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INDUCTION OF ANTIGEN SPECIFIC TOLERANCE TO BONE MARROW ALLOGRAFTS WITH $CD4^+CD25^+$ T LYMPHOCYTES

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Abbreviations: GvHD, Graft-versus-host disease; B6, C57BL/6; B6CBAF1, $(C57BL/6xCBA/J)F_1$; B6D2F1, $(C57BL/6xDBA/2)F_1$

Running title: $CD4^+CD25^+$ T lymphocytes induce specific tolerance

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Abstract

Thymus-derived regulatory T lymphocytes of $CD4^+CD25^+$ phenotype regulate a large variety of beneficial and deleterious immune-responses and can inhibit lethal graft-versus-host disease in rodents. *In vitro*, $CD4^+CD25^+$ T cells require specific MHC/peptide ligands for their activation, but once activated they act in an antigen-non-specific manner. *In vivo*, regulatory T cells are also activated in an antigen-specific fashion, but nothing is known about antigen-specificity of their suppressor effector function. Here we show that $CD4^+CD25^+$ regulatory T lymphocytes isolated from naive mice and activated *in vitro* with allogeneic APC, induced specific long-term tolerance to bone-marrow grafts disparate for major and minor histocompatibility antigens : Whereas "target" bone-marrow was protected, third-party bone-marrow was rejected. Importantly, in mice injected with a mix of target and third-party bone-marrows, protection and rejection processes took place simultaneously. These results indicate that $CD4^+CD25^+$ regulatory T cells can act in an antigen-specific manner *in vivo*. Our results suggest that $CD4^+CD25^+$ regulatory T cells could in the future be used in clinical settings to induce specific immunosuppression.

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Introduction

Due to the random rearrangements of genes encoding T and B-lymphocyte antigen-receptors, a significant number of autospecific and potentially autoreactive lymphocytes develop in primary lymphoid organs [1-3]. Central tolerance (i.e. induced in primary lymphoid organs) eliminates (by deletion) or functionally inactivates (by induction of anergy) such dangerous lymphocytes. In absence of central tolerance-induction, a strongly self-reactive T cell repertoire develops [4,5]. However, when central tolerance is partially defective, self-tolerance can be maintained by peripheral mechanisms [6-8]. Several types of peripheral tolerance mechanisms control lymphocytes having escaped central-tolerance and are known to play a crucial role in preventing autoimmunity (for reviews see refs. [6,9]).

One of these peripheral tolerance mechanisms was discovered using the day 3 thymectomy model of multi-organ autoimmunity in mice [10]. The pathology can be prevented by injection of $CD4^+CD25^+$ lymphocytes, which appear after day 3 of life in the peripheral lymphoid organs of normal mice. $CD4^+CD25^+$ regulatory T cells do not only inhibit autoimmunity; they can also inhibit experimental inflammatory bowel disease induced by injection of $CD4^+CD45RB^{high}$ cells into immunodeficient SCID mice or RAG-deficient animals [11]. Moreover, they contribute to the fine control of immunity to infectious agents such as parasites and viruses [12,13]. An undesired side-effect of the activity of $CD4^+CD25^+$ regulatory T lymphocytes is the occasional incapacity of the immune system to eliminate tumor cells [14,15]. Therefore, regulatory T lymphocytes play a crucial role in the pathophysiological maintenance of immunological tolerance (reviewed in refs. [11,16,17,18]).

Natural (i.e. thymus-derived) $CD4^+CD25^+$ regulatory T cells can induce transplantation-tolerance in mice. Lethal graft-versus-host disease (GvHD) can be significantly reduced by injection of freshly isolated or *ex vivo* cultured regulatory T cells [19-22]. In one report, freshly

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isolated $CD4^+CD25^+$ regulatory T cells from naive animals were shown to induce limited tolerance to minor-histocompatibility-antigen disparate skin grafts [23]. Intrathymic injection of donor-strain antigen results in a tolerant state which was shown to be due to development of $CD4^+CD25^+$ regulatory thymocytes [24].

Regulatory T cells with a $CD4^+CD25^+$ phenotype are also known to be involved in experimental systems in which tolerance to alloantigens is induced *in vivo* with antibodies to T cell surface-antigens (e.g. CD4, CD8, or CD154, refs. [23,25,26-29]) or with the active form of vitamin D3 and mycophenolate mofetil [30]. In these systems, however, it is not clear if thymus-derived regulatory T cells (that can be found in naive animals) are involved or if these cells are induced in the periphery [31,32].

Like all other TCRαβ-expressing T lymphocytes, thymus-derived $CD4^+CD25^+$ regulatory T lymphocytes are antigen-specific, at least during their activation-phase. These cells have been shown to proliferate in an antigen-specific manner *in vivo* [33-35]. Myelin basic protein-specific $CD4^+CD25^+$ regulatory T cells protect better against experimental autoimmune encephalomyelitis than regulatory T cells with a restricted (but non-myelin basic protein-specific) TCR-repertoire [36]. Regulatory T cells activated *in vitro* with host-type APC inhibit GvHD more potently than cells activated with third-party APC [21,22]. Also experimentally induced $CD4^+CD25^+$ regulatory T cells act in a specific manner and protect target but not third-party allografts [27,29]. *In vitro*, however, $CD4^+CD25^+$ regulatory T cells only require interaction with specific MHC/peptide complexes during their activation phase. Once activated, their suppressor effector function is completely non antigen-specific [37]. The antigen-specificity observed *in vivo* could therefore potentially be explained by the hypothesis that regulatory T cells are activated only in hosts presenting antigens for which they are specific. In mice in which these antigens are not expressed, the regulatory T cells are not activated and immunosuppression does not take place.

