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Oral Combined Therapy with Probiotics and Alloantigen Induces B Cell–Dependent Long-Lasting Specific Tolerance

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Allogeneic hematopoietic stem cell transplantation (aHSCT) is widely used for the treatment of hematologic malignancies. Although aHSCT provides a good response against the malignant cells (graft-versus-leukemia [GVL]), it also leads to the development of graft-versus-host disease (GVHD), a severe disease with high mortality and morbidity rates. Therapy for GVHD is commonly based on nonspecific immunosuppression of the transplanted recipient, resulting in the concomitant inhibition of the GVL effect. In this study, we propose an alternative approach to specifically suppress GVHD while sparing the GVL, based on oral treatment of transplant donors with recipient Ags, associated with the intake of probiotic Lactococcus lactis as tolerogenic adjuvant (combined therapy). We show that treatment of C57BL/6 mice with combined therapy before the transplant protects the recipients from clinical and pathological manifestations of disease, resulting in 100% survival rate. Importantly, the animals keep the immunological competence maintaining the GVL response as well as the response to third-party Ags. The protection is specific, long lasting and dependent on donor IL-10–sufficient B cells activity, which induces regulatory T cells in the host. These data suggest that combined therapy is a promising strategy for prevention of GVHD with preservation of GVL, opening new possibilities to treat human patients subjected to transplantation. The Journal of Immunology, 2014, 192: 1928–1937.

The main limitation of allogeneic hematopoietic stem cell transplantation (aHSCT) for the treatment of malignant and nonmalignant disease is the development of graft versus host disease (GVHD), a complex multiorgan inflammatory syndrome that results from recognition of genetically disparate tissues of the recipient patient by the incoming allogeneic donor T cells (1). GVHD treatment is based on use of immunosuppressive agents that block T cell function in a nonspecific way, leading to undesired consequences—because these alloreactive T cells also facilitate engraftment of hematopoietic stem cells, accelerate immune reconstitution (1–4), and contribute to the graft-versus-leukemia (GVL) effect (4). Because of the close association between GVH and GVL reactions (4), much effort has been put forth to develop therapies that reduce the first while maintaining the latter (1).

Oral tolerance is classically defined as the suppression of immune responses to Ags that have been previously administered by the oral route (5). Far from being just a matter of hyporesponsiveness, oral tolerance is an active immunologic event influenced by numerous factors such as Ag dose, microbiota, and costimulatory molecules, resulting from multiple mechanisms of action (6). Induction of oral tolerance has been shown to be effective in various experimental models of immune disorders (7–9) including GVHD (10–12). In fact, Nagler et al. (12) demonstrated that it is possible to alleviate acute GVHD (aGVHD) severity by the induction of oral tolerance in the recipient, although the maintenance of the GVL effect was not addressed in that study.

Administration of probiotics by the oral route also have been shown to modulate the immune response, favoring either pro- (13, 14) or anti-inflammatory (15, 16) responses. Recent studies suggest that TLRs and NF-κB signaling pathways play a crucial role in the outcome of the response (17–21). Also, probiotics can influence the integrity of the gut epithelial cell barrier through modulation of mucus, defensins, and IgA production, reduction of bacterial adhesion, and enhancement of tight junction and cell survival (22–24).

The relationship between the endogenous microbiota and development of aGVHD was proposed ∼40 y ago in experimental models (25) and confirmed in humans later on (26). As hypothesized by Hill et al. (27), damage of the host intestinal epithelium by the conditioning regimen results in systemic exposure to microbial Ags that ultimately lead to an inflammatory milieu that is adequate for donor T cell activation. With those results in mind, intestinal decontamination became a common practice in aHSCT (26, 28). More recently, Jenq et al. (29) showed that composition of the gut flora of recipient mice that developed GVHD had a dramatic loss of bacterial diversity and a distinct composition...
compared with recipient mice that did not develop GVHD. In addition, the authors revealed that antibiotic-treated mice showed an emergence of species that was associated with exacerbated GVHD. Interestingly, Gerbitz et al. (30) demonstrated that oral administration of *Lactobacillus rhamnosus* GG before and after transplantation not only improved survival and ameliorated aGVHD clinical scores but also reduced the translocation of enteric bacteria. Both works suggest that manipulation of the gut microbiota might be as good as decontamination with antibiotics to prevent GVHD.

Although promising, the clinical application of both protocols, oral tolerance induction (12) and probiotic treatment (30), cannot be used in transplanted patients. Once the conditioning regimen alters the intestinal epithelial barrier, these patients require special attention regarding their diet. As so, no live or raw food is permitted during the peritransplant phase, precluding oral administration of Ags or probiotics.

