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B cells “transduced” with TAT-fusion proteins can induce tolerance and protect mice from diabetes and EAE

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Abstract

Antigen-immunoglobulin fusion protein expressing B cells have been shown as excellent tolerogenic antigen-presenting cells in multiple disease models. Using efficient protein transduction by fusion with a HIV TAT protein transduction domain, we herein tested the TAT-fusion protein transduced B cells for their effects in antigen-specific tolerance induction in two animal models, experimental autoimmune encephalitis (EAE) and type 1 diabetes. We demonstrated that transfer of TAT-MOG₃₅₋₅₅ (myelin oligodendrocyte glycoprotein)-Ig ‘transduced B cells’ 10 days after EAE induction significantly protected mice from disease. Similarly, the onset of was delayed when NOD mice received insulin specific TAT-B₉₋₂₃- B cells. Surprisingly, no protection against EAE was observed in a prophylactic protocol when transduced B cells were given before disease induction. Moreover, TAT-ovalbumin transduced cells were tolerogenic in primed but not naïve mice. Our results suggest that TAT-fusion protein transduced B cells were tolerogenic in antigen primed recipients, a condition clinically relevant to autoimmune diseases.

Keywords

TAT fusion; B cells; Protein Therapy; Tolerance; T1D; EAE

1. Introduction

Autoimmune diseases, mediated by autoreactive T cells or auto-antibodies, are a variety of disorders caused by losing tolerance to self-antigens. To date, long-term cures for

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autoimmune diseases are not yet available. Among many strategies undergoing investigation, re-introducing tolerance to self-antigens via immunotherapy is very attractive and promising because antigen-specific tolerance may be achieved, without causing general suppression. B cells have been found to be excellent tolerogenic antigen presenting cells (APCs) both *in vitro* and *in vivo* [1; 2; 3]. Several groups, including ours, utilized this property and designed B-cell based gene therapies to induce tolerance in animal models for autoimmune diseases, allergy, and hemophilia [4; 5; 6; 7; 8; 9; 10; 11; 12; 13].

Experimental autoimmune encephalomyelitis (EAE) is an animal model of human multiple sclerosis. Chen *et al* found that adoptive transfer of proteolipid protein (PLP) expressing B cells induced PLP-specific hyporesponsiveness and protected mice from the induction of EAE [4]. Moreover, these B cells also prevented relapses in the diseased mice [5]. Ahangarani *et al* found that when retrovirally delivered with a fusion construct encoding the T cell epitope of a common allergen, Der P 2, and an endosomal targeting sequence, B cells were highly tolerogenic *in vivo*. Mice that were adoptively transferred with such B cells were protected from Der p 2-induced asthma due to the induction of Tr1 regulatory cells [12]. Our laboratory has generated antigen-specific tolerogenic B cells by retroviral gene transfer of B cells with a construct encoding the target antigen and IgG heavy chain (peptide-IgG). B cells expressing such peptide-IgG constructs either protected animals from disease or resulted in delayed disease onset in a number of models including EAE [9; 11], experimental autoimmune uveitis [8; 14], type 1 diabetes [9; 10], rheumatoid arthritis [15] and hemophilia [13]. Together, these findings from different groups indicate that B cells can be utilized as tolerogenic APCs in autoimmune therapies. The rationale of our peptide-IgG B-cell gene therapy for tolerance induction was based on the tolerogenic properties of immunoglobulin G [16; 17; 18]. We found that retrovirally transduced B cells process and present endogenously derived epitopes (from peptide-IgG fusion) via the endocytic pathway [19] and recruit regulatory T cells via B7:CTLA-4 interactions [20]. Indeed, we recently observed that tolerogenic B cells directly interact with target CD4⁺ T cells *in vivo* by using two-photon microscopy and anti-CTLA-4 antibody could block the conjugation between B and T cells (Su, Matheu *et al.*, in preparation). We previously demonstrated that tolerance induced by retrovirally transferred peptide-IgG B cells depends upon activation of natural CD4⁺CD25⁺ regulatory T cells (Tregs) [10; 13]. Recently, we extended these studies and identified the effects of our approach on the target CD4⁺ T cells *in vivo*. Using DO11.10 OVA₃₂₃₋₃₃₉ peptide-specific T-cell receptor (TCR) transgenic mice, we found that pOVA₃₂₃₋₃₃₉-IgG expressing B cells increased the percentage of antigen-specific FoxP3⁺ Tregs *in vivo*. Surprisingly, we found that the percentage of antigen-specific CD4⁺ effector T cells was reduced, suggesting tolerogenic B cells might directly induce effector T cell death [21]. Therefore, we propose that peptide-IgG expressing B cells induce tolerance by two mechanisms: induce or activate Tregs and induce effector T cell death, both require B/T direct contact via MHC class II and costimulatory signals.