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It remains therefore entirely unclear if $CD4^+CD25^+$ regulatory T cells are antigen-specific during their effector phase *in vivo*. Such antigen-specificity is required for a potential future use of these cells in induction of specific immunosuppression in patients. Here we present an experimental model in which transplanted bone marrow, disparate for major and minor histocompatibility antigens, is protected from rejection by host T cells through injection of $CD4^+CD25^+$ regulatory T cells cultured *ex vivo*. Using this model we show that $CD4^+CD25^+$ regulatory T cells can act in an alloantigen-specific manner during their effector phase. The model described here should prove useful in deciphering the *in vivo* mechanisms of specific immunosuppression mediated by $CD4^+CD25^+$ regulatory T lymphocytes. Moreover, our data suggest a clinical potential of these cells in protocols aimed at induction of specific tolerance to allogeneic grafts.

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Methods

Mice

All mice were used at 6-10 weeks of age. C57BL/6 ("B6": H-2^b), (C57BL/6xDBA/2)F_1 ("B6D2F1", H-2^{bd}) and (C57BL/6xCBA/J)F_1 ("B6CBAF1", H-2^{bk}) mice were purchased from Janvier (Le Genest St. Isle, France). All experiments involving animals were performed in compliance with the relevant laws and institutional guidelines (approval no. 31-13) and have been approved by the local ethics committee (ref. MP/01/31/10/03).

Purification of T-cell subsets

Erythrocyte-depleted (Lympholite-M, Cedarlane Laboratories, Hornby, Canada) splenocytes were enriched in $CD4^+$ T cells by magnetic depletion with sheep anti-rat antibody-coated Dynabeads (Dynal Biotech, Oslo, Norway) after incubation with a cocktail of the following rat mAb: anti-NK1.1 (PK136), anti-CD8 (53.6.7), anti-FcγRIII (2.4G2) and anti-MHC class II molecules (M5). The resulting population was then labeled with PE-labeled anti-CD25 mAb PC61 (BD PharMingen, Heidelberg, Germany; eBioscience, San Diego, CA) and $CD4^+CD25^+$ cells enriched with anti-PE microbeads using the AutoMACS (Miltenyi Biotec, Paris, France). Cell purity was checked by flow-cytometry on a FACSCalibur (BD Biosciences, San Jose, CA) using PE-labeled anti-CD25 mAb PC61 and FITC-labeled anti-CD4 (GK1.5). Positively sorted $CD4^+CD25^+$ T cells were routinely >95% pure.

$CD4^+$ or $CD8^+$ T cells were similarly isolated using negative selection with Dynabeads (PK136, 2.4G2, M5, and 53.6.7 or GK1.5) followed by positive selection on AutoMACS (with PE-labeled anti-CD4 mAb GK1.5 or anti-CD8 mAb 53.6.7 (BD Pharmingen; eBioscience)). Purity of populations routinely exceeded 98%.

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Ex vivo culture of $CD4^+CD25^+$ T Cells

$2x10^3$ $CD4^+CD25^+$ T cells/well from B6 mice were co-cultured with $5x10^5$ γ-irradiated (1700 Rad) total splenocytes from B6D2F1 or B6CBAF1 mice in 96-well round-bottom plates for 14 days. Cells were cultured in RPMI 1640 (Eurobio, Les Ulis, France) supplemented with 10% FCS, 2 mM L-glutamine, Penicillin, Streptomycin, 10 mM Hepes , 50 µM 2-ME, 1 mM non essential amino acids, 1 mM sodium pyruvate and 100 U/ml IL-2 (supernatant of PMA-stimulated EL4.IL-2 cells; ATCC, Manassas, VA). At day 7, 100µl of fresh medium was added and cells were cultured for another 7 days.

Bone marrow chimeras

Bone marrow from femurs and tibias was collected in DMEM (Eurobio, Les Ulis, France) supplemented with 10% FCS, 2 mM L-glutamine, Penicillin, Streptomycin. Single cell suspensions were washed in complete medium. $Thy1^+$ and $NK1.1^+$ cells were eliminated using AT83 and PK136 Abs, respectively, and rabbit complement (Saxon Europe, Suffolk, UK). $5x10^6$ cells from each donor were then injected i.v. into γ-irradiated hosts (850 Rad, ^{137}Cs source, 630 Rad/minute) that were kept on antibiotic-containing water (0.28% pediatric suspension of Bactrim, Roche, Basel, Switzerland) for the complete duration of the experiment. Effector and/or regulatory T cells were co-injected with bone-marrow preparations.

FACS analysis

For analysis of bone-marrow from femurs and tibias, mice were sacrificed 15 days after bone-marrow and T cell transfer. For PBMC-analysis, blood samples were taken at indicated

timepoints. Cells were labeled with anti-H-2K^{b}-FITC and anti-H-2K^{d}-PE or with anti-H-2K^{k}-FITC and anti-H-2K^{b}-PE (BD Pharmingen; eBioscience). The relative percentages of semiallogeneic cells shown in the figures are calculated as follows: (% of semiallogeneic cells in experimental mouse ÷ mean % of semiallogeneic cells in mice injected with bone-marrow only) x 100.