In this report, we reasoned if tolerance induction in the donor, rather than in the recipient (12, 30), could work as a strategy to dissociate GVH and GVL effects. We propose a new protocol that combines the induction of oral tolerance in bone marrow transplant donors to Ags from the recipient, with the use of the potential probiotic *Lactococcus lactis* NCDO2118 as tolerogenic adjuvant (combined therapy). Using a haploidentical model of aGVHD, we found that treatment of donor mice with the combined therapy protects recipients from aGVHD while maintaining the GVL effect. This protection is long lasting, Ag specific, and dependent on donor B cells, which induce regulatory T cells (Tregs) in the host.

**Materials and Methods**

**Animals**

Male 8- to 10-wk-old C57BL/6 (B6) (H-2<sup>b</sup>), B6 IL-10 knockout, or F<sub>1</sub> (BALB/c × B6)<sup>ko</sup> mice were used as cell donors for GVHD induction experiments and female 10- to 12-wk-old F<sub>1</sub> (BALB/c × B6 - H-2<sup>ko</sup>) mice were used as recipients. For skin transplants, male 12- to 14-wk-old F<sub>1</sub> (BALB/c × B6), B10.A (H-2<sup>b</sup>), or B6 mice were used as donors, and female B6 mice or F<sub>1</sub> (BALB/c × B6) mice were used as recipient. All animals were bred at the Brazilian National Cancer Institute animal facility (Rio de Janeiro, Brazil), housed in sterilized microisolators, and were handled according to the institutional guidelines. Animal care and all animal procedures were previously approved by Ethical Committee for Animal Experimentation of the Brazilian National Cancer Institute.

**Bacterial growth conditions and administration to donor mice**

The wild-type *L. lactis* ssp *lactis* NCDO2118 strain (obtained from Laboratório de Genética Celular e Molecular from Universidade Federal de Minas Gerais, MG/Brazil - taxonomy ID 1117941 at the National Center for Biotechnology Information database). The bacteria were grown in M17 broth (Difco) supplemented with 0.5% glucose at 30°C without agitation for 18 h. After centrifugation, the bacteria were resuspended on ice-cold PBS and filtered through a 40-µm cell strainer, and aliquots were stored at −80°C until use.

**Preparation of protein extract from recipient mice**

To prepare the protein extract, F<sub>1</sub> (BALB/c × B6) spleen cells were resuspended on ice-cold PBS and filtered through a 40-µm cell strainer, cells were disrupted by freezing and thawing five times and boiled for 10 min. Soluble fraction was obtained by centrifugation at 14,000 rpm, 30 min, at 4°C and used as oral treatment. Total protein was quantified by the Bio-Rad protein kit assay, according to the manufacturer’s specifications.

**Treatment of donor mouse with combined therapy**

Donor B6 mice received 50 µg F<sub>1</sub> (BALB/c × B6) spleen protein extract diluted in 100 µl PBS, by gavage, daily for 5 d. During this period, ad libitum doses of *L. lactis* NCDO2118 were offered in replacement of drinking water. Four days later, the animals were immunized with 10<sup>6</sup> F<sub>1</sub> splenocytes i.p. (31), and donor splenic cells were collected 1 wk after.

**Bone marrow transplantation**

Bone marrow transplantation (BMT) was modified from described previously (32). F<sub>1</sub> mice were lethally irradiated with 950 cGy total body irradiation (TH780C irradiator with a cobalt 60 [60Co] source), followed by the i.v. transfer of 5 × 10<sup>5</sup> bone marrow cells from healthy donors along with 5 × 10<sup>6</sup> T cells from total spleen of treated or untreated donors. When indicated, spleen cells were depleted of CD25<sup>+</sup> cells or CD19<sup>+</sup> cells. In GVL experiments, 10<sup>5</sup> P815 GFP-expressing mastocytoma cells were injected together with the bone marrow inoculum.

**GVHD clinical and histological score**

Mice were monitored daily for survival and weekly for GVHD clinical score. Clinical evaluation was modified from the literature (33, 34) and was based on six parameters: weight loss, fur texture, activity, pellage, femoral osteoporosis, and survival rate. For histopathological examination, samples of skin, liver, and colon collected at day 21 posttransplant were formalin preserved, paraffin embedded, sectioned, and stained with H&E. The pathologic score system was based on evaluation of individual parameters for each organ being the final score obtained by the sum of them. For skin sections, inflammatory infiltration, fibrosis, loss of appendages, epithelial changes, and ulceration were evaluated. For liver samples, parenchyma suffering, inflammatory infiltration, and portal space destruction were evaluated. For colon, lamina propria infiltration, deeper layer infiltration, structural changes, and damage extension were analyzed.