Despite these successes, safety and serious adverse events are still our major concerns of retrovirus-based gene therapy. Addressing these issues, we attempted to find an alternative strategy by asking whether exogenous antigen-pulsed B cells could act as functional tolerogenic APCs in both naïve and primed animals. Although OVA pulsed B cells are immunogenic but not tolerogenic *in vivo* (Su and Scott, unpublished observation), we surmised that the efficacy of exogenous antigen processing and presentation is critical to make B cells tolerogenic. HIV TAT protein is a key trans-activator of HIV-1 gene transcription and its major function is to enhance the processivity of transcribing polymerases [22]. It has been shown that the 11 amino acid peptide sequence from the TAT protein transduction domain, is able to mediate efficient cell entry of a variety of proteins when fused together [23; 24]. Taking advantage of this, we generated several TAT-peptide-

Ig fusion proteins to test whether TAT fusion protein transduced B cells are able to mediate tolerance induction *in vivo*.

In this study, we tested whether B cells pulsed with TAT fusions *in vitro* (TAT fusion transduction) are able to induce tolerance *in vivo*. Two animal models of autoimmunity, EAE and type 1 diabetes, were used. We found that TAT-fusion transduced B cells can protect mice from disease in both EAE and diabetes models. Furthermore, TAT-OVA transduced B cells were tolerogenic *in vivo* in OVA primed mice but not in naïve mice. In addition, we demonstrated that TAT fusion treated B cells induced specific CD4⁺ T cell apoptosis *in vitro*, suggesting a mechanism of cell-activation induced cell death (AICD). Taken together, our results provide insights into development of a novel strategy of protein therapy for inducing tolerance in autoimmunity.

2. Materials and Methods

2.1 Mice and Reagents

OT II and DO11.10 OVA₃₂₃₋₃₃₉ specific TCR transgenic mice, Balb/c, C57BL/6, and six-week-old NOD mice were purchased from Jackson Laboratory (Bar Harbor, ME). All mice were housed in pathogen-free conditions, and the animal experiments were approved by the University of Maryland Animal Care and Use Committee. OVA₃₂₃₋₃₃₉ peptide (pOVA) and MOG₃₅₋₅₅ peptide (pMOG) were synthesized by New England Peptide, Inc (Gardner, MA).

2.2 Expression, Purification, and Transduction of TAT-fusion Protein

The methods of cloning, expression, and purification of TAT-fusion protein were described in references from Dr. Dowdy's group [23; 25]. Briefly, DNA fragments encoding MOG₃₅₋₅₅-Ig, Insulin B₉₋₂₃-Ig, or OVA were subcloned into pTAT-HA vector. E-Coli (BL21 (DE3) pLysS) expressed TAT-fusion protein was purified using a Ni-NTA column. LPS activated B cells were cultured with 20–80 µg/ml indicated TAT-fusion proteins at 37°C for one hour. Cells were then washed at least three times with PBS before injection into recipients or culture *in vitro* with T cells.

