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In Vivo Induction of Type 1-Like Regulatory T Cells Using Genetically Modified B Cells Confers Long-Term IL-10-Dependent Antigen-Specific Unresponsiveness¹

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Regulatory T cells (Tregs) hold much promise for the therapy of allergy and autoimmunity, but their use is hampered by lack of Ag specificity (natural Tregs) and difficulty to expand in vitro or in vivo (adaptive Tregs). We designed a method for in vivo induction of Ag-specific Tregs, in BALB/c H-2d, that share characteristics with type 1 Tregs (Tr1). A retroviral vector was constructed encoding a major T cell epitope of a common allergen, Der p 2, fused to an endosomal targeting sequence (gp75) for efficient MHC class II presentation. B cells transduced with such construct were adoptively transferred to BALB/c mice before or after peptide immunization. Long-lasting Ag-specific immune tolerance was achieved in both cases. Genetically modified B cells constitutively expressed the transgene for at least 3 mo. B cells from IL-10^{-/-} mice were unable to induce tolerance. Upon transfer, B cells induced Foxp3⁺CD4⁺ T cells showing phenotypic and functional characteristics comparable to Tr1-cells, including production of IL-10 but not of TGF- β , and high expression of CTLA-4. Adoptive transfer of such T cells conferred unresponsiveness to allergen immunization and prevented the development of Der p 2-induced asthma. Functional Tr1-like cells can therefore be induced in vivo using retrovirally transduced B cells. *The Journal of Immunology*, 2009, 183: 8232–8243.

Establishing long-term Ag-specific unresponsiveness in a fully immunocompetent host is required to effectively treat autoimmune diseases and allergy and to prevent or suppress cellular and/or humoral immune responses following allogeneic transplantation and gene therapy. One possible way to establish such tolerance could be through the design of methods by which regulatory T cells (Tregs)³ can be elicited in vivo.

Tregs functionally inhibit a variety of immune responses, both in vitro and in vivo (1, 2). Natural Tregs occur naturally in vivo and are typically selected in the thymus. They predominantly use a diverse TCR repertoire involved in controlling responses to self-

Ags (3). However, Tregs can also be derived in vitro, and to a limited extent in vivo, under particular conditions of Ag or cytokine exposure (4). These induced or adaptive Tregs include mainly T regulatory 1 (Tr1) and Th3 cells. Th3 cells develop after oral administration of an Ag while Tr1 cells have essentially been described after in vitro activation of naive CD4⁺ T cells in the presence of IL-10, a combination of IL-4 and IL-10 (5), IL-10, and IFN- α (6), or in the presence of 1,25 (OH)2-vitamin D3 and dexamethasone (7). Tolerogenic dendritic cells (DC) or DC treated with IL-10 also help expanding Tr1 cells (8).

The potential of Tr1 cells for therapy is very appealing as reflected by the number of recently published reports (9). However, the main limitation for therapeutic applications is the lack of methods to expand Tr1 in vivo. Moreover, in vitro expansion of Tr1 cells can result in heterogeneous populations that may contain effector cells that could potentially exacerbate the underlying clinical condition or result in untoward side effects (5). To overcome these limitations and safety concerns, we therefore sought to develop an alternative strategy for Tr1 induction in vivo, based on the use of gene-modified APCs. Both DC and B lymphocytes have been explored as APCs for immune tolerance induction. DC are known to induce Ag-specific unresponsiveness or boost an immune response, depending on their state of maturation (10), which makes them particularly attractive for genetic modification. However, genetic modification of DC often results in the up-regulation of costimulatory molecules and MHC-II with a concomitant increase in their immune stimulatory properties (11). Moreover, their lifespan is limited in vivo because myeloid cells turnover relatively rapidly compared with lymphoid cells (12).

B lymphocytes offer an attractive alternative, due to their ability to survive for long periods of time after adoptive transfer into a recipient host. Moreover, they efficiently interact with T cells within lymph node and splenic germinal centers. However, the use of genetically modified B cells for immunomodulation has initially

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R. Roohi and W. Janssens designed and performed the research. V. Carlier analyzed FACS data. L. VanderElst adapted the vector to the specific purpose. T. VandenDriessche and M. Chuah designed the vectors and contributed to optimizing B cell transduction and experimental design and to the writing of the manuscript. B. Weynand (pathologist) examined the slides. J.A.J. Vanoirbeek performed lung function measurement in mice. M. Jacquemin developed the initial idea of using B cells. J.M. Saint-Remy designed the study and wrote the manuscript.

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³ Abbreviations used in this paper: Treg, regulatory T cell; Tr1, T regulatory 1; DC, dendritic cell; FVIII, coagulation factor VIII; gp75, glycoprotein 75; BALF, bronchioalveolar lavage fluid; AHR, airway hyperreactivity; AUC, area under the curve.

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been hampered by relatively poor gene transfer efficiency (13) and by their ability to tolerize only naive but not primed T cells (14). The low gene transfer efficiencies in primary B cells could be overcome using Moloney murine leukemia virus-based retroviral vectors instead. Indeed, we and others have shown that relatively high stable transduction efficiencies (>50%) could be achieved following retroviral transduction of primary murine B cells (15, 16). In addition, Scott and coworkers (16) recently showed that genetically modified B cells could be used to tolerize not only naive but also primed T cells. Indeed, B cells transduced with a retroviral vector expressing domains of coagulation factor VIII (FVIII) linked to an IgG H chain scaffold, suppress the ongoing production of anti-FVIII Abs upon transfer to a FVIII-immunized host. However, it was not known whether an IgG scaffold is absolutely necessary for immune tolerance induction and whether other approaches and mechanisms can result in Ag-specific immune tolerance.

Based on these findings, we evaluated whether B cells transduced with a retroviral construct that did not contain an IgG H chain scaffold could confer Ag-specific unresponsiveness after transfer to a naive or primed host. In particular, we wanted to test the hypothesis whether direct intracellular targeting of an Ag to late endosomes for MHC class-II presentation would endow B cells with tolerogenic properties on CD4⁺ specific T cells and, if so, whether the latter would share characteristics with Tr1.

As a model system, we used a peptide encompassing amino acid residues 21 to 35 (p21–35) from a major group 2 allergen, Der p 2, from the house dust mite *Dermatophagoides pteronyssinus* that is involved in the development of allergic diseases such as asthma. p21–35 has been extensively studied in our laboratory: it contains both a major promiscuous T cell epitope and a dominant B epitope (17), thereby providing a suitable sequence for the study of tolerance induction at B and T cell levels. Injection of p21–35 in conventional adjuvant elicits strong specific Ab responses, while the same peptide under tolerogenic conditions results in prevention and partial suppression of the immune response toward Der p 2 (18). To ensure efficient presentation of the Ag to CD4⁺ Th cells in the context of MHC class-II determinants, the p21–35 transgene was linked to a late endosome targeting sequence encoding the melanosomal glycoprotein 75 (gp75) (11).

We have previously demonstrated that retroviral transduction of B cells results in constitutive IL-10 production in vivo (R. Roohi Ahangarani, W. Janssens, V. Carlier, L. VanderElst, T. VandenDriessche, M. Chuah, M. Jacquemin, and J. M. Saint-Remy, submitted for publication). This IL-10 production could be ascribed to stable epigenetic chromatin modification at the STAT3-binding site in the cognate IL-10 promoter and required the activation of the TLR2 signaling cascade following retroviral transduction (R. Roohi Ahangarani, W. Janssens, V. Carlier, L. VanderElst, T. VandenDriessche, M. Chuah, M. Jacquemin, and J. M. Saint-Remy, submitted for publication). We now demonstrate efficient in vivo induction of Tr1-like cells following adoptive transfer of gene-modified B cells, which confers long-lasting and robust Ag-specific immune tolerance. This study also points to a novel mechanism whereby the production of IL-10 by these gene-engineered B cells is necessary to establish persistent Ag-specific immune unresponsiveness. This represents a promising new and robust approach to induce Tr1 in vivo with important clinical implications.

Materials and Methods

Animals and reagents

BALB/c (H-2^d) mice were obtained from the University animal facilities. B6.129P2-IL10^{tm1Cgn}/Crl IL-10 deficient mice (backcrossed for 18 generations on the BALB/cJ background) were purchased from Charles River

Laboratories. Smooth LPS from *Escherichia coli* (055:B5) was purchased from Sigma-Aldrich. Smartladder and 15-mer-peptide p21–35 derived from the Der p 2 sequence were purchased from Eurogentec. Peptide sequence was CHGSEPCIIHRGKPF with a purity >85%. TaqMan probe (5'-CCG-GCC-GCT-TGG-GTG-GAG-AG) and primers set for NeoR, forward primer (GAT-GGA-TTG-CAC-GCA-GGT-T) and reverse primer (GTG-CCC-AGT-CAT-AGC-CGA-ATA) were also purchased from Eurogentec. WEHI 231-cells were purchased from the European collection of cell cultures (ECACC). All experiments were conducted in accordance with rules established by the University Ethical Committee for animal experiments.