**GVL evaluation**

At day 25 posttransplant, samples of spleen, liver, bone marrow, total lymph nodes, and blood were analyzed by flow cytometry for the presence of P815-GFP<sup>+</sup> cell. For determination of tumor total elimination, recipients were monitored daily for tumor-related morbidity and mortality. Tumor-related death was determined if mice had paralysis (in that case they were euthanized immediately and examined) and if autopsy identified hepatosplenomegaly and presence of macroscopic tumor nodules on the liver, spleen, or bone marrow.

**Cell depletions**

For Treg depletion, spleen cells from donor mice were incubated with anti-CD25 (PC61.5) rat anti-mouse IgG Ab and positively selected using goat anti-rat IgG Dynabeads (Invitrogen, Carlsbad, CA). B cell depletion was done by positive selection using goat anti-mouse IgG Dynabeads (Invitrogen). After depletion, cell suspensions presented <1% of CD25<sup>+</sup> and 3% of CD19<sup>+</sup> cells. Both procedures were done accordingly to the manufacturer’s recommendations. For in vivo Treg depletion, recipient chimeras were injected i.p. with PC61 mAb (250 µg/mouse) at day 3 posttransplant. On day 14 posttransplant another i.p. dose of PC61 mAb were injected (100 µg/mice).

**Purification and adoptive transfer of B cells**

CD19<sup>+</sup> B cells were isolated from donor spleen by positive selection using anti-CD19 MACS microbeads (Miltenyi Biotec, Auburn, CA), according to the manufacturer’s recommendations. For adoptive transfer experiments, 1 × 10<sup>7</sup> purified CD19<sup>+</sup> B cells (purity > 95%) from treated or untreated donor were injected along with the graft. When indicated, the spleen used as T cell source was previously depleted of total B cells and reconstituted with purified CD19<sup>+</sup> B cells.

**Flow cytometry staining and analyses**

For surface staining, cell suspensions were preincubated with 2% normal mouse serum in PBS (Sigma-Aldrich, St. Louis, MO) on ice, followed by incubation with appropriate Ab mixture for 15 min. For Foxp3 staining, cells were labeled as specified in the ebioscience kit standard protocol. All samples were acquired in a FACScalibur (BD Biosciences, San Jose, CA) or in a FACSCanto (BD Biosciences). Acquisitions were performed using CellQuest (BD Biosciences) software and BD FACSDiva software, respectively. Analyses were performed using FlowJo software (Tree Star, Ashland, OR). For cell staining the following Abs were used: FITC or allophycocyanin anti-mouse CD4 (OKT1; 55-6.7); PE-Cy7 CD8a (55-6.7); PE anti-mouse CD25 (PC61.5, 7D4); allophycocyanin anti-rat/mouse Foxp3 (FJK-16); FITC or PECy7 anti-mouse CD3e (145-2C11); biotinylated anti-mouse CD11c (HL3); FITC anti-mouse CD11b (M1/70); PE-Cy5 anti-CD25, anti-mouse IgG1 (eB121-15F9); allophycocyanin anti-mouse IgD (11-26c [11-26]); FITC anti-mouse CD5 (53-6.7); PE anti-mouse CD1d (1B1); allophycocyanin-eFlour 780 anti-mouse CD21 (eBioSD9); PE anti-mouse CD43 (eBioR2/60); allophycocyanin-

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anti-mouse CD40 (1C10); FITC anti-mouse CD86 (GL1); biotinylated anti-mouse B7-H2 (HK5.3); allophycocyanin anti-mouse I-A/I-E (M5/114.15.2); biotinylated or allophycocyanin anti-mouse H-2Kb (AF6-88.5.5.3); PE anti-mouse H-2Kd (SF1-1.1.1) and FITC, PE, PerCP, or allophycocyanin streptavidins were all purchased from eBioscience (San Diego, CA). PE anti-mouse latency-associated polypeptide (LAP) (TW7-16B4) was purchased from BioLegend (San Diego, CA).

Statistical analysis

For grouped analysis, one-way or two-way ANOVA with Bonferroni posttest were used. Unpaired Student t test was applied for statistical differences between two groups. Survival data were analyzed with log-rank (Mantel–Cox) test. Error bars represent SD. Values of \( p < 0.05, < 0.01, \) and \(< 0.001 \) are referred to in the figure legends with symbols *, **, and ***, respectively.