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Results

Ex vivo expanded $CD4^+CD25^+$ regulatory T lymphocytes inhibit $CD4^+$ and $CD8^+$ T cell-mediated rejection of allogeneic bone-marrow

We have adapted our previously described mouse model for bone-marrow transplantation in which B6 (H-2^b) hosts are lethally irradiated and reconstituted with a 1/1 mixture of syngeneic (B6) and semi-allogeneic B6D2F1 (H-2^{bd}) bone marrow [4,8]. Equivalent percentages of syngeneic and allogeneic cells were found in the bone-marrow two weeks post-transfer (Fig. 1A). When host-type $CD4^+$ T cells were co-injected, the semi-allogeneic bone-marrow was eliminated and the retained syngeneic bone marrow allowed for survival of the host (Fig. 1B). Since we have been unable to induce tolerance to semi-allogeneic bone-marrow with freshly isolated regulatory T cells (data not shown), we evaluated the regulatory potential of $CD4^+CD25^+$ T cells activated and expanded *in vitro*. Purified B6 $CD4^+CD25^+$ splenic T cells were cultured *in vitro* with B6D2F1 APC in presence of IL-2. In 2-week cultures a 10 to 20-fold expansion was reproducibly obtained (data not shown). When such *in vitro*-cultured regulatory T cells were co-injected with B6 $CD4^+$ T lymphocytes, rejection of semi-allogeneic bone-marrow was inhibited very efficiently, even at a ratio of one regulatory to ten $CD4^+$ effector T cells (Figs. 1B and C).

Rejection of allogeneic grafts is also mediated by $CD8^+$ T lymphocytes. It was therefore important to assess if regulatory T cells could inhibit $CD8^+$ T cells *in vivo*. Lethally irradiated B6 mice were reconstituted with a mixture of syngeneic and semi-allogeneic bone-marrow cells. They were simultaneously injected with purified $CD8^+$ T lymphocytes with or without preactivated regulatory T cells. The data shown in figures 1B and 1D indicate that $CD8^+$ T lymphocytes fully rejected semi-allogeneic bone-marrow and that co-injection of *in vitro* expanded regulatory T cells (at a ratio of 10 $CD4^+CD25^+$ to 1 $CD8^+$ T cell) could inhibit this

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rejection. The higher ratios of regulatory T lymphocytes required to inhibit graft-rejection by $CD8^+$ (as compared to $CD4^+$) T cells are probably due to the much higher efficiency of $CD8^+$ cells in graft-rejection in our system (data not shown). In conclusion, $CD4^+CD25^+$ regulatory T lymphocytes are capable of inhibiting *in vivo* alloreactivity of $CD4^+$ as well as of $CD8^+$ T cells.

Regulatory T cells induce durable tolerance of total splenocytes to allogeneic bone-marrow

We then assessed if regulatory T lymphocytes could also inhibit semi-allogeneic bone-marrow rejection by total splenocytes. Lethally irradiated B6 hosts were reconstituted with a mixture of syngeneic and semi-allogeneic bone marrow cells, injected with B6 splenocytes, and co-injected (or not) with titrated numbers of B6 $CD4^+CD25^+$ T lymphocytes that had been preactivated with B6D2F1 APC. As shown in figures 2A and B, co-injected regulatory T cells inhibited semi-allogeneic bone-marrow rejection in a dose-dependent fashion. Already at a $CD4^+CD25^+$ regulatory T cell to splenocyte ration of 1/2 significant protection of semi-allogeneic bone-marrow was observed.

In these experiments, bone-marrow engraftment was analyzed after 2 weeks. To assess if the tolerance was durable, we reconstituted lethally irradiated B6 mice with syngeneic and semi-allogeneic bone-marrow, co-injected them with B6 splenocytes and preactivated B6 regulatory T cells, and analyzed peripheral blood mononuclear cells from 1 to 11 weeks post-reconstitution. Figure 2C shows that the tolerance was durable: up to 11 weeks after transfer no signs of rejection were observed at regulatory T cell/splenocyte ratios of 2 and 10. At a regulatory T cell/splenocyte ratio of 1:2 levels of protection were quite variable among the four animals analyzed. Interestingly, once established, the percentage of allogeneic cells in PBMC did not significantly change during the period in which the animals were analyzed.

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Therefore, even partial protection appears to be stable in time. We are currently investigating the underlying mechanisms.

Pre-activated regulatory T cells protect allogeneic bone-marrow in an MHC-haplotype specific manner

We next evaluated if the protection of allogeneic grafts by activated regulatory T cells was specific. B6 $CD4^+CD25^+$ T cells were pre-activated *in vitro* with B6D2F1 ($H\text{-}2^{bd}$) APC in presence of IL-2. These cells were then co-injected with B6 splenocytes, B6 bone marrow, and B6D2F1 ($H\text{-}2^{bd}$) or B6CBAF1 ($H\text{-}2^{bk}$) bone-marrow, into lethally irradiated B6 hosts. The results in figure 3A show that B6D2F1 bone marrow was more efficiently protected than B6CBAF1 cells, most notably at lower regulatory T cell to splenocyte ratios.

Since this specific protection might hypothetically be due to differences in the kinetics and/or potency of rejection of the two types of bone-marrow, we also performed the reciprocal experiment and tested if B6 regulatory T cells activated *in vitro* with B6CBAF1 APC acted in a specific manner (Fig. 3B). At a regulatory T cell to splenocyte ratio of 0.5, B6CBAF1 bone marrow was more efficiently protected than B6D2F1 cells. When using higher regulatory T cell to splenocyte ratios the specificity was lost.