2.3 Cell Purification and Culture

Splenic B cells were purified with anti-T cell antibody cocktail (anti-Thy1, anti-CD4, and anti-CD8) plus complement (Low Tox M, Cedarlane Laboratories, Accurate Chemical and Scientific Corporation, Westbury, NY). Purified B cells were cultured with RPMI 1640 medium (Gibco BRL, Invitrogen, Carlsbad, CA) supplemented with 5% FBS, 2 mM L-glutamine, and 2-mercaptoethanol. B cells were pre-stimulated with 1 µg/ml bacterial lipopolysaccharide (LPS, E. coli 055:B5, Sigma, St. Louis, MO) or anti-CD40 antibody (HM40-3, PharMingen, San Diego, CA). Splenic T cells were purified by depleting B220⁺ cells using goat anti rat IgG-magnetic beads (QIAGEN, Valencia, CA) and anti-B220 antibody (RA36B2).

2.4 Tolerance Induction to OVA₃₂₃₋₃₃₉ Peptide

Tolerance to pOVA was tested in two protocols. First, naïve C57BL/6 recipient animals were injected intraperitoneally with 10⁷ TAT-fusion protein transduced B cells. Seven days later, animals were immunized in a hind footpad and the base of tail with 25 µg pOVA emulsified in complete Freund's adjuvant (CFA). Two weeks later, mice were sacrificed and the cellular and humoral responses were examined. Alternatively, C57BL/6 mice were pre-immunized with 25 µg CFA emulsified pOVA. Ten days later, these mice were injected with 10⁷ TAT-fusion protein transduced B cells. Antibody and T cell responses were assayed four weeks after B cell transfer. Antibody titers were determined by the endpoint dilution ELISA method. ELISA plates were coated with 10 µg/ml full-length OVA protein. Cellular

responses were assayed by [³H] thymidine incorporation. Five×10⁵ cells from draining popliteal and inguinal lymph nodes were seeded per well in 96-well plate in the presence of indicated concentration of pOVA. Cultures were pulsed with 1μCi/well of [³H] thymidine (Amersham Life Sciences, Arlington Heights, IL) 48 hours later and incubated for an additional 16–20 hours. Cells were then harvested on glass fiber filters and incorporated [³H] thymidine was detected via gas scintillation counting in a Packard Matrix 9600 reader. Data are expressed as mean of Δcpm (count per minute minus background) ±SE.

2.5 EAE Induction

EAE was actively induced in 6–8 week old female C57BL/6 mice by subcutaneous immunization with 200μg of pMOG emulsified in CFA containing 4 mg/ml of *Mycobacterium tuberculosis* H37Ra (DIFCO, Detroit, MI). On the day of immunization (day 0) and 48 h later, mice also received 200ng of pertussis toxin (Sigma-Aldrich, St. Louis, MO) in 0.5 ml PBS intraperitoneally. On day 10 or 14 after EAE induction, recipients were i.p. transferred with 10⁷ TAT-MOG₃₅₋₅₅-IgG or -B₉₋₂₃-IgG transduced B cells. Clinical signs of EAE were assessed daily with a 0 to 5 scoring system [26]: 0, normal; 0.5, partially limp tail; 1, paralyzed tail; 2, loss in coordinated movement; 2.5, one hind limb paralyzed; 3, hind limbs paralyzed; 3.5, hind limbs paralyzed and forelimb weakness; 4, forelimbs paralyzed; 5, moribund.

2.6 Diabetes in NOD Mice

Spontaneous diabetes in female NOD mice begins to appear by 12 weeks of age (increasing to 70–90% incidence at 20–25 weeks of age) in our colony. Splenic B cells from NOD mice were pretreated with 1μg/ml anti-CD40 antibody overnight and then transduced with 20μg/ml TAT-B₉₋₂₃-IgG at 37°C for one hour. 10⁷ TAT-B₉₋₂₃-IgG transduced B cells were transferred into NOD female mice at seven weeks of age. Glucose levels in blood taken from tail snips were measured weekly after TAT-B₉₋₂₃-IgG B-cell therapy by standard methods using OneTouch Ultra Test strips and monitoring system (LifeScan Technologies, Milpitas, CA). Mice were considered diabetic when glucose levels reached >250 mg/dl. Animals were also evaluated daily for clinical signs of diabetes (e.g., weight loss, cage wetting, morbidity and mortality).