Results

Treatment of donor mice with combined therapy efficiently inhibits acute GVHD

Although it has been reported that induction of oral tolerance in the recipient can ameliorate aGVHD (12), there are no reports on whether it is possible to induce tolerance to host Ags in the donor before the transplant is performed. To determine whether donor T cells can be rendered tolerant to host tissues through the oral route, donor B6 mice received daily doses of host protein Ags by gavage, whereas \( L. \) lactis was offered ad libitum during 5 d (combined therapy). These animals were immunized i.p. with recipient spleen cells after 4 d and 1 wk later used as spleen cell donors along with normal B6 bone marrow to reconstitute lethally irradiated F1 (BALB/c × B6) mice (Fig. 1A). Fig. 1B shows that when combined therapy is used, an elevated survival rate was observed. Also, evaluation of clinical parameters reveals an important decrease in disease severity when compared with all other groups tested (Fig. 1C, Supplemental Fig. 1). Donor treatment based on gavage of recipient protein Ags (AgF1) or \( L. \) lactis administration ad libitum (Llactis), used separately, shows only a discrete protection (Fig. 1, Supplemental Fig. 1).

To confirm the reduction in aGVHD, we examined histopathologically the skin, colon, and liver of recipient mice 21 d after BMT. The pathologic scores were significantly lower in recipients transplanted with spleen cells from treated donors (AgF1/Ll group) when compared with those who received spleen cells from untreated donors (Ctr+) (Fig. 2), AgF1, or Llactis groups (Supplemental Fig. 2). This reduction might be related to a higher IL-10 production found on affected tissues at that same period (Supplemental Fig. 3).

Combined therapy maintains the GVL effect and immunocompetence in the recipient

The close association between GVHD and the GVL effect reflects the need for therapies that target the undesired reaction only (1, 4). To determine whether the combined therapy is able to decrease GVHD while maintaining the beneficial GVL effect, GFP-expressing P815 mastocytoma cells were injected along with the graft. Twenty-five days post-BMT, bone marrow, blood, lymph nodes, spleens, and livers of the recipients were examined for the presence of GFP+ cells. As expected by the absence of GVHD, GFP+ cells were found in all tissues analyzed of mice in the negative group.

FIGURE 1. Donor treatment with combined therapy reduces the clinical signs of acute GVHD. Donor mice (AgF1/Ll) received orally by gavage 50 \( \mu \)g F1 spleen cell protein extract and ad libitum doses of \( L. \) lactis daily for 5 d. Four days later, the animals were immunized with \( 10^6 \) F1 splenic cells i.p. After 6–7 d, BMT was done. Lethally irradiated F1 mice (BALB/c × B6) were reconstituted with \( 5 \times 10^6 \) bone marrow cells from B6 mice along with \( 5 \times 10^6 \) splenic T cells from control B6(Ctr+) or B6 mice orally treated with F1 cell extract (AgF1), with \( L. \) lactis (Llactis), or both (AgF1/Ll). (A) Schematic view of combined therapy treatment. (B) Survival rates. (C) Total clinical score. Data are representative of two experiments with five to eight mice per group. *\( p < 0.05, ***p < 0.001 \) for AgF1/Ll versus Llactis group. ***p < 0.001 for AgF1/Ll versus Ctr+ group. ***p < 0.001 for AgF1/Ll versus AgF1 group.
control disease group. In contrast, in the positive control disease group, where GVHD developed, no GFP+ cells were observed (Fig. 3A). However, despite being protected from aGVHD, mice in the experimental AgF1/Ll group were capable of eliminating the tumors cells from all tissues. The protected chimeras remained free of tumor for >15 wk post-BMT (Fig. 3B), whereas chimeras from negative and positive control disease groups died with clear signals of tumor growth and GVHD development, respectively.

Because the therapy proposed in this study combines two treatments with immunomodulatory effects, the protection observed could be a result of a general state of immunosuppression that would interfere with recipient response to other Ags. To test whether the immunosuppression induced by combined therapy was specific for host Ags, recipients protected for >1 y post-BMT received skin grafts from third-party and from BALB/c donors. The protected chimeras specifically rejected the unrelated skin graft but accepted the BALB/c graft (Supplemental Fig. 4A).