Retroviral vector generation

The murine leukemia virus retroviral vector plasmid pMND-GFP was generated by cloning the GFP gene into the pMND-MFG-SN vector (provided by Dr. D. Kohn, Children's Hospital of Los Angeles). The chimeric construct p21gp75, encoding peptide 21–35 connected to the trans-membrane and cytosolic part of gp75 (amino acid 488–539) via a linker Ser-Gly-Gly-Ser-Gly-Gly-Ser-Gly-Gly, was made by PCR using partially overlapping primers (5'-CCGGAATCCCAACCATGGATTG CCAATGGTCAGAACCATGTATCATCTCGTTGGTAAACCATCTCT CTGGTGGTTCCGGAGGTTTCAGGAGG-3', 5'-CAGACAAGAAGC AACCCCGAAAATGGCAGCTACAAGTAACAACGCAGCCACTAC AGCAATGGTAATGATGCCTCTGAACCTCCGGAACC-3', 5'-GC ATAGCGTTGATAGTGATCAGTGAGGAGGAGGCTGGTTGGCTTCA TTCTGGTGCTTCTAGAACGGATCAGACAGAAGAAGCAACCCCG-3' and 5'-GTATCTCTCGAGTCAGACCATGGAGTGGTTAGGATT CCGGAGCTCCTCATAGTCTCAGCATAGCGTTGATAGTGATCA G-3'). The pMND-p21gp75 and pMND-p21–35 retroviral vectors were generated by restricting the p21gp75 and p21–35 fragments with EcoR I/XhoI and subsequent cloning into the respective sites of pMND-GFP. Stable retroviral vector producer cell lines that produced the MND-GFP, MND-p21–35, and MND-p21gp75 retroviral vector particles were obtained by introducing the corresponding constructs into GPE86 (19) packaging cells and subsequent selection in G418 (800 µg/ml), as described previously (15). The resulting packaging cell lines, designated as GPE-MND-GFP, GPE-MND-p21–35, and GPE-MND-p21gp75, were grown in DMEM (Life Technologies) supplemented with 10% heat-inactivated FBS (Life Technologies), 100 U/ml penicillin, 100 µg/ml streptomycin, and 200 mM L-glutamine (designated as D10 medium). Conditioned medium was collected over 24 h from confluent vector producer cell lines. This vector-containing supernatant was concentrated 200-fold using centricon ultrafiltration (Millipore Corporation). By using quantitative real-time PCR with TaqMan's set of specific primers and the probe for the amplification of neoR gene (20), vector genome copies in concentrated supernatants of packaging cell lines for the GFP, p21–35 and p21gp75 construct were established at 9×10^9 , 3.7×10^{10} , and 3.9×10^{10} per ml, respectively.

B cell transduction

Murine B lymphocytes were obtained from spleens of naive BALB/c mice by Ficoll density gradient purification (Nycomed Pharma) and subsequently enriched by positive selection using magnetic beads coupled with Abs to CD19 (Miltenyi Biotec). Purified B lymphocytes were cultured in the presence of 50 µg/ml LPS in enriched RPMI 1640 medium (Life Technologies), containing 10% FCS, 50 µM 2-ME, 100 U/ml penicillin, and 100 µg/ml streptomycin. The next day, concentrated viral vector-containing supernatant (200 µl; preparation as described above) supplemented with 6-µg/ml polybrene was added to the cells. To enhance transduction efficiency, cells were subjected to two successive rounds of centrifugation with vector-containing medium for 1 h at 2600 rpm and 20°C, separated by a 4 h interval. In some experiments, GFP-transduced B cells were loaded with p21–35 by overnight incubation with soluble peptide (30 µg/ml). GFP-transduced B cells were stained with anti-mouse CD19 PE-conjugated mAbs (BD Biosciences). Isotype-matched PE-conjugated mAb were used as negative controls. After staining and washing, 10,000 events were acquired with a FACSCalibur and analyzed by CellQuest software (BD Biosciences). Dying cells were excluded by gating and GFP fluorescence was measured to assess gene transfer efficiency. B cell survival was monitored by propidium iodide staining and cytofluorimetric analysis. All results were triplicates of independent samples and significance was calculated by one-way ANOVA.

T cell activation

T cells were obtained by Ficoll density gradient purification of splenocytes and negative selection (purity 85–90%) using magnetic beads against CD11c, CD11b, and CD45R (Miltenyi Biotec). Pools of T cells from four

mice were tested for cell surface Ag expression by direct immunofluorescence and flow cytometric analysis. T cells collected from lymph nodes and spleen 10 days after the second immunization were cocultured (10^6 cells/ $200\ \mu\text{l}$) in flat-bottom 96-well plates with peptide (p21–35, $30\ \mu\text{M}$). APCs were prepared by total B and T cell depletion from splenocytes and lymph node cells with CD19 and CD90 beads, respectively (Miltenyi Biotec). Supernatants were collected 24 and 48 h later for cytokine assay. IFN- γ , IL-2, and IL-10 were assayed using the BD OptEIA Set Mouse (BD Biosciences).

Ab responses

To determine the anti-p21–35 Ab titers, neutravidin microtitration plates (Perbio Science) were coated with biotin-labeled p21–35 ($2\ \mu\text{g}/\text{ml}$ in PBS, $100\ \mu\text{l}$) by overnight incubation at 4°C . Plates were washed with PBS-0.05% Tween and residual binding sites were blocked by $100\ \mu\text{l}$ of PBS-0.5% BSA for 1 h at RT. Fifty μl of serially diluted sera (1/200, 1/1000, 1/5000) were then added to the plates and incubated for 2 h at RT. Specific Ab binding was detected by addition of $50\ \mu\text{l}$ peroxidase-conjugated goat anti-mouse Ab (BD Biosciences) to either IgG2a or IgG1 and o-phenylenediamine. Anti-p21–35 IgG1 and IgG2a Abs were titrated against a standard curve established with a specific mAb of the same isotype produced in our laboratory. As control, microtitration plates (MaxiSorp Surface; NUNC) were incubated at 4°C overnight with $100\ \mu\text{l}$ PBS containing $5\ \mu\text{g}/\text{ml}$ tetanus toxoid (Aventis Pasteur). Specific Ab binding was detected as described for p21–35 specific Abs.

MHC-class II presentation of the p21gp75 Ag

To validate that the p21gp75 fusion peptide was presented in association with MHC-class II determinants, a proliferation assay was conducted using WEHI-231 cells (B cell lymphoma of BALB/c $\times$ NXB F_1 origin) transduced with the MND-p21gp75, and an MHC-II restricted cytotoxic T cell line (G-121) specific to p21–35 (21). p21gp75-transduced and nontransduced WEHI cells were cocultured for 32 h with T cell clone G-121 at a 1:1 ratio (5×10^4 cells/well). The G-121 clone did not proliferate under those conditions (21). In some conditions, 15-mer-peptides were added corresponding to peptides p21–35 and p71–85. Proliferation of the transduced or nontransduced WEHI cells was evaluated by measuring thymidine incorporation, following addition of $1\ \mu\text{Ci}/\text{well}$ of [^3H] thymidine during the last 18 h of incubation.

In vivo studies

For prevention experiments, B cells stably transduced with the MND-p21gp75 vector ($1.5 \times 10^7/\text{mouse}$) were injected i.p. into naive 6–8 wk-old female BALB/c mice. Ten days after cell transfer, recipient mice were challenged with a s.c. immunization of p21–35 ($25\ \mu\text{g}/100\ \mu\text{l}$) in CFA and boosted 14 days later with the same dose in IFA. Ten days after the second immunization, the animals were bled and sera were assessed for p21–35 specific Abs (IgG1 and IgG2a) by ELISA. Three control groups were added and submitted to the same immunization protocol with p21–35. To assess the specificity of the suppression, a group of four mice was treated according to the same protocol, but received a s.c. injection of tetanus toxoid ($50\ \mu\text{g}$ in $100\ \mu\text{l}$ in CFA/IFA) each time the p21–35 peptide was administered. For suppression experiments, an immune response was first established by two consecutive immunizations with p21–35 ($25\ \mu\text{g}/\text{mouse}$) in CFA/IFA. Successful immunization was confirmed at day 28 by assaying anti-p21–35 Ab titers. At day 30, mice were randomly separated into two groups (seven mice/group); group A was injected i.p. with 2×10^7 LPS-activated B cells transduced with the MND-P21gp75 and the control group B received 2×10^7 LPS-activated but not transduced B cells. Sixty days after cell transfer, all mice received a boost injection with p21–35 in IFA. The anti-p21–35 Ab titers were determined by ELISA.

Expression of p21gp75 in transduced B-cells in vivo

Expression from the p21gp75 construct in the transduced B cells in vivo was analyzed by RT-PCR analysis. Three months after adoptive transfer, mice were euthanized and B cells were purified from the spleen by Ficoll density gradient purification and enriched by positive selection using magnetic beads coupled with Abs to CD19. mRNA was extracted using the QuickPrep Micro mRNA purification kit (Amersham Biosciences) and converted into cDNA using the First Strand cDNA synthesis kit (Amersham Biosciences). PCR was conducted on cDNA using retroviral vector-specific primers, 5'-CCCTTTATCCAGCCCTCACTC-3' and 5'CCTGGGACTTTCCACACCC-3'. RT-PCR products were separated by gel electrophoresis and visualized with a Stratagene Eagle Eye II (Stratagene).

CD4⁺CD25[−] T cells purification and transfer

CD4⁺CD25⁺ and CD4⁺CD25[−] T cells were separated by magnetic cell sorting (CD4⁺CD25⁺ T cells isolation kit, Miltenyi Biotec) according to manufacturer's instructions. Both cell populations were $>90\%$ pure as assessed by flow cytometry. CD4⁺CD25[−] T cells were injected i.v. (7×10^7 cells/mouse) into 6–8 wk recipient female BALB/c mice.

FACS analysis

Flow cytometry was conducted on a Becton Dickson FACScan. Fluorescence-conjugated Abs were purchased from eBioscience: CD25 (PE; clone 7D4), CD28 (PE; clone 37.51), ICOS (PE; clone 7E.17G9), CTLA-4 (PE; clone UC10–4F10–11), IA/IE (FITC; clone 2Gg), rat anti-mouse CD4 (FITC; RM4–5), Cy5.5 anti-mouse CD25 (PerCP; PC61), and anti-mouse Foxp3 (PE; Clone: FJK-165). Data were analyzed using the CellQuest software.