2.7 In vitro Culture of TAT-fusion Transduced B cells and Specific T cells

Splenic B cells from Balb/c mice were cultured with 1μg/ml LPS overnight and then pulsed with 100μg/ml TAT-OVA or TAT-B₉₋₂₃-IgG for one hour at 37°C. After extensive washing with PBS, B cells were cultured with freshly isolated splenic DO11.10 T cells at an 1:1 E:T ratio in 24-well plate in the presence of 30ng/ml pOVA. After 12h, cells were collected and assayed for apoptosis by examining the binding of Annexin V.

2.8 Antibodies and Fluorescence-Activated Cell Sorting (FACS) Analysis

PE-anti-mouse-CD4 and APC-Annexin V mAbs were purchased from Pharmingen (San Diego, CA) or eBioscience (San Diego, CA). For flow cytometry assay, cells were incubated with 2.4G2 mAb (anti-CD16/32) for 15 minutes to block FcR then stained with indicated Abs for 40 minutes at 4°C. Cells were analyzed on a FACScan or FACScalibur flow cytometer (Becton-Dickinson). Data were analyzed by FlowJo software (Tree Star Inc. San Carlos, CA).

2.9 Statistics

Equal variances one-tailed Student's T test statistics were applied where indicated with differences considered significant at *, p < 0.05; **, p < 0.01.

3. Results

3.1 B Cells Can Be Transduced With TAT-fusion Protein

We previously showed that retrovirally gene transferred B cells process and present endogenously produced peptide-IgG in the class II MHC pathway [19]. To enhance antigen presentation by B cells, we utilized the protein transduction property of TAT peptide and made three TAT- fusion proteins, TAT-MOG₃₅₋₅₅-IgG, TAT-B₉₋₂₃-IgG, and TAT-OVA. We first confirmed that B cells can be efficiently transduced with TAT-fusion protein *in vitro*. TAT-MOG₃₅₋₅₅-IgG, conjugated with FITC, was incubated with LPS activated B cells *in vitro* for one hour; after washing, the cells were examined by FACS. As shown in Figure 1a, TAT-MOG₃₅₋₅₅-IgG-FITC can be transduced to B cells at a very high efficiency. Furthermore, we validated that TAT-fusion transduced B cells can effectively activate specific T cells *in vitro*. T cells from two strains of pOVA₃₂₃₋₃₃₉ TCR transgenic mice, DO11.10 and OT II, were used as target responders and cultured with irradiated TAT-OVA transduced Balb/c or C57BL/6 B cells at different ratios, respectively. As shown in Figure 1b, TAT-OVA transduced Balb/c B cells exhibited a robust effect in activating DO11.10 T-cell proliferation in a dose dependent manner. Similar results were found when TAT-OVA transduced C57BL/6 B cells were used to stimulate OT II T cells *in vitro* (data not shown). Together these suggested that TAT-fusion transduced B cells are functional APCs *in vitro*.

3.2 Application of B Cell Delivered TAT-fusion Protein As A Strategy In Autoimmunity

Next, we investigated whether TAT-fusion transduced B cells can be used *in vivo* for tolerance induction in two autoimmune disease models, EAE and type 1 diabetes. First, we asked whether TAT-MOG₃₅₋₅₅-IgG B cells are tolerogenic in MOG₃₅₋₅₅-induced EAE. Six-week old C57BL/6 mice were immunized subcutaneously with MOG₃₅₋₅₅/CFA to actively induce EAE. Ten days later, these mice were injected with 1×10^7 LPS activated TAT-MOG₃₅₋₅₅-IgG transduced B cells. As shown in Figure 2a, mice that received TAT-MOG₃₅₋₅₅-IgG transduced B cells exhibited significantly suppressed disease severity as compared with the control mice that received TAT-B₉₋₂₃-IgG B cells, indicating that TAT-MOG₃₅₋₅₅-IgG transduced B cells were tolerogenic APCs for active EAE *in vivo*. In contrast, we found that TAT-MOG₃₅₋₅₅-IgG transduced B cells have no effect in disease protection in a pre-treatment protocol. When we transferred TAT-MOG₃₅₋₅₅-IgG B cells into naïve mice and induced EAE one week later, the disease development was identical in both experiment and control groups (Fig. 2b). Taken together, our data suggest that TAT-MOG₃₅₋₅₅-IgG B cells have no suppressive effect in naïve animals but can protect mice from EAE in the therapeutic protocol, a more significant clinical result.

