10, IL-12, IFN- γ , and TNF- α ; Th2-related cytokines such as IL-2, IL-4, IL-5, and IL-10; and Th17-related cytokines such as IL-13, IL-6, IL-17A, IL-23, TNF- α , and TGF- β were detected using appropriate detection antibodies. The table in A) shows the observed values for all cytokines (pg/ml). Bar graphs in B) depict relative levels of detectable cytokines in serum between differently immunized mice in the absence or presence of DC depletion. Absorbance was then read at 450 nm within 30 min of stopping the reaction. ND, not determined.