Experimental asthma model

BALB/c mice were sensitized by i.p. injection of alum-adsorbed recombinant allergen (rDer p 2) followed by intranasal instillation as described (22). Briefly, one day after, pulmonary function was assessed by whole body plethysmography upon challenge with increasing doses of methacholine. A second series of nasal instillations was conducted 10 days later followed by pulmonary function evaluation. The enhanced pause (Penh) was used as a parameter for airway responsiveness (18). Three days later mice were sacrificed for bronchioalveolar lavage fluid (BALF) collection. Cell composition was established on cytopins and cytokine concentrations determined by ELISA. Formaldehyde-fixed lungs were embedded in paraffin for sectioning ($7\ \mu\text{m}$ -thick slides) and staining. Eosinophils and goblet cells were detected by May-Grünwald Giemsa and periodic acid-Schiff reaction, respectively. All slides were examined by two persons, including a pathologist (B.W.) who was unaware of the groups to which mice belonged. Anti-Der p 2 Ab concentrations were determined using Neutravidin-coated ELISA plates (Pierce) coated with $2\ \mu\text{g}/\text{ml}$ biotin-labeled rDer p 2. Isotype-specific Abs were used for ELISA determination of IgG1, IgG2a, and IgE allergen-specific Abs.

Airway hyperreactivity (AHR) assay

AHR responses were measured in six mice per group using a forced oscillation technique (FlexiVent, SCIREQ) (23). Airway resistance (R) was assessed after exposure to increasing doses of methacholine. Mice were anesthetized with an intraperitoneal injection of pentobarbital ($70\ \text{mg}/\text{kg}$). The trachea was exposed and a 19-gauge metal needle was inserted into the trachea. Mice were connected to a computer-controlled small animal ventilator and quasi-sinusoidally ventilated with a tidal volume of $10\ \text{ml}/\text{kg}$ at a frequency of 150 breaths/minute and a positive end-expiratory pressure of $2\ \text{cm H}_2\text{O}$ to achieve a mean lung volume close to that during spontaneous breathing. After measurement of a baseline, each mouse was challenged with methacholine aerosol, generated with an in-line nebulizer, and administered directly through the ventilator for 5 s, with increasing concentrations (0, 0.625, 1.25, 2.5, 5, and $10\ \text{mg}/\text{ml}$). Airway resistance (R) was measured using a "snapshot" protocol each 20 s for 2 min. The mean of these six values was used for each methacholine concentration, unless the COD (coefficient of determination) of a measurement was smaller than 0.95. For each mouse, R was plotted against methacholine concentration (from 0 to $10\ \text{mg}/\text{ml}$) and the area under the curve (AUC) was calculated. Results are expressed as mean Penh values ($\pm$ SEM) measured every 30 s over 3 min after challenge with each of the indicated concentrations of methacholine.

Statistical analysis

Statistical evaluation was conducted with the Instat 3 software (GraphPad Software). Triplicates of FACS, thymidine incorporation, and thymidine release results were expressed as mean with SD. Differences in Ab titers were evaluated by Kruskal Wallis, Mann-Whitney, or paired Wilcoxon test, where appropriate. AHR data are shown as means and SEM. AUC data of the resistance are shown as individual values and groups mean. Data on airway resistance (R) upon methacholine provocation were analyzed using a two-way ANOVA, while de AUC of R was analyzed using a Student *t* test (GraphPad Prism 4.01, GraphPad Software). A level of $p < 0.05$ (two-tailed) was considered significant.

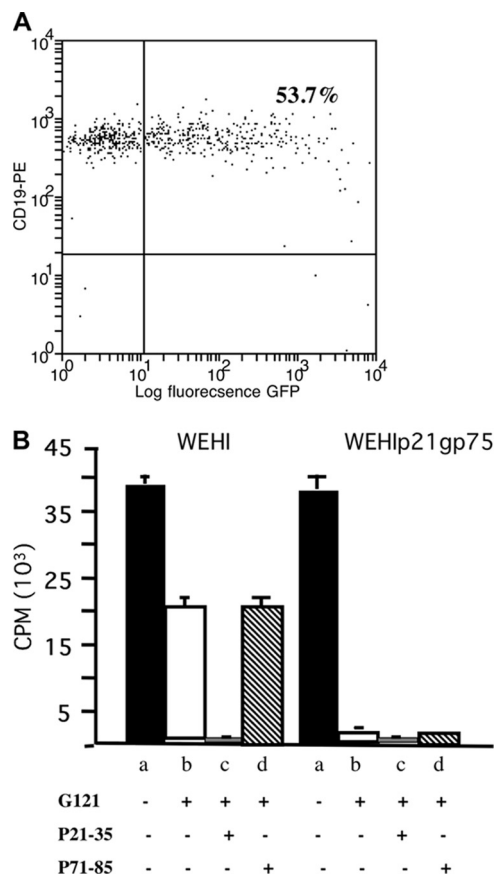


FIGURE 1. B cells are efficiently transduced and present p21–35 by MHC class II determinants. *A*, FACS analysis of CD19-positive cells activated by LPS and transduced by two runs of centrifugation with the MND-GFP vector. The percentage of GFP-positive B cells is illustrated as obtained 24 h after transduction. *B*, Lysis of nonirradiated WEHI-231 cells (*left*) or WEHI cells transduced with the p21gp75 vector (*right*) in the presence of a p21–35 specific cytotoxic T cell line (G-121). Thymidine incorporation (cpm) was used as a parameter reflecting cell lysis. WEHI cells were incubated alone (black column; a), in the presence of the G-121 T cell line without added p21–35 peptide (white column; b), with the G-121 T cell line and addition of p21–35 (gray column; c), or with the G-121 T cell line and addition of a control p71–85 peptide (striped column; d). Peptides were added at 18 μ M/ml. Cocultures were seeded at 1:1 target:effector ratio and harvested 32 h later. Data shown are means and SD from triplicate wells.

Results

B cells are efficiently transduced for p21–35 presentation by MHC class II determinants

BALB/c mouse B cells were activated with LPS and transduced with a Moloney murine leukemia virus-based retroviral vector encoding the Der p 2-derived T cell epitope p21–35 fused to the gp75 protein (MND-p21gp75). Transduction efficiency was checked with a GFP control vector (MND-GFP). Fig. 1*A* shows that 200 μ l of a highly concentrated vector preparation applied to LPS-activated B cells typically resulted in >50% transduction efficiency. We subsequently confirmed that the p21–35 peptide was actually presented within the context of MHC-class II determinants using stably transduced WEHI-231 cells and a MHC-class II restricted T cell line (G-121) known to lyse target cells after cognate interaction with p21–35 (21) (Fig. 1*B*). In contrast, nontransduced WEHI control cells were lysed by the G-121 T cell line only after addition of soluble p21–35 peptide.

p21gp75 transduced B cells prevent Ab production upon peptide immunization

The p21–35 sequence, which contained a B cell epitope and an immunodominant T cell epitope (24), was used for assessing the effect of transduced B cells on specific Ab production. Fig. 2*A* outlines the experimental protocol followed to evaluate whether p21gp75 transduced B cells would prevent an immune response toward p21–35. Four groups of BALB/c mice ($n = 8$) were adoptively transferred (i.p. injection, 1.5×10^7 cells/mouse) with B lymphocytes. Group A mice were adoptively transferred with B cells loaded with the p21–35 peptide. Mice in group B or C were adoptively transferred with B cells activated with LPS or p21gp75 transduced B cells, respectively. Group D mice received GFP-transduced B cells. Persistence of the p21–35gp75 transcript was assessed in group C mice. All animals were then immunized by three s.c. injections of p21–35 in CFA/IFA.

Fig. 2*B* shows the results of specific IgG1 and IgG2a Ab titers. Median Ab values are given in Fig. 2*C*. An increase in p21–35 specific IgG1 and IgG2a Abs was observed following each immunization in mice having received LPS-activated nontransduced B cells with (group A) or without loaded peptide (group B), or GFP-transduced B cells (group D). Interestingly, Ab concentrations at the time of first bleeding (day 24) were significantly higher in group A mice (B cells loaded with p21–35) than in group B or D (Kruskal-Wallis test, $p < 0.0003$ for IgG1 and for IgG2a). In contrast, mice receiving B cells transduced with the p21gp75 construct (group C) showed significantly reduced values (at days 24, 40, and 70) for both IgG1 and IgG2a Abs (Kruskal-Wallis test, $p < 0.01$ for IgG1 and IgG2a). Yet in vitro, peptide-loaded, and transduced B cells presented the peptide with comparable efficacy as evaluated by p21–35 specific activation of a CD4⁺ T cell effector clone (data not shown).

RT-PCR analysis revealed that the integrated vector genome stably expressed the p21–35gp75 mRNA for at least 3 mo after-injection of the genetically modified B cells in six of seven mice (Fig. 2*D*). Only one mouse (number 4 in the Fig. 2*D*) failed to express p21–35gp75, which likely explains why no reduction in Ab titer was apparent in that mouse (open symbols in Fig. 2*B*, column c). These data suggest that the gene-modified B cells may not have engrafted in that mouse and hereby further establish a causal relationship between long-term persistence of gene-modified B cells and lasting tolerance induction. These observations show that transduced B-lymphocytes expressing the T cell epitope of p21–35 in MHC class II determinants effectively prevented a specific immune response even after recurring peptide injections with a potent adjuvant.