However, after 1 y, we cannot exclude the participation of newly generated T cells because the thymic function of the chimeras, at that posttransplant period, already had been restored and the newly emergent T cells are tolerant to the BALB/c Ags. To test the specificity of the tolerance induced for host Ags, B6 mice treated or not with the combined therapy received skin grafts from third-party and from F1 donors. As shown in Supplemental Fig. 4B, both treated or untreated B6 recipients rejected the skin grafts. However, when female B6 mice were treated with the combined therapy using male B6 splenic protein extract on gavage, they did not reject male skin graft unlike the untreated females (Supplemental Fig. 4C). It suggests that combined therapy induces a more effective tolerance to minor rather than major histocompatibility

FIGURE 2. Donor treatment with combined therapy reduces the histopathological manifestations of acute GVHD. Donor mice treatment with combined therapy and HSCT were performed as described in Fig. 1. Representative micrographs of skin (A), colon (C), and liver (E) from negative control disease group (left panels), positive control disease group (middle panels), and experimental AgF1/Ll group (right panels) are shown. H&E staining. Total histopathological score of skin (B), colon (D), and liver (F). Data were combined from different experiments with 14–18 mice. *p < 0.05, ***p < 0.001.

FIGURE 3. Combined therapy preserves the graft versus tumor effect. Donor mice treatment with combined therapy and HSCT were performed as described previously. Along with hematopoietic stem cells, 10^7 mastocitoma tumor cells (P815) expressing GFP were injected. (A) Twenty-five days posttransplant, spleen, liver, bone marrow (BM), total lymph nodes (tLn), and blood from the chimeras were analyzed by flow cytometry for the presence of GFP+ cells. (B) Survival rate of chimeras that also received tumor cells. Data are representative of two experiments, with five mice individually analyzed per group. ***p < 0.001 for AgF1/Ll (+P815) versus Ctr− (+P815) group. ***p < 0.001 for AgF1/Ll (+P815) versus Ctr+ (+P815) group.
Ags, which may explain how it is able to protect from GVHD preserving the GVL effect.

Altogether, the data show that treatment of donor mice with combined therapy efficiently reduces clinical and pathological aGVHD development on a specific and long-lasting fashion, without immunosuppressing the recipient and preserving the GVL effect.

**Protection against aGVHD by the combined therapy is not mediated by donor Foxp3+ Tregs**

The phenomenon of oral tolerance can occur by different mechanisms being the expansion of regulatory-suppressive T cells generally related to repeated lower doses of Ag (5, 6). To investigate the mechanism by which the combined therapy exerts the protection against aGVHD, spleen cells from treated and untreated donors were analyzed. No significant differences were observed in absolute number or frequency of splenic cell subpopulations, including Foxp3+ T cells (Fig. 4A, 4B). To further exclude the participation of donor Tregs, CD25+ T cells were depleted from spleen cells of treated donors before transplantation. Depletion of donor Tregs did not abrogate the protection, and the elevated survival rate was maintained (Fig. 4C, 4D), confirming that these cells are not necessary for the protective effect.

**Donor B cells tolerized by the combined therapy are responsible for GVHD protection**

Several recent studies in both mice and humans have showed the participation of B cell on immunoregulation and protection against acquired inflammatory syndromes (35). In models of oral tolerance induction, B cells seem to play an important role in tolerance induced with low doses of Ag (36). Because the combined therapy is based on induction of oral tolerance with small amounts of recipient Ag, we evaluated the participation of donor B cells in the combined therapy protocol. Spleen cells from combined therapy–treated donors were depleted of B lymphocytes before transplantation. As indicated in Fig. 5A, elimination of B cells completely abrogated the protection from aGVHD, leading to a higher total clinical score and an elevated mortality rate in this group (AgF1/L1 [-LØ B]) when compared with non-B cell–depleted one (AgF1/L1). Because the role of B cells in GVHD pathophysiology is still controversial (37), one may argue that loss of protection could be related not only to the absence of B cells tolerized by treatment but also being exacerbated by the absence of B lymphocytes at all.

To overcome this issue, spleen cells from combined therapy–treated donors were reconstituted with purified splenic CD19+ B cells from untreated donors. The reconstitution had no effect, and the recipients still developed aGVHD. However, when CD19+ B cells from a treated donor were used to reconstitute the combined therapy B-depleted spleen, protection was completely restored (Fig. 5A).

We next tested whether tolerized B cells were sufficient to inhibit aGVHD by transferring CD19+ B cells from a treated donor along with spleen cells (and bone marrow) from a control untreated C57BL/6 donor (Fig. 5B). Of interest, depletion of B lymphocytes from untreated donor spleen cells ameliorated the GVHD, suggesting that B cells from untreated mice, at least in this model, have a pathogenic role in GVHD development. Taken together, the data show that protection of recipient mice against aGVHD is mediated by B lymphocytes with regulatory functions generated by the combined therapy treatment of donor.