Regulatory T cells protect target bone marrow while allowing third party alloreactivity to develop simultaneously

Since the observed specificity in the protection of allogeneic bone-marrow might be due to several factors other than specificity in the effector function of regulatory T cells (e.g. differential survival, homeostatic expansion, or activation in the distinct experimental mice), we then tested if the specificity was maintained when both types of semi-allogeneic bone marrow (i.e. B6D2F1 and B6CBAF1) were transferred (together with syngeneic bone-

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marrow) into the same host. When the three types of bone-marrow were injected without effector T cells, practically equivalent percentages of syngeneic (B6) and the two types of allogeneic (B6D2F1 and B6CBAF1) cells were found in bone marrow two weeks later (Fig. 4A). As shown in figures 4B (top) and 4C, addition of syngeneic effector splenocytes resulted in the clearance of both allogeneic populations, and regulatory T cells preactivated with B6D2F1 APC preferentially inhibited rejection of B6D2F1 (as compared to B6CBAF1) bone marrow, especially at lower regulatory to effector T cell ratios. At higher regulatory to effector T cell ratios, specificity was gradually lost, and cells of both semi-allogeneic origins were efficiently preserved.

Again, to exclude the hypothetical possibility that the observed specificity was due to differences in the kinetics and/or potency of rejection of the two types of bone-marrow, we then performed the reciprocal experiment. Regulatory T cells preactivated with B6CBAF1 cells preferentially protected B6CBAF1 (as compared to B6D2F1) bone marrow, again most notably at lower regulatory to effector T cell ratios (Figs. 4B (bottom) and C). These results definitively show that preactivated regulatory T cells can act in an antigen specific manner during their effector function *in vivo*.

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Discussion

Like all other T lymphocytes, $CD4^+CD25^+$ regulatory T cells are antigen-specific and are thought to act in an antigen-specific manner *in vivo*. *In vitro,* however, antigen-specificity of these cells is limited to the activation phase while the effector phase is completely antigen-non-specific. While in several *in vivo* models regulatory T cells have been shown to act in an antigen-specific manner, in none of these systems has a distinction been made between antigen-specific activation and antigen-specific suppressor effector function.

Using a novel experimental system in which allogeneic bone marrow is durably protected from rejection by *in vitro* activated regulatory T cells, we show here that regulatory T cells can act in an antigen-specific manner during their effector phase *in vivo*. Our results suggest that $CD4^+CD25^+$ regulatory T cells could become a powerful tool to induce specific tolerance to allogeneic grafts in clinical settings.

$CD4^+CD25^+$ regulatory T cells have been used in a variety of systems to inhibit immunopathology. All these models have in common that injection of T lymphocytes must be followed by homeostatic proliferation. In the colitis model, disease is induced in lymphopenic mice [11]. GvHD models rely on lethally irradiated recipients, which are therefore lymphopenic [19-21,38]. The induction of transplantation-tolerance (GvHD or graft-rejection) with experimentally induced $CD4^+CD25^+$ regulatory T cells has also been studied in lymphopenic mice [23,25-29]. In the experimental models of autoimmunity induced by day 3 thymectomy or in nude mice, homeostatic expansion of injected T lymphocytes also certainly occurs [10]. Since regulatory T cells are known to regulate homeostatic expansion and to expand themselves in immunodeficient mice [39,40], it has been suggested that regulatory T cells could outcompete pathogenic T cells during the expansion-phase and thus inhibit immunopathology [41]. Such a scenario could also explain the regulatory T cell-induced tolerance we observed when mice

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were injected with only one type of allogeneic bone-marrow (target or third party). In experiments in which the mice were co-injected with two types of allogeneic bone-marrow, however, target bone marrow was preferentially protected (notably at low regulatory to effector T cell ratios). Therefore, in contrast to the abovementioned reports, in our system tolerance cannot be explained by differential homeostatic expansion of alloreactive *versus* innocuous regulatory T cells.

T-cell tolerance induced by $CD4^+CD25^+$ regulatory T lymphocytes has never before been shown to be antigen-specific in the effector phase. In GvHD and transplantation models, tolerance was studied in separate hosts [21,22,27,29]. Since homeostatic proliferation of regulatory T cells depends upon MHC class II expression [40] and potentially even upon interaction with specific antigen [33], antigen-specificity *in vivo* may be due to lack of homeostatic proliferation in animals lacking the "target" tissue. In these models, antigen-specificity is probably also due to the absence of specific ligands capable of activating the suppressor-effector function of regulatory T-lymphocytes. These hypothetical explanations may also apply to the specific tolerance we observed when target and third party bone marrows were injected (together with splenocytes and regulatory T cells) into separate hosts. However, we also observed preferential protection of target bone marrow in mice in which third party cells were simultaneously rejected. One could argue that the specific protection was due to differences in the kinetics and/or potency of rejection of the two types of bone marrow but such objections can be ruled out since identical results were obtained in reciprocal experiments. Since target bone marrow was protected in these mice, regulatory T cells had clearly been activated, but protected third-party bone marrow much less efficiently. Therefore, our data directly show that $CD4^+CD25^+$ regulatory T cells can act in an antigen-specific manner during their effector phase.

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Several reason(s) could explain why protection is not entirely antigen-specific, especially at higher regulatory to effector T cell ratios. Since this lack of specificity was also observed in mice in which target bone-marrow was absent, regulatory T cells are sufficiently cross-reactive with third-party antigens to cause partial protection. Such a cross-reactivity may in part be due to "indirect antigen presentation" by host-type MHC molecules that are expressed by both types of semi-allogeneic bone marrows. Direct cross-reactivity towards allogeneic MHC molecules most likely also plays an important role [3]. In contrast to the C57BL/6 hosts we used, both donor strains (B6D2F1 and B6CBAF1) present several mouse mammary tumor virus-encoded endogenous superantigens [42]. Therefore, superantigen-reactivity of injected regulatory T cells may cause very significant cross-reactivity. In the mice in which we injected both types of allogeneic bone-marrow a contribution of non antigen-specific "bystander" mechanisms formally cannot be excluded. In any case, our data directly indicate that immunosuppression mediated by $CD4^+CD25^+$ regulatory T cells can act in an antigen-specific manner, not only in the activation but very importantly also during the *in vivo* effector phase. It will be important to assess if the antigen-specific suppressor effector function of $CD4^+CD25^+$ T cells will allow for clearance of infection of donor-type cells (e.g. by viruses).