To verify that unresponsiveness to immunization was specific for the peptide, an additional group of four mice was treated according to the same protocol except for coimmunization with tetanus toxoid. Titers of both p21–35 and tetanus toxoid specific Abs were evaluated and compared with those obtained in naive mice. Fig. 2*E* shows IgG1 Ab titers for p21–35 and tetanus toxoid 10 days after immunization. As expected, we found a significant decrease in the titer of anti-p21 IgG1 (Mann-Whitney U test, $p < 0.03$), but no difference in the titer of tetanus toxoid IgG1.

To determine whether the presentation of p21–35 by MHC class II determinants was important for preventing specific Ab formation, we conducted an additional experiment in which B cells were transduced with a construct devoid of gp75, the late endosome targeting sequence. This should significantly reduce the amount of peptide loaded on class II molecules, though not abolishing it, and thereby reduce the efficiency of specific Ab formation. Thus, mice transferred with B cells transduced with either the gp75-containing

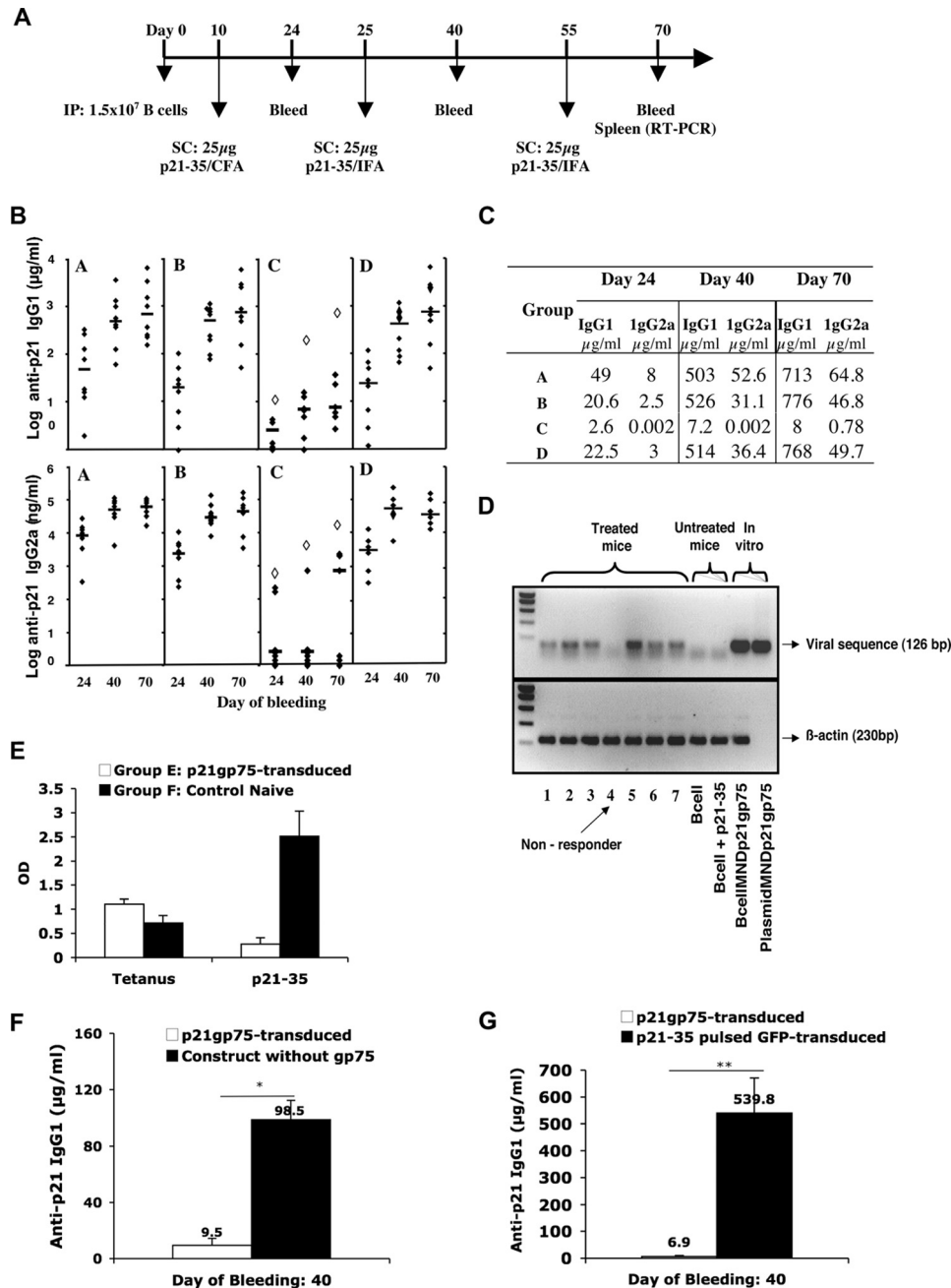


FIGURE 2. Prevention of specific Ab response in transduced B cell recipient mice. *A*, Outline of the protocol for B cell adoptive transfer and p21–35 immunization. *B*, Concentrations of anti p21–35 IgG1 and IgG2a Abs measured by ELISA after each of three consecutive immunizations. Group A mice ($n = 8$) were transferred with LPS-activated B cells loaded with p21–35; group B mice ($n = 8$) were transferred with naive LPS-activated B cells; and group D mice ($n = 8$) were transferred with GFP-transduced B cells. Group C mice ($n = 8$) were transferred with p21gp75 transduced B cells. *Upper panel*, Individual anti-p21–35 IgG1 titers ($\mu\text{g/ml}$) and means after each immunization. *Lower panel*, Individual anti-p21–35 IgG2a titers (ng/ml) and means. *C*, Median values of anti p21–35 Abs. *D*, RT-PCR analysis demonstrating the presence of transgenic B cells in recipient mice 3 mo after adoptive transfer. mRNA was extracted from individual mouse spleen cells ($n = 7$) adoptively transferred with transduced B cells and immunized with p21–35 (group C). As a control, mRNA was extracted from B cells freshly transduced with the p21gp75 vector. RT-PCR products were separated by electrophoresis on a 1.5% agarose gel and the size of the products was compared with that of plasmid pMND-p21gp75. Lower bands represent amplified β -actin. A MW marker is shown on the left. *E*, p21–35 and tetanus toxoid specific IgG1 Abs obtained in mice adoptively transferred with p21gp75 transduced B cells (group E; $n = 4$) and in control naive mice (group F; $n = 4$). Mice were immunized simultaneously by s.c. injections of p21–35 and tetanus toxoid in IFA. Data are presented as means $\pm$ SEM. *F* and *G*, Concentrations of anti-p21–35 IgG1 Ab ($\mu\text{g/ml}$) measured by ELISA after two immunizations. *F*, Mice ($n = 6/\text{group}$) were transferred with B cells transduced either with a full construct containing a gp75 sequence (p21gp75-transduced) or the same construct in the absence of gp75. *G*, Mice ($n = 6/\text{group}$) were transferred with p21gp75 transduced B cells (p21gp75-transduced) or GFP-transduced B cells pulsed with p21–35 peptide (p21–35 pulsed GFP-transduced). Bars represent means $\pm$ SEM. *, $p < 0.05$; **, $p < 0.01$.

construct or not ($n = 6$ in each group) were then immunized twice with p21–35 and the concentration of specific Abs evaluated thereafter as described above. Fig. 2*F* indicates that the production of

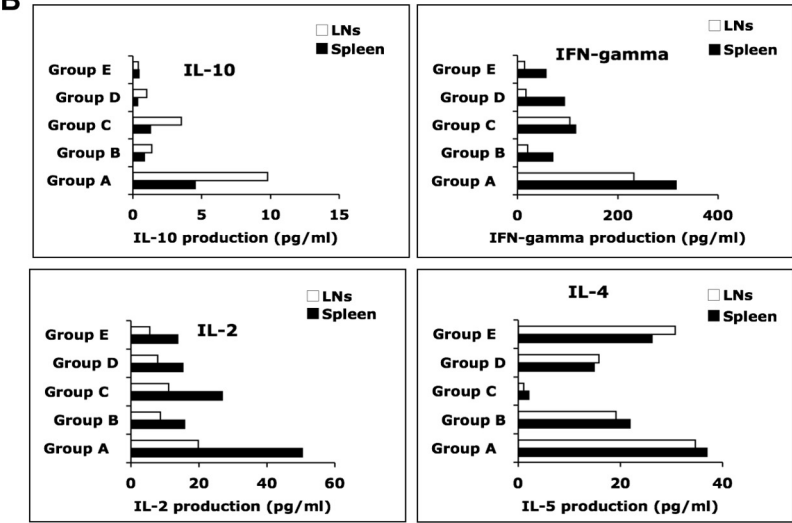
specific IgG1 Abs (day 40) was ~ 10 -fold higher in mice transferred with B cells containing no gp75 than in the group receiving B cells transduced with a full gp75-containing construct. Yet, Ab

FIGURE 3. Transduced B cells expand a population of T cells expressing CTLA-4 and producing IFN- γ and IL-10. **A**, BALB/c mice were adoptively transferred with LPS-activated B cells either loaded with p21–35 (group A), with no peptide (group B), transduced with p21–35 (group C), or transduced with GFP (group D) as in Fig. 2. CD4⁺ spleen T cells obtained 10 days after B cell transfer and p21–35 injection were analyzed by FACS for expression of five activation markers. Figures represent percentages of positive cells in pools of four spleens. nd: not detected. **B**, IFN- γ , IL-2, IL-10, and IL-4 production by spleen and lymph node cells (pooled from four mice) measured by ELISA in supernatants of cells incubated with 30 μ M p21–35 for 48 h. Mouse groups are as in **A**. A group of mice not receiving B cells was added as a control (group E). Results are given in pg/ml. **C**, Density plots of expression of Foxp3 assessed by FACS analysis in CD4⁺ T cells from mice transferred with p21gp75-transduced B cells: percentage of Foxp3⁺ cells in the CD4⁺CD25⁺ (left) and in the CD4⁺CD25[−] (right) T cells.