**Oral administration of recipient protein Ag with L. lactis induces an increase in splenic B cells compatible with Bregs**

To better characterize the B cells involved in the regulatory activity over aGVHD development, donor mice were treated or not with the combined therapy and spleen cells were analyzed 6 d after the end of the oral therapy. Nontreated mice were used as controls. (A) Frequency of spleen cell subpopulations. (B) Absolute number of spleen cell subpopulations. (C) Total clinical score. (D) Survival rates. Data are representative of five pools of three mice per group in (A) and (B), and a total of 10 mice was individually analyzed per group in (C) and (D). ***p < 0.001 for AgF1/L1 (-CD25) versus Ctr+.

![FIGURE 4.](http://www.jimmunol.org/)
combined therapy and their splenic B cells analyzed by flow cytometry.

When the B lymphocytes were grouped according to the expression of IgM and IgD, we found a higher frequency of transitional B cells (CD19+IgM+IgD) in treated animals. No differences were observed among the immature (CD19+IgM-IgD+) and mature (CD19+IgM-IgD) B cells subpopulations (Fig. 6A), suggesting that combined therapy induced an increase in transitional Bregs. However, several different phenotypes have been attributed to B cells with regulatory functions and at least two subpopulations were described: transitional Breg, mentioned above, and the B10 cells (37). In common, both express CD21 and CD1d molecules, being that the B10 is also positive for CD5 (38). Despite the increase in overall CD21 expression in splenic B cells from treated donors (Fig. 6B), we observed that CD19CD21+CD1d− and CD19CD21+CD1d+ CD5+ subpopulations were reduced on these animals (Fig. 6C, 6D).

Although there is not a unique marker, or set of markers, that exclusively identifies regulatory B cells (Bregs), expression of molecules such as CD86 (39) and class II MHC (40) are necessary for most of their regulatory functions, regardless of the specific B cell immunophenotype. In fact, splenic B lymphocytes from combined therapy–treated donor express high amounts of both molecules (Fig. 6C, 6D).

Recently, B cells with strong suppressive functions in vitro and in vivo were generated by the incubation of naïve B cells with a relevant Ag conjugated to cholera toxin B subunit. In this scenario, tolerance was shown to be at least, partially dependent on B cell expressing the LAP/TGF-β. On the basis of these data, we looked at LAP expression in CD19+ cells and found a higher expression of this molecule in B cells from treated when compared with untreated mice (Fig. 6G, 6H).

FIGURE 5. Protection of recipient mice against GVHD is dependent on donor B cells generated by combined therapy. Donor mice were treated with the combined therapy as described previously. Before transplantation, B cells were depleted from combined therapy treated or untreated donor spleen. (A) Total clinical score and survival rates of mice reconstituted with untreated splenocytes from treated donors (AgF1/Ll group), splenocytes depleted of B cells (AgF1/Ll−LoB group), splenocytes depleted of B cells, and reconstituted with B cells from treated (AgF1/Ll−LoB+LoBAg/Ll group) or untreated donors (AgF1/Ll−LoB+LoB B6 group). (B) Total clinical score and survival rates of mice reconstituted with untreated splenocytes from untreated donors added (Ctr+−LoB)+LoBAg/Ll) or not with B cells from treated donors (Ctr+) and spleen depleted of B cells (Ctr+−LoB). Data are representative of two experiments with 6–10 mice/group. *p < 0.05, **p < 0.01, ***p < 0.001 for (AgF1/Ll) versus (AgF1/Ll−LoB) group, splenocytes depleted of B cells (AgF1/Ll−LoB) group, splenocytes depleted of B cells, and reconstituted with B cells from treated (AgF1/Ll−LoB+LoBAg/Ll group) or untreated donors (AgF1/Ll−LoB+LoB B6 group). Data are representative of two experiments with 6–10 mice/group. *p < 0.05, **p < 0.01, ***p < 0.001 for (AgF1/Ll) versus (AgF1/Ll−LoB). Data are repre-
Donor mice do not have higher numbers of Tregs nor do the spleen cells depend on the Tregs to exert their inhibitory effect (Fig. 4). Reinforcing these data, in vivo depletion of CD25+ cells from recipient reconstituted with combined therapy–treated donors totally abrogated the protection (Fig. 9A, 9B).

Taken together, the results show that tolerized B cells from the donors mediate the protection against GVHD by inducing Foxp3+ cells in the transplant recipients.