In parallel, we asked whether this approach can be applied in another autoimmune disease, type 1 diabetes. Splenic B cells from NOD mice (7 weeks old) were treated with 1µg/ml of anti-CD40 antibody overnight and then pulsed with TAT-B₉₋₂₃-IgG or TAT-OVA as control. Anti-CD40 has the same effects as LPS in B-cell activation and is routinely used to activate B cells in our laboratory in the diabetes model. One $\times 10^7$ TAT-transduced B cells were transferred into seven-week old female NOD recipients. Blood glucose was monitored weekly. As shown in figure 3, mice that received TAT-B₉₋₂₃-IgG transduced B cells exhibited a delayed onset of diabetes. This result, again, suggests that this strategy is feasible and effective as a tolerance inducing therapy in autoimmunity.

3.3 TAT-OVA B Cells Are Tolerogenic In Pre-sensitized But Not In Naïve Mice

To further investigate the mechanism by which TAT-fusion transduced B cells are tolerogenic, we utilized TAT-OVA to transduce B cells and examined the *in vivo* effects of these OVA-expressing B cells in two protocols. We first examined tolerance induction to pOVA by TAT-OVA transduced B cells in naïve mice. LPS activated C57BL/6 B cells were

incubated with 80µg/ml of TAT-OVA for one hour *in vitro*. Then 1×10^7 such B cells were injected into naïve C57BL/6 recipients. Seven days later, these recipients were immunized with 25µg pOVA emulsified in CFA on a footpad and the base of tail. Two weeks after immunization, T cell and antibody responses were assayed. Under these conditions, TAT-OVA expressing B cells were immunogenic rather than tolerogenic in naïve mice *in vivo*. As shown in Figure 4a, mice that received control B cells only developed a trace amount of anti-OVA IgG antibody two weeks after pOVA challenge, but mice that received TAT-OVA B cells exhibited a robust anti-OVA antibody response. In addition, T cell proliferation data also suggest that TAT-OVA B cells primed rather than tolerized mice *in vivo* (Fig. 4b).

Successes in the therapeutic protocol in EAE and type I diabetes models and failure of inducing tolerance in the prevention protocol led us to hypothesize that this approach only has protective effects when primed T cells exist in the hosts. Therefore, we changed our therapy protocol by transferring TAT-OVA B cells into pre-sensitized mice. C57BL/6 mice were immunized with pOVA/CFA. Ten days later, these mice were transferred with TAT-OVA B cells. Four weeks after B-cell transfer, we assayed T cell and antibody responses and found that the immune responses to OVA in the OVA primed mice were reduced in both cellular and humoral levels. As shown in Figure 4c, antibody titers in the TAT-OVA group were significantly reduced compared to the control treated mice. In addition, the T cell response against pOVA was also reduced in the TAT-OVA B cells transferred mice (Fig 4d).

3.4 TAT-fusion B Cells Induce Specific Target T Cell Apoptosis

Based on these data, we proposed that TAT-fusion protein transduced B cells might induce specific tolerance by inducing T cell apoptosis via activation induced cell death (AICD). To further investigate this process as a mechanism of tolerance induction by TAT-fusion transduced B cells, we asked whether TAT-fusion transduced B cells can induce specific T cell apoptosis *in vitro*. Splenic T cells from DO11.10 mice were cultured with LPS activated, TAT-OVA transduced Balb/c B cells *in vitro* in the presence of 30ng/ml pOVA. TAT-B₉₋₂₃-IgG transduced or untransduced B cells were used as controls. After 12 hours, we collected cells and examined T cell apoptosis by Annexin V staining. As shown in Figure 5, untransduced or TAT-B₉₋₂₃-IgG transduced B cells had no effect on T-cell death. In contrast, CD4⁺ T cells cultured with TAT-OVA B cells exhibited a high expression of Annexin V, indicating that many of these T cells were undergoing apoptosis. This result suggests that TAT fusion- transduced B cells can induce target T cell death *in vitro*.