Allogeneic bone-marrow or hematopoietic stem cell transplantation is extensively used to correct hereditary defects such as primary immunodeficiencies, metabolic diseases, β-thalassemia major, and sickle cell anemia (for review see ref. [43]). These protocols require very frequent transfusions and/or immunosuppression to obtain and maintain hematopoietic chimerism. Since immunosuppressive drugs do not act in an antigen specific manner, their use is associated with opportunistic infections and increased tumor-incidence. Patients suffering from the above-mentioned congenital diseases would benefit from the availability of specific immunosuppressive protocols. The results we have obtained with our experimental model

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indicate that specific immunosuppression can be achieved with $CD4^+CD25^+$ regulatory T lymphocytes. They should open the way to development of clinical approaches aimed at induction of specific and durable tolerance to bone-marrow and hematopoietic stem cell allografts. Moreover, regulatory T cells could potentially also be used to induce specific and durable immunological tolerance to organ-allografts, and we are currently investigating this intriguing possibility.

In conclusion, the data presented here indicate that regulatory T cells can induce antigen-specific immunosuppression. It will be interesting to assess how antigen-specific *in vivo* tolerance is achieved. The model we describe here may prove particularly useful in addressing this and other questions concerning the *in vivo* functioning of $CD4^+CD25^+$ regulatory T cells. Moreover, our results suggest the feasibility of development of clinical protocols in which $CD4^+CD25^+$ regulatory T lymphocytes would be used to induce specific tolerance to allogeneic hematopoietic stem cell or organ-grafts.

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Legends to the Figures

Figure 1.

$CD4^+CD25^+$ regulatory T cells preactivated in vitro induce $CD4^+$ and $CD8^+$ T cell-tolerance to semiallogeneic bone-marrow

B6 mice were lethally irradiated and grafted with a 1/1 mixture of B6 (H-2^b) and B6D2F1 (H-2^{bd}) bone-marrow ($5x10^6$ cells each). Two weeks later equivalent percentages of syngeneic and semi-allogeneic bone-marrow were observed in bone marrow (A). Irradiated mice were co-injected with the two types of bone-marrow and $3x10^5$ $CD4^+$ (B left, C) or $CD8^+$ T cells (B right, D) with (B bottom) or without (B top) $CD4^+CD25^+$ regulatory T cells from B6 mice *in vitro* preactivated with B6D2F1 APC (B, regulatory to effector T cell ratio = 10, C, D, as indicated). Mice were analyzed two weeks later. **(A top, B)** FACS analysis of bone-marrow, **(C)(D)** Relative percentage (see Material and Methods) of semiallogeneic bone marrow. Squares indicate individual mice from a representative experiment of 4 performed.

Figure 2.

Preactivated $CD4^+CD25^+$ regulatory T cells durably prevent semiallogeneic bone-marrow rejection by total splenocytes

B6 mice were lethally irradiated and grafted with a 1/1 mixture of B6 (H-2^b) and B6D2F1 (H-2^{bd}) bone-marrow ($5x10^6$ cells each) and co-injected with 10^5 B6 splenocytes and regulatory T cells preactivated with B6D2F1 APC *in vitro* (regulatory to effector T cell ratio as indicated). Bone marrow was analyzed 2 weeks later (A, B, combined data from 4 independent experiments), PBMC at indicated timepoints (C, representative individual mice). **(A)** FACS analysis of bone-marrow, **(B) (C)** Relative percentage of semiallogeneic cells (see Material and Methods). *** $p<0.001$ (Student's t-test).

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Figure 3.

Prevention of semiallogeneic bone-marrow rejection mediated by $CD4^+CD25^+$ regulatory T cells is MHC-haplotype specific

B6 mice were lethally irradiated, grafted with a 1/1 mixture of B6 (H-2^b) plus B6D2F1 (H-2^{bd}) or B6CBAF1 (H-2^{bk}) bone-marrow ($5x10^6$ cells each), and co-injected with 10^5 B6 splenocytes and with regulatory T cells from B6 mice preactivated with B6D2F1 **(A)** or B6CBAF1 **(B)** APC *in vitro* (regulatory to effector T cell ratios as indicated). Bone marrow was analyzed 2 weeks later. Relative percentage of semiallogeneic H-2^{bd} (□) or H-2^{bk} (■) cells in the individual mice is indicated (see Material and Methods). * $p< 0.05$ (Student's t-test). Combined data from 3 independent experiments are shown.

Figure 4.

Simultaneous protection of target bone marrow and rejection of third-party cells.

B6 mice were lethally irradiated, grafted with a 1/1/1 mixture of B6 (H-2^b), B6D2F1 (H-2^{bd}), and B6CBAF1 (H-2^{bk}) bone-marrow ($5x10^6$ cells each), and co-injected with 10^5 B6 splenocytes and regulatory T cells preactivated with B6D2F1 or B6CBAF1 APC *in vitro* (as indicated, regulatory T cell to splenocyte ratios as indicated). Bone marrow was analyzed 2 weeks later. **(A)** Percentages of the three types of bone marrow recovered from control mice (i.e. mice that had not received splenocytes). **(B)** H-$2K^d$ and H-$2K^k$ expression by bone-marrow cells recovered from individual mice that had received bone-marrow, splenocytes, and titrated numbers of preactivated regulatory T cells, as indicated. **(C)** Summary of all data depicted as ratios of relative percentages of H-2^{bk} to H-2bd bone-marrow cells. (□) Mice injected with regulatory T cells preactivated with H-2^{bd} APC, (■) Mice injected with regulatory T cells activated with H-2^{bk} APC. ** $p< 0.01$ (Wilcoxon test). Combined data from 3 independent experiments are shown.