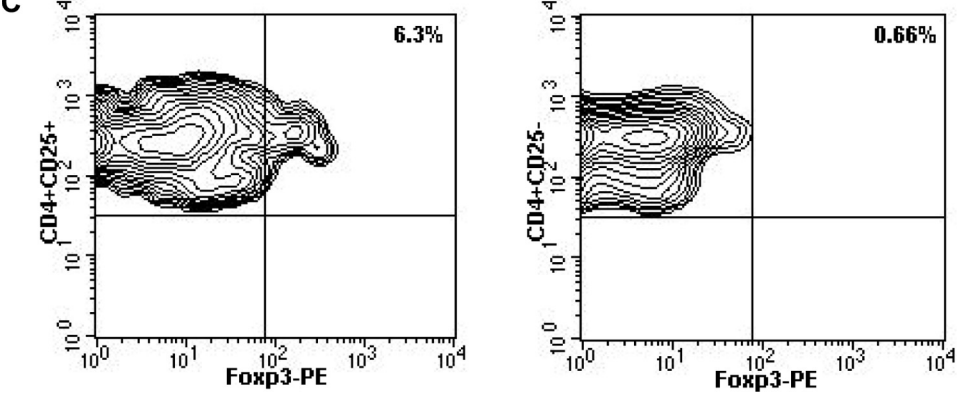
A

Group	Surface Markers					
	CD25	Icos	FasL	CD28	CTLA-4 (Intracellular)	CTLA-4 (surface)
A	10.4	39.1	3	1.3	nd	2
B	8.8	39.7	2.7	1.7	nd	2.5
C	8.9	31.2	5.9	2.4	10.74	3.2
D	9.6	37.9	3	1.5	nd	1.5

B



C



concentrations were ~5-fold lower than in mice receiving, for instance GFP-transduced B cells (see Fig. 2C, group D). These data showed that in the absence of gp75, reduced class II-driven p21–35 presentation was sufficient to significantly reduce the efficiency of Ab suppression.

To further confirm that Ag persistence was important for the prevention of Ab formation, we pulsed GFP-transduced B cells with p21–35 in vitro before transferring them to a group of naive BALB/c mice ($n = 6$), which were then immunized twice with the peptide as described above. Fig. 2G shows that the concentration of specific IgG1 Abs in mice transferred with pulsed B cells was similar (day 40) to that obtained in naive mice. Taken together, these experiments indicated that stable expression of Ag within the context of MHC class II molecules was essential for efficient suppression of specific Ab production.

We next determined whether the transfer of transduced B cells affected the Ag-specific T cell response. Four groups of mice were

treated according to the protocol described in Fig. 2A, but mice were sacrificed 10 days after the second peptide injection. Purified CD4⁺ T cells were obtained by negative selection from splenocytes and used to assess expression of five surface activation markers by FACS analysis. The results are shown in Fig. 3A. T cells expressing intracellular CTLA-4 were observed only in group C mice, which had been transferred with p21gp75 transduced B cells but no significant difference was observed between the groups for surface expression of CTLA-4. A doubling of FasL⁺ cell numbers was also observed in group C mice. Th1 and Th2 cytokines were evaluated in supernatants of T cells prepared from spleen or peripheral lymph nodes after 48 h of culture with peptide. A group of mice that had received no B cells was added (group E) as a control. As shown in Fig. 3B, an increased production of IL-2, IL-10, and IFN- γ was observed only with cells obtained from animals treated with p21gp75 transduced B cells (group C), while such cells did not produce IL-4 or TGF- β (data not shown).

To determine whether in vivo exposure to transduced B cells resulted in expansion of p21–35 specific CD4⁺ cells expressing Foxp3, purified CD4⁺ T cells were first activated in vitro by two cycles of exposure to p21–35-loaded APCs, in the presence of 10 IU/ml IL-2 for the second stimulation cycle. On day 7 after the second expansion, Foxp3 expression was detected by FACS with gating on CD25⁺ population. Fig. 3C shows that CD4⁺CD25⁺ cells did not express Foxp3 (*right panel*), while 6.3% of CD4⁺CD25⁺ cells were Foxp3⁺, representing the expected proportion of natural regulatory T cells. Our results therefore indicated that induction of T cell tolerance by transduced B cells did not involve expression of Foxp3.

Taken together, these data suggest that B cells transduced with the p21gp75 construct induced activation of Tr1 (25).

p21gp75 transduced B cells suppress ongoing specific Ab production

We next evaluated whether transduced B cells also suppressed an established immune response toward the peptide. To this end, BALB/c mice (*n* = 14) were immunized by two s.c. injections of p21–35 in CFA/IFA and randomly separated into 2 groups at day 30, just before cell transfer. Group A and B mice received p21gp75 transduced B cells or LPS-activated B cells (Fig. 4A). Serum anti-p21–35 Abs were titrated 2 wk after peptide immunization and their titers compared with those obtained before B cell transfer. Group B mice showed an increase of specific Abs after immunization, as expected (Fig. 4, B and C). By contrast, mice from group A had a significant decrease in median anti-p21–35 IgG1 and IgG2a titers after transfer of transduced cells (paired Wilcoxon test, *p* < 0.02 for both Ab isotypes). These observations indicate that transgenic B cells could, at least partly, suppress an established p21–35 immune response, even when potent adjuvants were used.

Cytofluorimetric analysis of T cell surface markers from spleen cells obtained at the end of the experimental protocol showed an increased proportion of cells carrying intracellular CTLA-4 and FasL (Fig. 5A) and no secreted TGF- β production (data not shown) in the group of mice transferred with transduced B cells, confirming the data shown in prevention experiments. The production of IL-2, IL-10, IL-4, and IFN- γ after 48 h of culture with p21–35 was determined as described for prevention experiments (Fig. 2A). As shown in Fig. 5B, an increase in IL-10 and IFN- γ and the absence of IL-4 was observed in mice transferred with p21gp75 transduced B cells which is again consistent with a Tr1-like phenotype.

IL-10^{-/-} transduced B cells do not confer tolerance

The results so far suggested that transduced B cells exerted a long-lasting prevention and/or suppression effect on specific Ab production via T cells. In addition, T cells showed up-regulation of CTLA-4 and FasL expression together with IL-10 production, both in prevention and suppression experiments. To ascertain that IL-10 production was necessary to confer Ag-specific unresponsiveness, splenic B cells from homozygous IL-10 deficient (IL-10^{-/-}) BALB/c mice were transduced with the MNDp21gp75 vector, according to the same protocol as described above for wild-type B cells. Expression of p21gp75 in the IL10^{-/-} B cells was confirmed by RT-PCR (data not shown). Transduced IL10^{-/-} B cells were transferred to naive wild-type BALB/c mice, followed 10 days later by a s.c. immunization of p21–35 (25 μ g/100 μ l) in CFA and two boost injections at days 25 and 55 with the same dose of peptide in IFA (see protocol outline in Fig. 2A). The production of specific Abs was then evaluated.

The results illustrated in Fig. 6, A and C show that the titers of anti-p21–35 IgG1 and IgG2a Abs were not reduced at any time