4. Discussion

Re-establishing specific tolerance to self antigens is a desirable goal to treat patients with autoimmune diseases. Utilizing the translocation properties of TAT peptide, we designed a novel B-cell based protein therapy. Herein we demonstrated that adoptive transfer of B cells transduced with TAT-fusions can protect mice in two models of autoimmunity, EAE and type 1 diabetes. Our data suggest that TAT-peptide B cells can tolerize and protect recipients possibly by inducing primed autoreactive T cell death via AICD.

We have previously reported that retrovirally transduced B cells expressing a fusion peptide-IgG construct could mediate antigen-specific tolerance in multiple animal models of autoimmunity and hemophilia A. For example, engineered MOG-IgG- or MBP-IgG- B cells through retroviral gene transfer could protect mice from EAE induced by MOG₃₅₋₅₅ and MBP peptide Ac₁₋₁₁, respectively [9; 11]. Both Th1 and Th2 responses and specific antibody production were inhibited with this B-cell gene therapy. Similarly, when retrovirally modified GAD65-IgG- or (Pro) Insulin-IgG- B cells were employed in the diabetes model, treatment delayed the onset and decreased the incidence of diabetes in

young NOD female mice [9; 10]. Furthermore, we have shown that B cells process endogenously produced peptide-IgG in the lysosome/endosome pathway and then subsequently present the immunodominant epitopes onto class II MHC grooves [19]. We also found that the expression of class II MHC and B7 costimulatory molecules on tolerogenic B cells is essential for tolerance induction [20; 27]. Together, these findings suggest that retrovirally transduced peptide-IgG B cells tolerize specific CD4⁺ T cells via class II MHC presentation. Moreover, we have demonstrated that tolerance induction in our system requires CD25⁺ natural Tregs [10; 13], as well as the recruitment and activation of adaptive Tregs [21]. These successes in application and mechanistic studies indicate that B cells can be educated *in vitro* and then serve as efficient tolerogenic APCs *in vivo*.

However, the safety of retroviral transduction is the major concern in gene therapy approaches because of the incorporation of the viral DNA carrying target gene into oncogenic hot spots in the host genome. In addition, we have previously shown that retroviral transduction efficiency of B cells is about 10–40% [28]. Although this transduction efficiency is sufficient to induce tolerance, we predict that a stronger or longer hyporesponsiveness could be achieved with enhanced transduction efficiency. Thus, to translate the B-cell gene therapy approach into the clinic, it is important to find alternative strategies to deliver the relevant antigen fusion proteins into B cells in a safer and more efficient manner. The rapid membrane translocation property of TAT transduction domain has been utilized to deliver foreign proteins, DNA, and drugs in anti-cancer therapies [29]. It has been found that truncated TAT containing the basic region still retains the membrane translocation and nuclear localization properties [30]. Therefore, we combined the transduction property of TAT peptide and advantages of B cells as tolerogenic APCs and designed this B-cell based protein therapy.

In this study, we generated several fusion proteins containing the minimal transduction domain of TAT-47-57 (YGRKKRRQRRR) and focused on the feasibility of applying this TAT-fusion protein therapy on autoimmune diseases. We demonstrated here that TAT-fusion transduced B cells are tolerogenic in two models, EAE and type 1 diabetes. We showed that a single injection of TAT-MOG₃₅₋₅₅-IgG transduced B cells 10 days after EAE induction ameliorated clinical disease and that TAT-B₉₋₂₃-IgG B-cell treatment delayed the onset of diabetes in young NOD mice. In addition, TAT-OVA B cells prevented the immune response to OVA in pre-immunized mice at both humoral and cellular levels. Achievement in these models indicates that our novel B-cell delivered TAT-fusion protein therapy has therapeutic potential in clinical treatment of autoimmunity. However, it is worth noting that the tolerogenic effect of TAT-MOG₃₅₋₅₅-IgG B cells was only achieved during the early phase after EAE induction. Indeed, we did not see the suppression of disease in animals that received therapy 14 days after EAE induction (data not shown), at which point severe disease symptoms had already developed. One possible reason of failure of tolerance induction under this condition is that, after a certain time of EAE induction, injury of central nervous system in the recipients may be irreversible even though autoreactive T cells can be deleted.