Figure 1

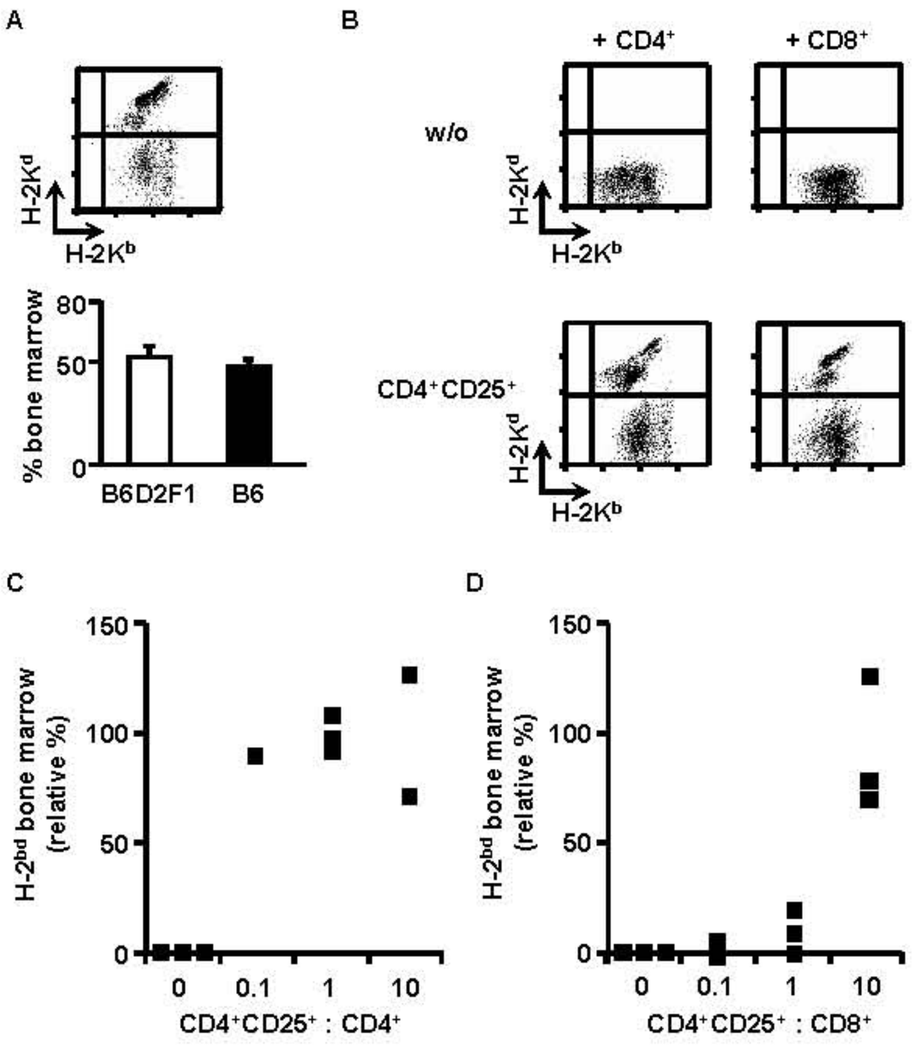

A
H-2K^d
H-2K^b
% bone marrow
80
50
0
B6D2F1
B6
B
+ CD4+
+ CD8+
w/o
CD4+CD25+
H-2K^d
H-2K^b
C
H-2^bd bone marrow (relative %)
150
100
50
0
0
0.1
1
10
CD4+CD25+ : CD4+
D
H-2^bd bone marrow (relative %)
150
100
50
0
0
0.1
1
10
CD4+CD25+ : CD8+