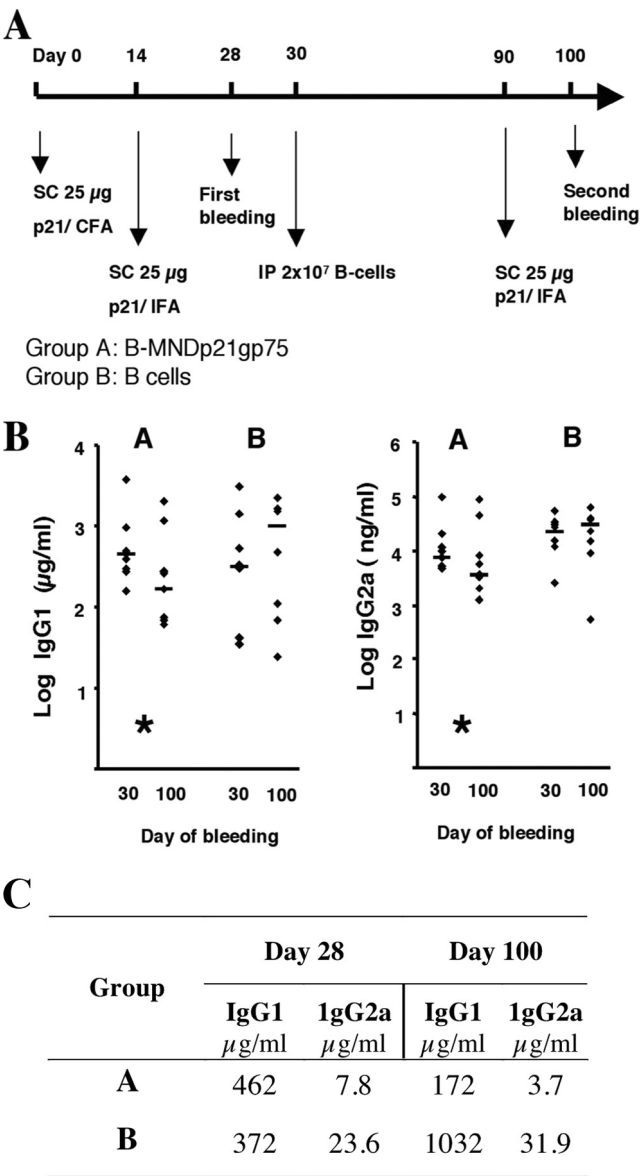


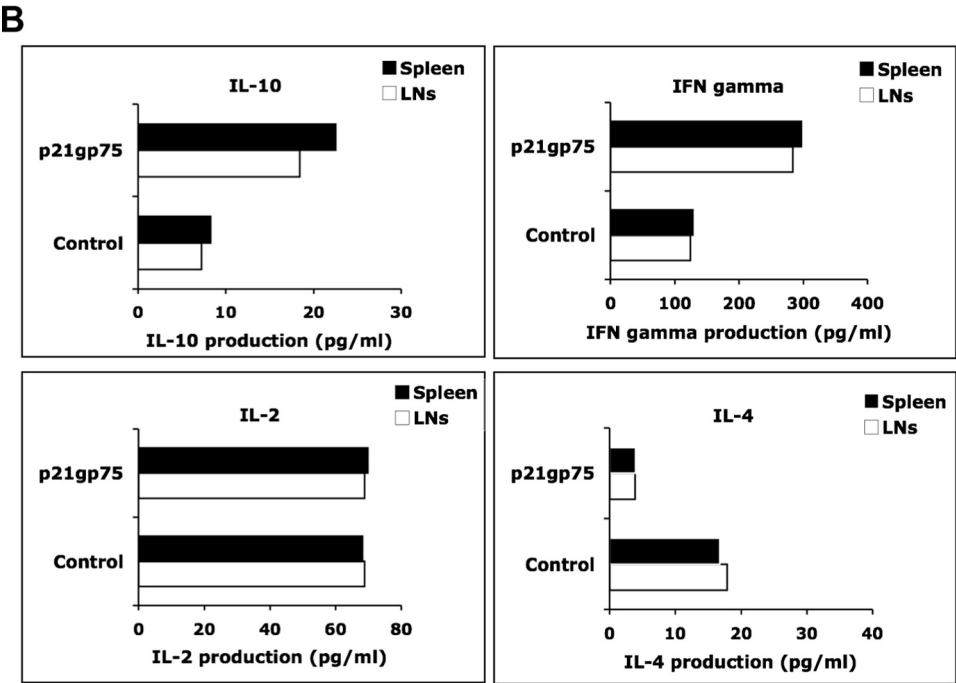
FIGURE 4. Suppression of specific Ab response in transduced B cells recipient mice immunized with p21–35. **A**, Outline of the protocol for p21–35 immunization and B cell adoptive transfer. **B**, Concentrations of anti p21–35 IgG1 and IgG2a Abs measured by ELISA after two immunizations and before cell transfer, and after the third immunization. Group A mice (*n* = 8) were transferred with p21gp75 transduced B cells and group B mice (*n* = 8) were transferred with naive LPS-activated B cells. Individual values and medians of anti p21–35 IgG1 titers (*left*, μ g/ml) and IgG2a (*right*, ng/ml) obtained after two and three immunizations are shown, statistic significance is demonstrated by an asterisk. **C**, Median values of anti p21–35 Abs.

point in mice receiving IL10^{-/-} B cells transduced with the MNDp21gp75 vector, as compared with mice that received wild-type B cells transduced with the same vector (Fig. 6, B and D). The inability of the MND-p21gp75 transduced IL-10^{-/-} B cells to inhibit the specific Ab response was not due to impaired homing or elimination of the genetically modified cells to or from lymphoid organs, as mRNA for p21gp75 from IL-10^{-/-} B-cells could readily be detected by RT-PCR in the spleen of recipient mice 3 mo post transplantation (data not shown), consistent with the long-term persistence of gene modified B cells in wild-type BALB/c mice. This confirms that the elevated IL-10 production following transduction of B cells with MND-p21gp75 was necessary to confer Ag-specific unresponsiveness in BALB/c mice.

A

Surface Markers					
Group	CD25	Icos	FasL	CD28	CTLA-4
A	3.28	18.84	2.33	3.9	7
B	1.28	15.36	0.27	6.7	2.9

FIGURE 5. p21–35 immunized recipients of transduced B cells show increased CTLA-4 expression by T cells and production of IFN- γ and IL-10. **A**, BALB/c mice ($n = 4$) were immunized by s.c. administration of p21–35 followed by adoptive transfer of either LPS-activated p21-transduced B cells (group A) or B cells activated by LPS (group B). CD4⁺ spleen T cells obtained 10 days after cell transfer were analyzed by FACS for expression of five activation markers. Figures represent percentages of positive cells in pooled from four spleens. **B**, IL-10, IFN- γ , IL-2, and IL-4 production by spleen and lymph node cells (pool of four mice for lymph nodes) were measured by ELISA in supernatants of cells incubated with 30 μ M p21–35 for 48 h. Results are given in pg/ml.



Transfer of CD4⁺ T cells prevents experimental allergic asthma

We next wanted to determine whether CD4⁺ T cells elicited by the transfer of p21gp75-transduced B cells were able to prevent the induction of experimental asthma. Thus, BALB/c mice were adoptively transferred by i.v. injection of either 7×10^6 CD4⁺CD25⁺ T cells or 7×10^6 CD4⁺CD25⁻ T cells obtained from splenocytes of genetically modified B cells recipients after p21–53 immunization and sorted using magnetic beads. Allergen sensitization and nasal instillation was then initiated. Specific anti-Der p 2 Abs in serum, BALF cell and cytokine contents, lung histology, and airway response to methacholine were then evaluated. Results were compared with a validated control model established in our laboratory.

Fig. 7 provides a direct comparison of the results obtained upon transfer of CD4⁺CD25⁺ T cells (namely, natural regulatory T cells) with those obtained in mice receiving CD4⁺CD25⁻ T cells that contained the induced Tr1-like cells. Although the CD4⁺CD25⁺ population contained a majority of cells with regulatory properties while the CD4⁺CD25⁻ T cells should contain only a minor proportion of Ag-specific Tr1-like cells, the data provided in Fig. 7 shows significantly stronger suppression with Tr1-like cells. Thus, the concentration of Der p 2 specific IgG1 Abs in peripheral blood, the number of cells, and in particular that of eosinophils in BALF and in lung tissue, as well as signs of nonspecific reactivity measured by methacholine inhalation were all significantly lower

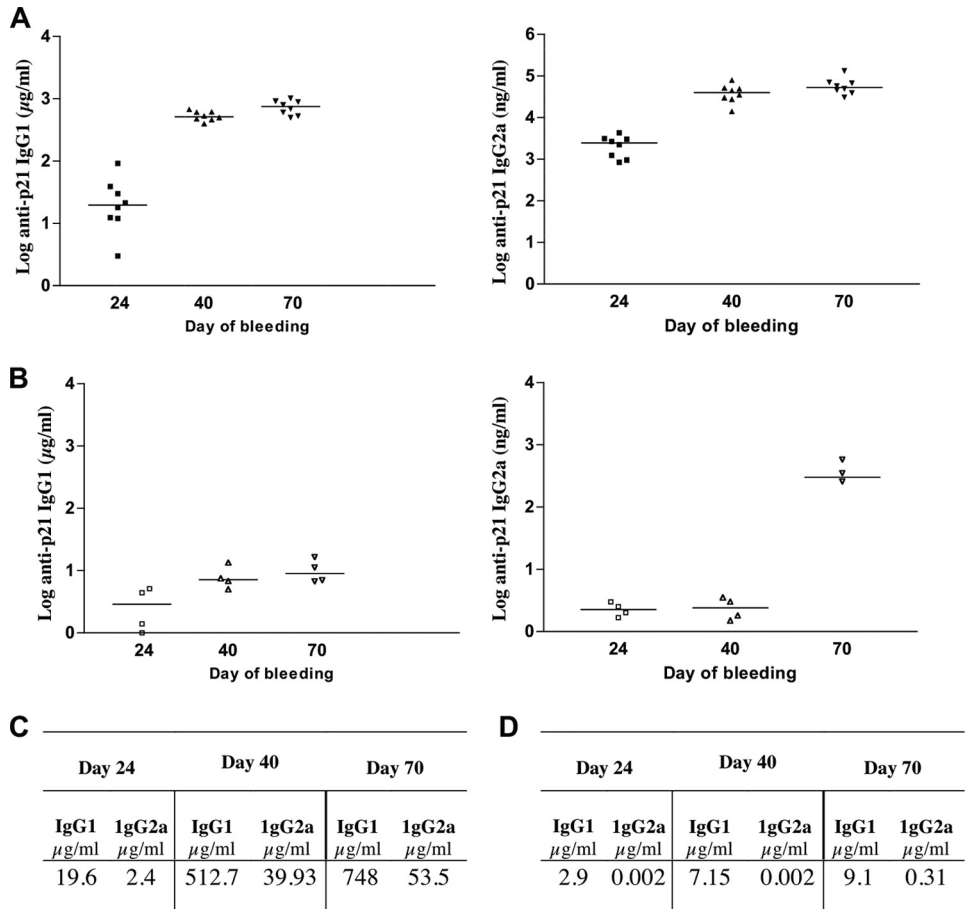
than levels obtained with the transfer of the same number of CD4⁺CD25⁺ T cells. In addition a trend toward more IL-10 production in BALF was observed with Tr1-like cells.

Additional experiments were conducted to exclude that the mere activation of B cells with LPS (without any transduction) would not elicit the same preventive effects on Ab production or asthma induction. B cells were activated by LPS and transferred in a first BALB/c mouse. CD4⁺ T cells were then recovered from the mice and separated into CD25⁺ and CD25⁻ population by cell sorting. CD4⁺CD25⁻ T cells were then used to reconstitute a second host mouse, which was then immunized twice with p21–35. A control group of mice was reconstituted with CD25⁻ T cells obtained from mice reconstituted with p21gp75-transduced B cells. We measured the concentrations of p21–35 specific Abs in the second host. Data shown in supplementary Fig. 1⁴ indicate that mice belonging to the group receiving T cells from hosts transferred with LPS-activated B cells produced concentrations of Abs comparable to those obtained in naive animals, which had received no T cells.