**Discussion**

HSCT is an immunotherapy for treatment of malignant and non-malignant diseases that is seriously limited by GVHD, an immune syndrome with high morbidity and mortality rates (1–3). Prophylaxis and treatment of GVHD is based on nonspecific immunosuppressive agents that, among other complications, eliminate GVLr, which elevates the risk of tumor relapse (4). Education of the immune system by induction of oral tolerance (12) and manipulation of gut microbiota by ingestion of probiotic bacteria (30) represent rationally viable and noninvasive therapies to reduce the development of GVHD. Nonetheless, their clinical application has been limited because the HSCT recipients are subjected to conditioning regimens that require a strict diet in which live or raw food is precluded.

In this study, we demonstrate that treatment of donor mice with combined therapy reduces clinical and pathological manifestations of aGVHD, improving the survival rates to 100%. This phenomenon is specific and long lasting, and it is not a result of a non-specific immunosuppressive milieu. Maintenance of beneficial GVL effect is as important as the reduction of GVHD when HSCT is used to treat a malignant disease (45–47). In humans, although it has been suggested that GVL and GVHD are temporally separate (48) and that the severity of GVHD does not implicate in im-

![FIGURE 6](http://www.jimmunol.org/)

**FIGURE 6.** B cells generated by combined therapy in the donors are phenotypically compatible with Bregs. Donor mice were treated with combined therapy as previously described, and splenocytes were analyzed at the end of treatment. (A) Frequency of immature (CD19+IgMhiIgDlo), transitional (CD19+IgMloIgDhi), and mature (CD19+IgMloIgDhi) B cells. (B) CD21 expression on CD19+ B cells (mean fluorescence intensity [MFI]). (C) CD86 expression on CD19+ B cells (MFI). (D) Class II MHC expression on CD19+ B cells (MFI). (E) Frequency of splenic CD19+CD21+CD1d+ subpopulation. (F) Frequency of splenic CD19+CD21+CD1d+CD5+ subpopulation. (G) Frequency of LAP+ B cells. (H) Quantification of (G). Data are representative of 10 mice individually analyzed per group. *p < 0.05, **p < 0.01, ***p < 0.001.

![FIGURE 7](http://www.jimmunol.org/)

**FIGURE 7.** Protection of recipient mice against GVHD is dependent on IL-10 produced by tolerized donor B cells. Lethally irradiated F1 mice (BALB/c × B6) were reconstituted with $5 \times 10^6$ bone marrow cells from B6 mice along with $5 \times 10^6$ splenic T cells from control B6 (Ct+). Splenic CD19+ cells (LoB) from untreated mice (Ct+LoB B6), from combined therapy treated wild-type (Ct+LoB AgF1/LI) or IL-10–deficient (Ct+LoB IL10KO) donors were transferred along with the bone marrow and spleen cells to assess the role of IL-10 in GVHD protection. (A) Total clinical score. (B) Survival rates. A total of 10 mice was individually analyzed per group. *p < 0.05, **p < 0.01, ***p < 0.001. *(Ct+) versus (Ct+LoB AgF1/LI).
proved GVL effect (49), both reactions are clinically associated (4), and there is no specific therapeutic approach to target the undesired reaction only. In our approach, recipients reconstituted with spleen cells from combined therapy–treated donors develop less severe aGVHD and are still capable of eliminating tumor cells injected along with the graft, showing that the GVLr is maintained. This dual capacity may be related with the fact that combined therapy induces a more effective tolerance to minor histocompatibility Ags. Actually, it is expected because the Ags are administered in the form of soluble proteins that will be captured and processed, losing the entire MHC peptide complexes.