Figure 2

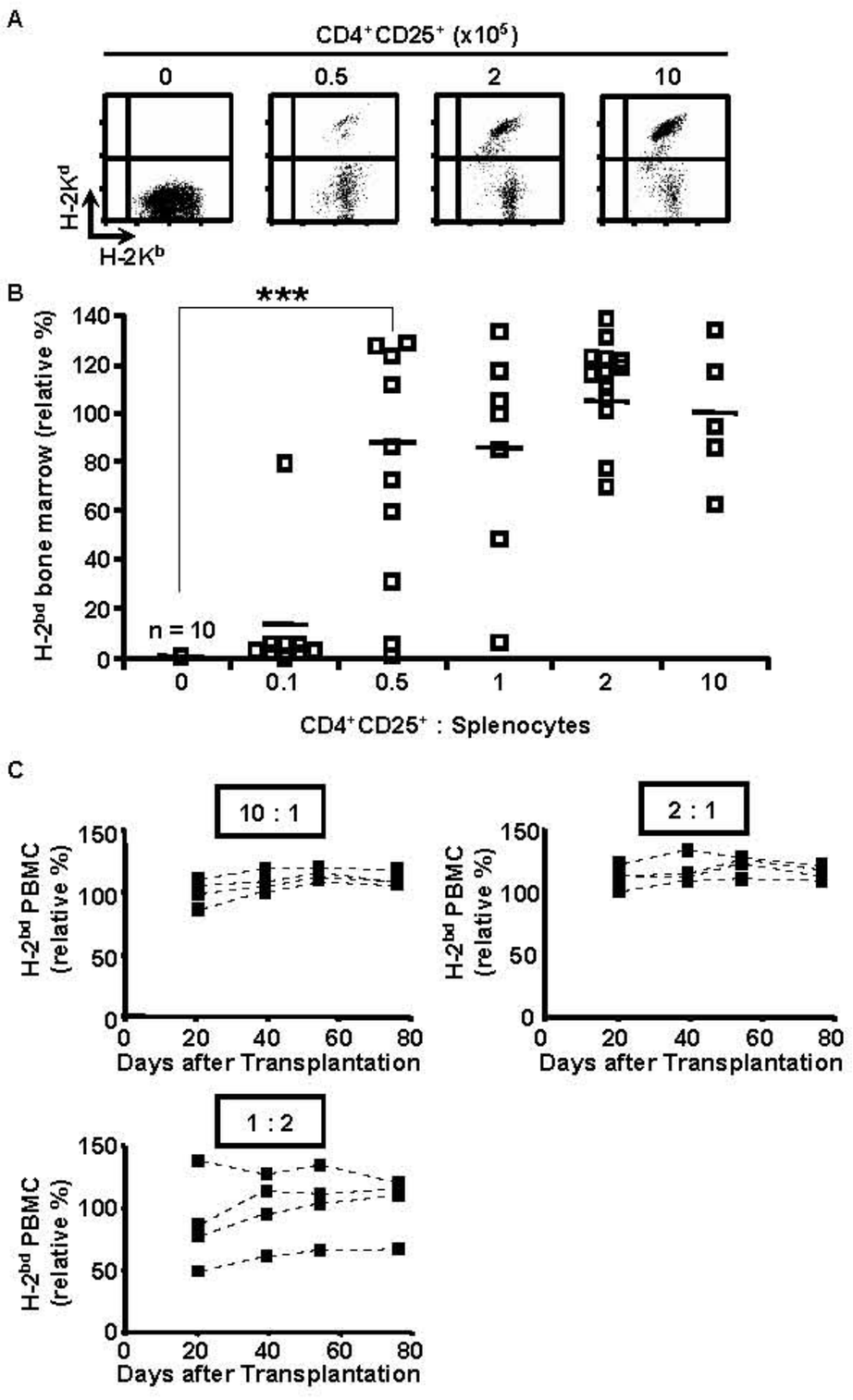

A
CD4+CD25+ (x10^5)
0
0.5
2
10
H-2K^d
H-2K^b
B
***
H-2^bd bone marrow (relative %)
n = 10
CD4+CD25+ : Splenocytes
C
10 : 1
2 : 1
1 : 2
H-2^bd PBMC (relative %)
Days after Transplantation