Interestingly, in contrast to retroviral B-cell gene therapy, which had shown efficacy in both naïve and primed animals [8; 9; 13], TAT-MOG₃₅₋₅₅-IgG and TAT-OVA transduced B cells only protected primed, but not naïve animals. Failure to induce tolerance in the naïve mice using a pretreatment protocol suggests the mechanism(s) of tolerance induction by TAT-fusion B cells might be different from that of retrovirus based peptide-IgG gene therapy, which requires CD4⁺CD25⁺ Tregs. A certain amount of activated autoreactive T cells is the prerequisites of initiation of T-cell mediated autoimmune diseases. Thus, under diseased conditions, TAT-fusion transduced B cells might give activated pathogenic target CD4⁺ T cells activation signals and as a result, these pathogenic T cells undergo apoptosis, which

results in amelioration of disease symptoms. We tested this hypothesis by *in vitro* culturing TAT-fusion transduced B cells with specific T cells. Indeed, *in vitro* culture of OVA specific TCR transgenic T cells with TAT-OVA transduced B cells resulted in CD4⁺ T-cell apoptosis, suggesting that TAT-fusion transduced B cells might function by interacting with CD4⁺ effector T cells and then inducing them to die via AICD. In addition, the TAT-OVA construct was initially designed as a size comparable control to TAT-MOG₃₅₋₅₅-IgG and TAT-B₉₋₂₃-IgG. As we previously showed in the retroviral peptide-IgG gene therapy, the IgG heavy chain plays an important role in increasing the efficacy of tolerance induction; however, without the IgG heavy chain, tolerance is still achievable, albeit less effective [28; 31]. In this study, our data confirmed these observations as in the absence of IgG heavy chain TAT-OVA transduced B cells can also protect mice in pre-sensitized condition.

We showed that TAT-fusion transduced B cells are functional APCs and have either immunogenic or tolerogenic activity *in vivo*, which depends on when diseases are initiated. Nevertheless, the mechanisms of TAT-fusion protein processing and presentation by B cells are not fully understood. In the current study, we demonstrated that B cells can be transduced with TAT-fusion protein very efficiently. It has been found that TAT-mediated transduction mainly through the mechanism of lipid raft macropinocytosis [32]. Thus, we speculate in our system, TAT-fusions might be internalized into B cells via the same or similar mechanism. We also observed that a small portion of the TAT fusion protein could physically associate with the cell surface during the transduction (data not shown). Whether or not these surface-associated TAT-fusion proteins contribute to tolerance induction needs to be determined in the future.

In conclusion, we found that TAT-fusion transduced B cells have protective effects in two autoimmune disease models, EAE and type 1 diabetes. The mechanism(s) of tolerance induction by this approach is not likely the same as those of our well established retroviral B-cell gene therapy. Rather than inducing or activating regulatory T cells, TAT-fusion transduced B cells seem to induce target CD4⁺ effector T cell death via apoptosis (AICD). Our results indicate that this novel strategy using TAT-fusion transduced B cells as a vehicle to induce tolerance might be a feasible approach for treating autoimmune diseases.

