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Role of Regulatory T Cell Populations in Controlling Graft vs Host Disease

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Abstract

Immune function is critical in health and disease. The control and regulation of immune reactions is an area of intense investigation that has important implications for allogeneic hematopoietic cell transplantation. Immune reactions are regulated in a number of important ways.

Compartmentalization of immune responses and the production of both pro-inflammatory and anti-inflammatory cytokines play a major role. More recently several populations of T cells that regulate immune responses termed regulatory T cells have been identified. This manuscript will focus on CD4⁺CD25⁺FoxP3⁺ natural regulatory T cells (T_{reg}) and αβ TCR⁺CD4⁺NK1.1⁺ natural killer T (NK-T) cells which both suppress graft vs host disease but appear to function by distinct mechanisms.

Keywords

Graft vs host disease; regulatory T cells; NK-T cells

Allogeneic hematopoietic cell transplantation (HCT) has proven to be an effective therapy for a broad range of hematologic malignancies and selected solid tumors as well as genetic abnormalities. The major benefits of allogeneic HCT is through the introduction of the hemtolymphoid system of the donor which can correct errors of hematopoiesis and generate and immune response against the underlying malignancy. However, allogeneic HCT is limited by graft vs host disease (GVHD) which is caused by alloreactive donor derived immune effector cells. GVHD is characterized by dysregulation and excessive immune activation resulting in tissue damage primarily to the skin, gastrointestinal tract and liver. Control of GVHD holds great promise not only for improving allogeneic HCT as it is currently practiced but also to allow for extension to other potential indications such as for the treatment of patients with severe autoimmune disease or for the induction of tolerance to organ transplantation. To date a number of different populations of cells capable of regulating immune responses have been isolated and characterized in murine and other model systems. Here I will focus on the role of CD4⁺CD25⁺FoxP3⁺ natural regulatory T cells (T_{reg}) and αβTCR⁺CD4⁺DX5⁺ natural killer T (NK-T) cells.

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Isolation and purification of regulatory T cell populations has been possible through magnetic cell separation and cell sorting such that highly purified (95–99% purity) populations of both T_{reg} and NK-T cells can be readily obtained and utilized in experimental models to elucidate function. The discovery of T_{reg} was facilitated by the development of the mixed lymphocyte reaction (MLR). In this reaction, irradiated stimulator cell populations are mixed with responding cells for several days and then pulsed with 3H -thymidine. An alternative approach involves the use of responding cells that are labeled with the dye CFSE that is diluted upon cell proliferation. Responses are quantified by the incorporation of 3H -thymidine or dilution of the CFSE during cell division. The addition of T_{reg} resulted in a marked suppression of the proliferation of the responding cells in a dose dependent manner. Typically, a 1:1 ratio between T_{reg} and conventional CD4 and CD8 cells (T_{con}) is required for full suppression in the MLR. Interestingly, *in vitro* the T_{reg} do not proliferate. A number of groups have demonstrated that the addition of T_{reg} to T_{con} in animal models of allogeneic hematopoietic cell transplantation, both across major and minor histocompatibility barriers, resulted in suppression of graft-versus-host disease (GVHD) and improved survival as compared to animals that received T_{con} alone^{1–4}. These studies have demonstrated the ability of T_{reg} to have biological function *in vivo*.

Using bioluminescent imaging (BLI), we have demonstrated that tumor cells marked with luciferase will actively proliferate in these *in vivo* models when T cell depleted bone marrow is used alone disease progresses and animals die after ~6–8 weeks of progressive leukemia⁵. When CD4 and CD8⁺ T_{con} alone are added, animals have control of disease, however, these animals die very rapidly due to GVHD with 2–3 weeks. Importantly, when T_{reg} are added to the T_{con} , there is a marked suppression of GVHD yet GVT reactions are maintained. Maintenance of GVT was demonstrated in two different tumor model systems using both A20 and BCL₁ tumor models that has been reproduced by others. In these studies when T_{reg} are added to T cell depleted bone marrow (TCD-BM), there is no GVHD yet no GVT effects. The GVT reaction is dependent upon the T_{con} that is maintained in the presence of T_{reg} ^{5, 6}. The understanding of this apparent separation of GVHD and GVT effects involved the dissection of T_{con} function in the presence or absence of T_{reg} . In these studies the most striking findings were that T_{reg} suppress T_{con} proliferation demonstrated by BLI and assessment of the number of donor-derived T_{con} in different tissues following HCT⁵. These studies have demonstrated that *in vivo* T_{reg} do not block T_{con} activation, but block their proliferation. In the setting such as GVHD, T_{con} proliferation is required for the full impact of GVHD mediated infiltration and destruction of target tissues whereas in these models proliferation of alloreactive or tumor reactive T cells is not required for GVT effects. These observations are likely dependent upon the precursor frequency of donor derived alloreactive T cells as well as the tumor burden. The opposite situation would be a setting where immunization is being performed for a very low frequency T cell population where significant T cell proliferation is required for example during immunization with a tumor vaccine. Here T_{reg} would block the manifestation of this immune reaction through suppression of T_{con} proliferation and depletion of T_{reg} could be potentially beneficial.