**FIGURE 8.** B cells generated by the combined therapy increase the frequency of donor Foxp3⁺ cells in the recipient mice. Donor mice were treated with the combined therapy as described previously. Before transplantation, B cells were depleted from combined therapy treated or untreated donor spleen. Four days posttransplant, the recipients were euthanized, and the presence of H-2Kᵇ-H-2Kᵈ Foxp3⁺ was analyzed in the spleen and mesenterial and peripheral lymph nodes. Representative data of Foxp3⁺ cells on peripheral and mesenteric lymph nodes (A) and spleen (C) gated on H-2Kᵇ-H-2Kᵈ cells. Frequency and absolute number of H-2Kᵇ-H-2Kᵈ Foxp3⁺ in peripheral or mesenteric lymph nodes (B) and in the spleen (D). Data are representative of the individual analysis of six to eight mice per group for the spleen and analysis of three to four pools of five animals for lymph nodes. *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 9.** Posttransplant in vivo depletion of CD25⁺ T cells abrogates the protection. Donor mice treatment with combined therapy and HSCT was performed as described previously. On third day posttransplant, chimeras reconstituted with combined therapy treated donor spleen received an i.p. dose of PC61 mAb (250 µg/mice) (54) for depletion of CD25⁺ cells. On day 14 posttransplant, a reinforced i.p. dose of PC61 mAb was injected in recipient chimeras (100 µg/mice). (A) Total clinical score. (B) Survival rates. A total of 10 mice was individually analyzed per group. ***p < 0.001 for AgF1/LI versus AgF1/LI (CD25). **p < 0.01, ***p < 0.001 for AgF1/LI versus Ctr⁺. **p < 0.01 for AgF1/LI (CD25⁺) versus Ctr⁺.
Recently, B lymphocytes subset able to modulate inflammatory responses were described previously (35). These Bregs seem to play an essential role in oral tolerance induced with low amounts of Ags (36). In accordance to that, protection obtained with combined therapy is totally abrogated in the absence of B lymphocytes. Moreover, CD19+ cells from treated donors, but not from untreated ones, are sufficient to inhibit aGVHD in recipients reconstituted with control spleen cells, showing that combined therapy induces the generation of a B cell subpopulation with regulatory functions.

Generation of specific Foxp3+ Tregs by allogeneic primary B cells (50) and by LAP/TGF-β+ B cells (41) have been reported previously. Indeed, our data show that combined therapy not only increases the CD19+LAP+ population but also induces donor Foxp3+ Tregs. Interestingly, in our model, the expansion of Tregs did not occur in treated donors because we did not observe differences in frequency or absolute number of donor splenic Foxp3+ cells. Moreover, elimination of CD25+ population in the graft before the transplant does not abrogate the protective effect from combined therapy. The induction of donor Foxp3+ cells, possibly from effector alloreactive T cells, happens on recipient after the transplant and is dependent on those B cells generated by the treatment, indicating that Ag-specific presentation and recognition are important steps for acquisition of a regulatory phenotype by allo-T cells and for their following activation. The abrogation of protection after in vivo depletion of CD25+ cells on recipient supports this hypothesis. On the basis of these data, we propose a model in which B cells from orally treated mice present cognate Ags to allospecific responder T cells responsible for the GVH reaction, explaining the Ag-specific suppression toward aGVHD only. These results are in accordance with previous reports showing that the generation of specific Tregs after allogestion in vitro inhibits aGVHD and spare GVL/GVT responses in some experimental models (51, 52).

The heterogeneity among Bregs and the absence of a master transcriptional factor associated with this subpopulation strongly suggest that, like their T cell counterpart, they can be divided into different regulatory subsets originated from ontogenically distinct subsets (53). However, some features such as the production of IL-10 (35) and expression of molecules like CD86 (39) and class II MHC (40) along with an immature phenotype are common among the different subsets. In fact, we observed increased IL-10 in protected recipients. Moreover, combined therapy induces a high frequency of transitional B lymphocytes, with high expression of CD21, CD86, and class II MHC, a phenotype compatible with Bregs. In addition, we demonstrated that IL-10 production by splenic B cells from treated donors is necessary for the protection observed after combined therapy, indicating the B10 cells, the IL-10–producing Breg (42), is in the fact the key player in this setting. The contribution of a distinct cell type in the immediate induction of B cell with regulatory function cannot be excluded, although to our knowledge there is no report showing the dependence of other cells, such as dendritic cells, on Breg activation.

Finally, B cell differentiation into a regulatory subtype was described by Lampopoulos et al. (43) as being dependent on two signals. A first signal delivered by TLR initiates IL-10 production by B cells. In a second phase, engagement of receptors classically involved with B cell survival and expansion, such as the BCR, amplifies the initial population of IL-10–producing B cells, allowing the effective suppression. Altogether, these data might support the idea that the ingestion of L. lactis gives the innate stimulus necessary to the acquisition of a regulatory phenotype by the B cells, which is dependent on IL-10 in our model. The recipient Ags in the protein extract would then provide BCR stimuli that would enable specific B cells to present the cognate Ags to T cells, generating Tregs. Activity of these alloreactive Tregs would dampen the response to recipient Ags but preserve the response to Ags derived from the tumor cells (51, 52).

Our results suggest that oral tolerance induction by the use of a probiotic bacteria and the recipient protein extract, on HSC-T donors before transplantation, might prove safe, simple, and effective for preventing GVHD in human patients.

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Disclosures

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References


Corrections


The eighth author’s name was omitted from the article. The corrected author and affiliation lines are shown below.


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