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Fig. 1. TAT fusion protein transduced B cells could serve as functional APC *in vitro*

(a) LPS activated B cells are efficiently transduced with TAT-MOG₃₅₋₅₅-Ig fusion protein. C57BL/6 splenic B cells were treated with 1 μg/ml LPS for 24h and then pulsed with FITC conjugated TAT-MOG₃₅₋₅₅-IgG fusion protein for an hour at 37°C, followed by extensive washing. The FITC levels on TAT-MOG₃₅₋₅₅-IgG transduced (line) or untransduced B cells (solid) were analyzed by FACS. (b) LPS activated splenic B cells from Balb/c mice were transduced with TAT-OVA fusion protein for an hour at 37°C and then irradiated at 800rad to prevent further proliferation. These B cells (effectors) were cultured with purified DO11.10 T cells (targets) for 48h at an E:T ratio of 1:5 or 1:1. Then the cells were pulsed with [³H] thymidine, and T cell proliferation was assayed by [³H] thymidine incorporation 20h later. Data are presented by mean (CPM) ± Standard Error from quadruplicate samples.

Fig. 2. TAT-MOG₃₅₋₅₅-IgG transduced B cells protect mice from EAE

(a) Six week old C57BL/6 mice (n=5) were immunized for EAE by subcutaneous immunization with MOG₃₅₋₅₅/CFA. The same day and 48 hours later, these mice also received 200ng of pertussis toxin in PBS via i.p. injection. Ten days later, 10⁷ TAT-MOG₃₅₋₅₅-IgG transduced B cells were transferred into these recipients (TAT-B₉₋₂₃-IgG as control). This is a representative from two independent experiments with same trends. * P<0.05 at individual time point. (b) TAT-MOG₃₅₋₅₅-IgG transduced B cells do not protect mice from EAE in a pre-treatment protocol. Six week old C57BL/6 mice (n=5) were transferred with TAT-MOG₃₅₋₅₅-IgG B cells. Seven days after B-cell transfer, these mice were actively induced with EAE as described above.

Fig. 3. NOD mice that received TAT-B₉₋₂₃-IgG transduced B cells exhibit delayed onset of diabetes

Splenic B cells from female NOD mice were treated with 1 μ g/ml anti CD40 antibody overnight and then pulsed with TAT-B₉₋₂₃-IgG or TAT-OVA as control. 10⁷ B cells were transferred into female NOD recipients at 7 weeks of age (n= 8). Blood glucose was monitored weekly. This is a representative of two independent experiments.

Fig. 4. TAT-OVA transduced B cells are immunogenic in naïve mice and are tolerogenic in pre-sensitized mice

(a, b) LPS-activated C57BL/6 B cells were transduced with 80 μ g/ml TAT-OVA (TAT-B9-23-IgG as control) for an hour at 37°C and then 10⁷ B cells were transferred into naïve C57BL/6 recipients (n=4). Seven days later, recipient mice were immunized with pOVA/CFA on one footpad and the base of tail. Two weeks later, humoral and cellular responses were measured by ELISA (a) and T-cell proliferation assay against pOVA (b), respectively. This is a representative of two independent experiments. (c, d) C57BL/6 mice were immunized with pOVA/CFA on one footpad and the base of tail. After 10 days, recipients were injected with 10⁷ LPS-activated, TAT-OVA transduced B cells (n=4). Four weeks after B-cell transfer, mice were sacrificed and assayed for humoral and cellular responses by ELISA (c) and T-cell proliferation assay against pOVA (d), respectively. This is a representative of two independent experiments. Data are presented by mean $\pm$ Standard Error. * P<0.05; ** P<0.01.

Fig. 5. TAT-OVA transduced B cells induce DO11.10 T cell apoptosis *in vitro*

Balb/c B cells were pretreated with 1 μ g/ml LPS overnight and then transduced with TAT-OVA or TAT-B₉₋₂₃-IgG. Freshly isolated DO11.10 T cells were co-cultured with untransduced, TAT-OVA or TAT-B₉₋₂₃-IgG transduced B cells for 12h in the presence of 30ng/ml pOVA. Cells were stained with CD4-PE, Annexin V-APC, and PI. Annexin V binding on PI⁻CD4⁺ cells was shown. Solid, untransduced B cells; dark line, TAT-B₉₋₂₃-IgG transduced B cells; light grey line, TAT-OVA transduced B cells. This is a representative of two similar experiments.