Figure 3

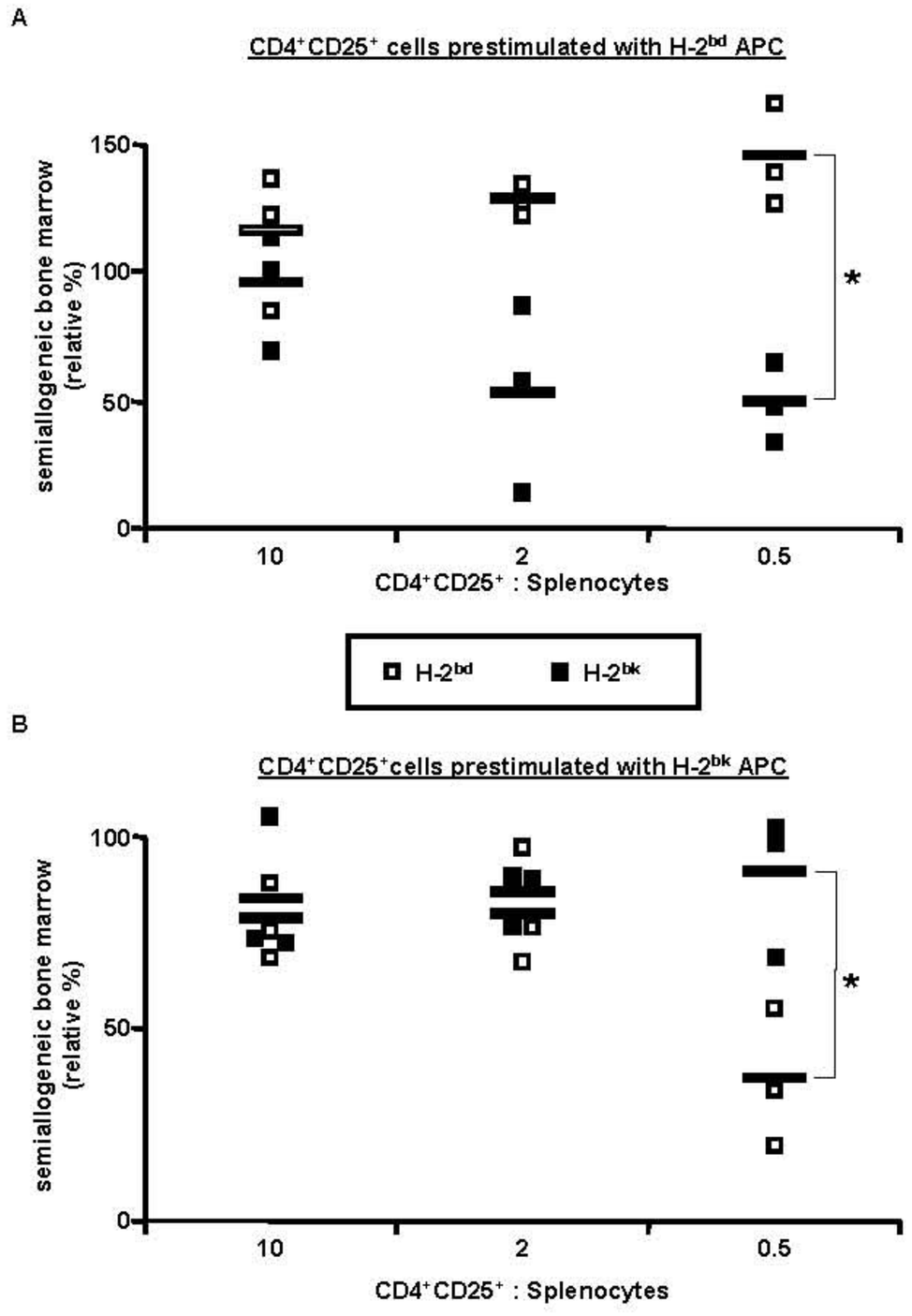
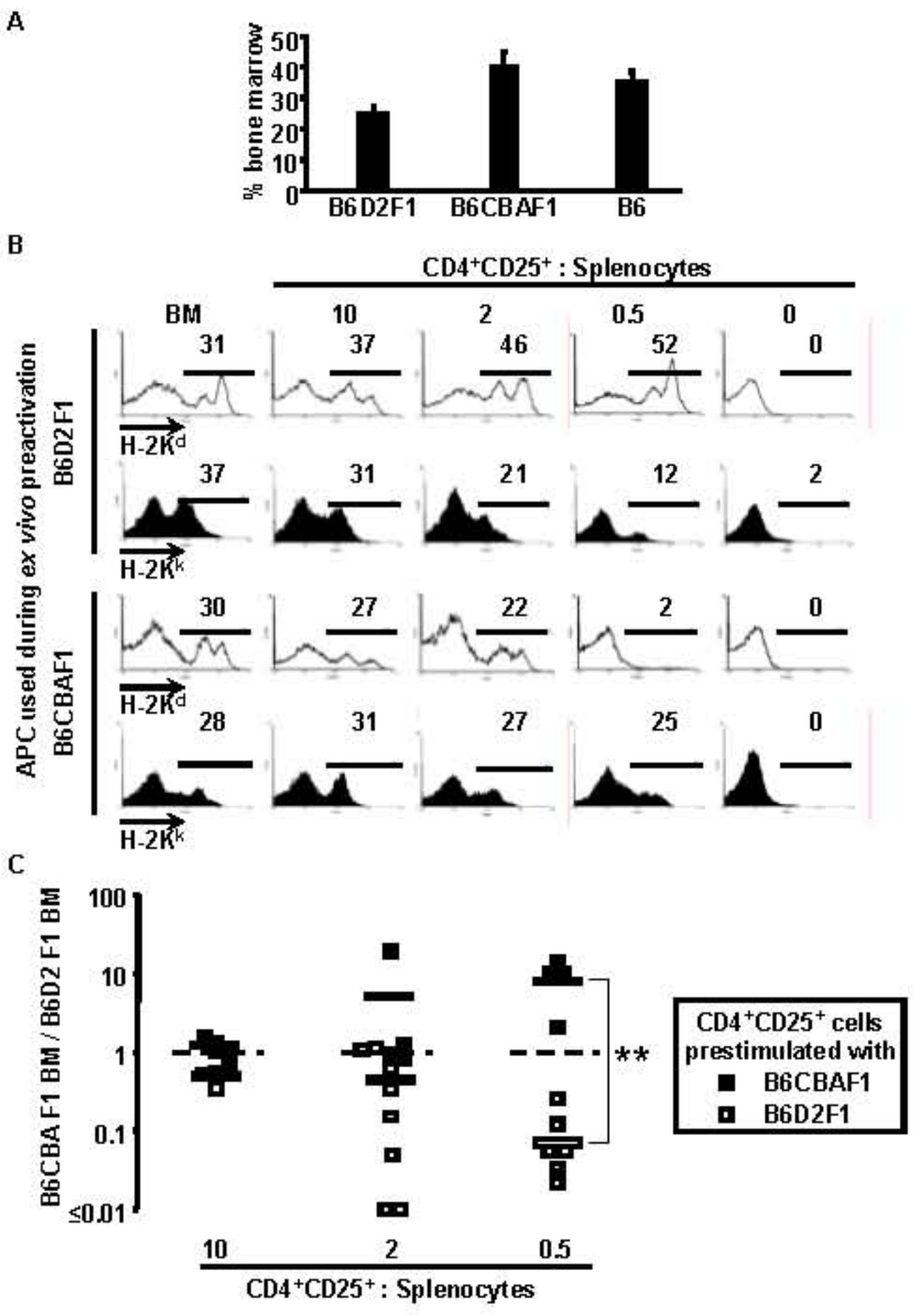

A
CD4+CD25+ cells prestimulated with H-2bd APC
semiallogeneic bone marrow (relative %)
150
100
50
0
10
2
0.5
CD4+CD25+ : Splenocytes
*
H-2bd
H-2bk
B
CD4+CD25+cells prestimulated with H-2bk APC
semiallogeneic bone marrow (relative %)
100
50
0
10
2
0.5
CD4+CD25+ : Splenocytes
*

Figure 4


A
% bone marrow
50
40
30
20
10
0
B6D2F1
B6CBAF1
B6
B
CD4+CD25+ : Splenocytes
BM
10
2
0.5
0
ex vivo preactivation
B6D2F1
31
37
46
52
0
H-2Kd
37
31
21
12
2
H-2Kk
APC used during
B6CBAF1
30
27
22
2
0
H-2Kd
28
31
27
25
0
H-2Kk
C
B6CBA F1 BM / B6D2 F1 BM
100
10
1
0.1
≤0.01
**
CD4+CD25+ cells
prestimulated with
B6CBAF1
B6D2F1
10
2
0.5
CD4+CD25+ : Splenocytes