We also assessed the capacity to induce asthma-like alterations directly in the first host mouse. In such a case, two groups of BALB/c mice were reconstituted with B cells either p21gp75-transduced or B cells only activated by LPS. All mice were sensitized with IP Der p 2, and then submitted to nasal instillation.

⁴ The online version of this article contains supplementary material.

FIGURE 6. p21gp75 transduced B cells from BALB/c IL-10^{-/-} mice do not confer specific unresponsiveness. BALB/c mice (*n* = 8) were transferred with LPS-activated p21gp75 transduced B cells from IL-10^{-/-} (A) or wild-type (B) animals, and concentrations of anti p21–35 IgG1 and IgG2a Abs were measured by ELISA 2 wk after each of three consecutive immunizations in both groups. A and B, Left panel, Individual anti-p21–35 IgG1 titers (μg/ml) and means after each immunization. Right panel, Individual anti-p21–35 IgG2a titers (ng/ml) and means in the recipients of IL-10^{-/-} (A) and wild-type (B) transduced B cells, respectively. C and D, Median values of anti p21–35 Abs for the recipients of IL-10^{-/-} (A) and wild-type (B) transduced B cells, respectively.



Results of such experiments are given in supplementary Fig. 2, indicating that mice receiving LPS-activated B cells showed alterations comparable to those of naive mice receiving no B cells, while mice receiving fully transduced B cells showed no specific Ab production, no eosinophil in BALF and no increased airway reactivity to methacholine. These data therefore confirmed that B cell activation by LPS only did not suffice to elicit tolerance.

Taken together, the transfer of Tr1-like cells fully abrogated both lung and BALF accumulation of eosinophils and reduced airway hyperreactivity down to levels observed in naive animals. These data established the physiological relevance of induced Tr1-like cell efficacy in preclinical model of allergic asthma.

Discussion

The present data establish proof of concept that gene-modified B cells presenting a peptide in the context of MHC class II determinants induce an Ag-specific and long-lasting state of unresponsiveness when such B cells are transferred either before or after immunization. To our knowledge, this is the first study showing that endosomal retargeting in B-cells can induce robust long-lasting immune tolerance in both prophylactic and therapeutic settings, even in the face of recurrent immunizations with adjuvant. Evidence is presented showing that unresponsiveness to peptide immunization correlates with production of IL-10 by transduced B cells and elicitation of Tr1-like regulatory T cells.

The induction of Ag-specific immune tolerance using gene-modified B cells required a robust gene transfer approach, permitting stable expression of the cognate Ag. To achieve this, retroviral vectors were used as they are ideally suited to constitutively express the transgene following their stable genomic integration into the target cell genome. Though B cells are notoriously difficult to transduce, a robust trans-

duction efficiency of >50% was achieved, contingent upon LPS activation and the use of an optimized transduction protocol (15). As it was known that the long terminal repeats of retroviral vectors can be transcriptionally silenced, a modified promoter (MND) was used which is less prone to transcriptional silencing (26). Indeed, our results demonstrate that prolonged transgene expression could be obtained in vivo for at least 3 mo after adoptive transfer of the gene-modified B cells. The retroviral construct was designed to express a peptide encompassing a major T cell epitope of the Der p 2 allergen (p21–35) linked to the endosomal targeting peptide gp75. Using an MHC-II-restricted cytotoxic T cell line (21), we demonstrated that the intracellularly expressed p21–35 peptide was effectively retargeted toward the endocytic pathway for efficient expression within MHC-class II determinants.

The use of retrovirally transduced B cells expressing p21–35 was an obligate step for efficient induction of unresponsiveness. Though the number of B cells used to induce tolerance was relatively high, it is unlikely that this contributed to an specific effect, because control mice injected with an equal number of nontransduced or GFP-transduced B cells showed no induction of an immune response. Moreover, in earlier studies performed by others (27, 28), a comparable number of B cells (15–20 × 10⁶) were transferred to confer Ag-specific immune response.

Efficient induction of unresponsiveness by p21gp75 transduced B cells was consistent with a robust decrease in p21–35 Ab titers. In contrast, nontransduced LPS-activated B and transduced LPS-activated B cells expressing GFP did not induce p21–35 specific unresponsiveness, as comparable high-titer p21–35 specific Abs were detected in these cohorts. In fact, LPS-activated B cells loaded with peptide showed an opposite effect on the p21–35 immune response as compared with transduced B cells expressing

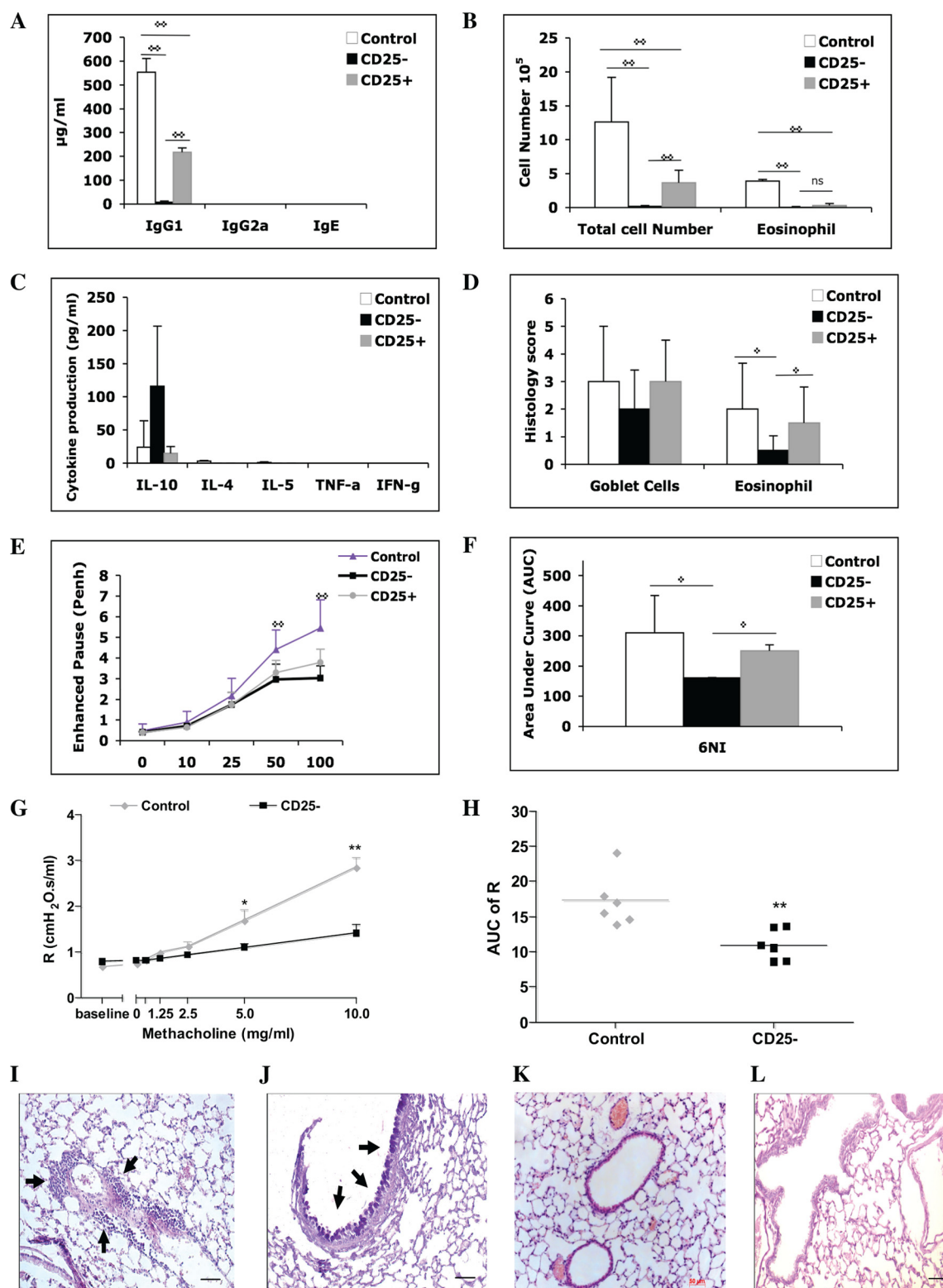


FIGURE 7. Evaluation of experimental asthma in BALB/c mice. **A**, Derp 2-specific serum Ab levels obtained after two series of rDer p 2 nasal instillations in BALB/c mice presensitized by i.p. immunization. Bars represent mean $\pm$ SEM; *, $p < 0.05$; **, $p < 0.01$. **B**, Comparison of BALF cell content in BALB/c mice sensitized with Der p 2 with or without CD4⁺ T cell transfer. BALF total cell and eosinophil numbers on day 56, namely 3 days after the last series of nasal instillations. Cells obtained by bronchoalveolar lavage were counted and identified on cytopins; ns, not significant. **C**, Comparison of BALF cytokine concentrations (pg/ml; mean $\pm$ SEM). **D**, Histological scoring. Scores were established for eosinophil and goblet cells, on a scale from 0 to 6 as a function of increased infiltration. Bars represent mean $\pm$ SEM. **E**, Methacholine airway responsiveness, Penh values ($\pm$ SEM). **F**, Means $\pm$ SD of areas under the curve (AUC) for Penh values. **G** and **H**, Airway reactivity, expressed as resistance (R), measured by the Flexivent perturbation Snapshot (**G**) and mean R and individual values and mean area under the curve (AUC) of R (**H**). *, $p < 0.05$ and **, $p < 0.01$ compared with control mice. **I-L**, Der p 2-induced lung inflammation and bronchial mucus production. Lung sections from control group (**I** and **J**) and mice transferred with CD4⁺ T cells (**K** and **L**). Lung cryosection slides were stained with HE (**I** and **K**) or periodic acid-Schiff (**J** and **L**). Scale bars represent 50 µm.

p21–35gp75, as Ab production increased. This indicates that intracellular expression of p21–35 and MHC-II presentation is necessary to confer Ag-specific unresponsiveness.

Viral vector-mediated gene transfer may however alter cell biology in part by triggering virus-responding intracellular pathways (29). It was therefore mandatory to verify that induction of unresponsiveness was specific for the Ag. Coimmunization with p21–35 and tetanus toxoid of mice adoptively transferred with p21gp75 transduced B cells did not affect the response toward tetanus toxoid, while drastically reducing the response to the peptide. Additional experiments (data not shown) using an unrelated peptide derived from a coagulation factor showed essentially the same results, namely no detectable alteration of the response to the alternative peptide in recipients of p21gp75 transduced B cells.

One property of B cells that makes them intrinsically attractive for establishing specific unresponsiveness is their ability to migrate toward T cell-rich compartments of the spleen and persist there for prolonged periods of time (30). We show in this study that retrovirally transduced B cells were still detectable in the spleen at least 3 mo after transfer (Fig. 2D). We have already shown (R. Roohi Ahangarani, W. Janssens, V. Carlier, L. VanderElst, T. VandenDriessche, M. Chuah, M. Jacquemin and J. M. Saint-Remy, submitted for publication) that SNARF-1 labeled transduced B cells localize within the spleen white pulp marked with MOMA-1 Ab specific for metallophilic macrophages, which suggested that IL-10 producing B cells were located in an optimal microenvironment that fostered T-B cell interactions. It is interesting to point out that the only mouse in the prevention experiment (Fig. 2) that showed a normal response to injection of peptide had no detectable transgenic B cells in the spleen. Long-term survival and potential outgrowth of the transduced B cell population in vivo has already been demonstrated in other studies (28), as well as in B cell-deficient or SCID mice (31). Persistence of transgene-producing B cells are thought to be key in the maintenance of unresponsiveness even in the face of strong and recurring immunization.

Tr1 cells are defined by their unique cytokine production profile (i.e., IL-10²⁺, IL5⁺, TGF- β ⁺, IL-4⁺, IL-2^{low}, IFN- γ ^{low}) (32), up-regulation of CTLA-4 and suppress immune responses via cell-cell interaction and/or production of IL-10 and TGF- β (25). Evidence supporting the in vivo induction of Tr1 cells in the present study includes strict IL-10 dependency, the cytokine secretion pattern and phenotypic characteristics, as well as functional activity. Indeed, CD4⁺ T cells from tolerized mice showed increased production of IL-10, low production of IL-2 and IFN- γ , no production of IL-4 and up-regulation of intracellular CTLA-4. Interestingly, however, the Tr1-like cells described in this study are distinct from bona fide TR1 cells by virtue of the absence of TGF- β production. Transfer of these Tr1-like cells to a second host prevents the development of allergic asthma, both inflammatory changes and methacholine response. Though this pre-clinical evidence is preliminary, we believe it constitutes the first demonstration that functional Tr1-like cells can be induced directly in vivo to control asthma and pulmonary inflammation in an experimental model. In this context, it is worth pointing out that, the Tr1-like cells elicited in the present study showed much more efficiency in controlling immune response as compared with natural Tregs.

Tr1 cells are important for controlling immune responses to non-self-Ags, including alloantigens (33), allergens (34), contact sensitizer (35) or food Ags (36). Consequently, much effort has been devoted to determine under which conditions Tr1-like cells could be induced either ex vivo (5, 37) or in vivo (33, 36, 38, 39) both in mice and humans (28). For instance, using IL-10 producing B cells, Mauri et al. (40) prevented experimental arthritis, a result that was not observed with B cells from IL-10^{-/-} mice. Generating Tr1 cells in vitro has, however, proved difficult because culture conditions also invariably induce significant number of Th1 and Th2 cells (41, 42), and attempts

to improve homogeneity of IL-10 producing T cell populations by use of IFN- α (6) have not met with much success. In contrast, in the present model, genetically modified B cells with constitutive production of IL-10 could improve induction of a homogeneous population of regulatory T cells.

Genetically modified B cells were adoptively transferred immediately after activation and transduction. The present results and the work of Hussain et al. (39) consistent with that of others (28, 43, 44), therefore challenge the proposal that only resting B cells elicit T cell unresponsiveness (45).

Other studies have clearly demonstrated that transduced B cells induced unresponsiveness by targeting T cells. Thus, Chen et al. (46) have shown decreased T cell proliferation in a model of experimental autoimmune encephalitis, using a methodology comparable to ours. Scott and colleagues (47) observed a clear down-regulation of the T cell compartment in autoimmune disease. It should be stressed, however, that their approach significantly differed from ours, insofar as they made use of an IgG secretory scaffold instead of the gp75-driven MHC class-II presentation used in the present study. Scott et al. (48) nevertheless showed that MHC class II presentation was required with their construct, but that IL-10 was dispensable, in contrast to our present data, suggesting that a different Tr1-independent mechanism may be involved. Genetically modified B cells expressing Ags linked to IgG scaffold suppress symptoms associated with ongoing disease in models of diabetes and multiple sclerosis (43, 47), and more recently in the immune response toward coagulation Factor VIII (16), indicating that primed T cells are also amenable to tolerance induction, by contrast to earlier evidence (14, 49). It is not known whether Tr1-like cells and/or IL-10 are implicated in the control of immune response in those studies.

The seemingly exquisite specificity of our strategy, and the fact that a single injection of retrovirally transduced B cells is sufficient for long term prevention but also suppression of Ab production, hold much promise for clinical applications. The retroviral vector used in this study can accommodate much longer sequences than p21–35, such as the full-length allergen from which the peptide was derived. However, this might not be required as the present data shows that a peptide encompassing a T cell epitope is sufficient to induce tolerance to a full Ag. Some studies have indicated that ectopic transgene expression in APCs following gene therapy can increase the risk of develop an immune response to the transgene product and/or the genetically modified cells (50–52). It seems that the use of gene modified B cells according to the present strategy exerts an opposite effect by diminishing the risk of Ab development.

Altogether, the data presented in this study suggests that our model system could be of use not only in the prevention but also in the treatment of established diseases such as allergic bronchial asthma and some autoimmune diseases. Importantly, the data shows that the efficacy in controlling signs of disease far outweighs the effect observed by transfer of natural regulatory T cells. It may also represent a novel mechanism whereby the immune response toward a therapeutically relevant protein can be suppressed or prevented following gene therapy, distinct from what has been reported previously following hepatic gene delivery (53). Moreover, the present approach could also be of use for gene vaccination. It has indeed been demonstrated that presensitization to a viral vector, for instance adenovirus, is ominous for the outcome of vaccination (54); our strategy could help eliminating such presensitization.

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Disclosures

The authors have no financial conflict of interest.

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Figure legends

Supplementary Figure 1. Prevention of specific antibody response after immunization with p21-35 in recipient mice of CD25⁻ T cells from tolerized mice

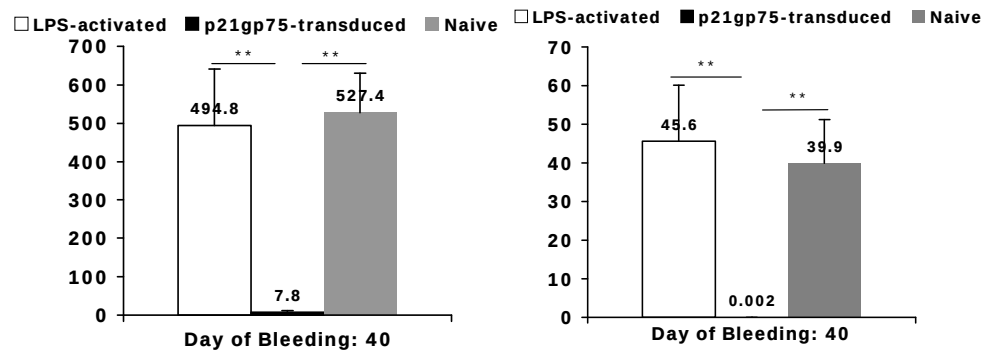
Concentrations of anti p21-35 IgG1 ($\mu\text{g/ml}$) and IgG2a ($\mu\text{g/ml}$) antibodies measured by ELISA after 2 consecutive immunizations in three groups of mice ($n=6/\text{group}$). Group A: mice reconstituted with CD4⁺CD25⁻ from recipients of non-transduced LPS-activated B cells; Group B: mice reconstituted with CD4⁺CD25⁻ from recipients mice of p21gp75-transduced B cells; and Group C: naïve mice. Bars represent medians $\pm$ SEM; ** $p<0.01$.

Supplementary Figure 2. Evaluation of experimental asthma in B cell-recipient BALB/c mice

Comparison of antibody titers, BALF cell content and methacholine reactivity in groups of BALB/c mice transferred with non-transduced LPS-activated B cells or with fully transduced B cells. A control group of naïve mice is also shown. All mice were then submitted to the asthma-inducing protocol as described in Material and Methods.

A. Derp 2-specific serum antibody levels. Bars represent mean $\pm$ SEM; **B.** BALF total cell and eosinophil content, 3 days after last exposure to allergen. **C.** Methacholine airway responsiveness, Penh values ($\pm$ SEM). * $p<0.05$; ** $p<0.01$.

Supplementary Figure 1



Supplementary Figure 2