An important question is what are the trafficking and survival characteristics of T_{reg} compared to T_{con} . We have addressed this question using BLI and created transgenic animals which constitutively express luciferase⁷. In these models the trafficking and survival of different populations of cells can be readily assessed by isolating cell populations such as T_{reg} and T_{con} from the *luc*⁺ transgenic mice and transplanting these cells along with TCD-BM into irradiated wild type recipient mice. When comparing T_{reg} to T_{con} both cell populations actively proliferate in allogeneic recipients, especially following total body irradiation. Both cell populations initially migrate to nodal sites such as the cervical lymph nodes, mesenteric lymph nodes, and the spleen, actively proliferate in these sites and then infiltrate GVHD target organs. Interestingly, T_{reg} do not cause GVHD. Within the first 10

days following transplantation, T_{reg} proliferate as actively as T_{con} , yet T_{con} continue to proliferate and progress resulting in GVHD whereas T_{reg} proliferation wanes after ~4–6 weeks and do not cause significant pathology⁸. The reasons that T_{reg} proliferation is limited remain active areas of on-going investigation.

These studies suggested to us that the addition of T_{reg} prior to T_{con} , which could be readily accomplished both experimentally and in the clinic, may allow for infusion of lower numbers of T_{reg} for control of GVHD if these cells were allowed to traffic to nodal sites and begin to proliferate. If successful this could help solve one of the major problems in translating these concepts to the clinic namely the relatively low numbers of T_{reg} available. In fact, the ratio of T_{reg} to T_{con} of 1:1 required when both cell populations are given the same day can be reduced to 1:3 or even 1:10 if the T_{reg} are given 48 hours prior to the T_{con} ⁹. These studies demonstrated that the administration of T_{reg} prior to T_{con} was more effective and have influenced the design of the clinical trials extending these observations to the clinic.

One of the major concerns relating to the use of T_{reg} clinically is the question of whether the T_{reg} will result in a state of global immunosuppression increasing the risks of opportunistic infections. We addressed this concern by exploring the immune reconstitution following both T_{con} and T_{con} plus T_{reg} infusions. One of the major sites of GVHD induced tissue destruction are nodal tissues where it could be demonstrated that in the presence of T_{con} , both lymph nodes and the thymus were damaged resulting in smaller atrophied organs with disrupted architecture. In these animals, CD4 and CD8 T cell reconstitution was impaired. To assess the functional capabilities of these animals an infectious agent in this case, murine CMV (mCMV) was used to infect the recipient animals resulted in lethality when the mCMV was given at days 14, 30 and 63. When T_{con} and T_{reg} were both utilized, this resulted in preservation of thymic and nodal architecture, improved immune reconstitution and ability to better control this infectious challenge⁹. The T_{con} plus T_{reg} treated animals were capable of mounting an immune response demonstrated by ELISPOT analysis against mCMV. These studies demonstrate that the combined use of T_{con} plus T_{reg} results in improved immune recovery due to blocking the GVHD-induced damage to nodal structures which is required for immune reconstitution. These studies have important implications for clinical translation.

Another population of immune regulatory cells termed NK-T cells has also been implicated in the control of GVHD¹⁰. These cells express markers typically found on T cells ($\alpha\beta$ TCR and CD4), as well as NK cells (NK1.1 and DX5). The potential role of NK-T cells in the control of GVHD has been highlighted by the development of the total lymphoid irradiation and anti-thymocyte globulin (TLI/ATG) regimen which has been effective in both murine models and patients¹¹. In these murine systems, as well as in clinical translational studies, following TLI and ATG there is an increase in the number of NK-T cells which produce the immunoregulatory cytokine IL-4 required for the suppression of GVHD. Animals prepared with TLI/ATG can tolerate up to 1,000 times the number of T cells as TBI prepared animals. We evaluated the adoptive transfer of NK-T cells from donor animals in the same model systems described above for the analysis of T_{reg} . NK-T cells could also be highly purified by magnetic bead separation and FACS to >98% purity. The NK-T cell population is composed of both a TCR variant and an invariant population of cells both present at ~50% of the cells which can be identified by the reactivity to CD1d and can be separated using a CD1d tetramer. Using BLI, we demonstrated that NK-T cells also actively proliferate in these animal models, yet also do not cause GVHD. NK-T cells persist much longer than T_{reg} , over 100 days, and traffic to many of the same sites as conventional T cells yet don't infiltrate target tissues as readily and are retained more in nodal structures. Interestingly, when NK-T cells were added to the same models of GVHD across major histocompatibility barriers

induced by donor derived T_{con} as few as 10–20,000 NK-T cells were capable of significantly improving animal survival as compared to control animals who received T_{con} alone¹². Paradoxically, increasing numbers of NK-T cells were less effective in these animal models for reasons that remain to be elucidated. Both variant and invariant NK-T cells appeared to function in these models and IL-4 production was a critically important feature of the NK-T cells. As previously described, T_{reg} appear to control GVHD by suppressing T_{con} proliferation which can be demonstrated by BLI. When NK-T cells were evaluated in the BLI experiments these cells did not suppress luc^+ T_{con} proliferation which actively proliferated in the presence or absence of NK-T cells. This suggested that NK-T cells control GVHD through a different mechanism not related to suppression of T cell proliferation and related to IL-4 production. The mechanisms by which these different populations of regulatory cells suppress the GVHD reaction are areas of ongoing investigation (Table 1).

These studies, as well as many others from a variety of different laboratories, have suggested that these regulatory cell populations could be ideal populations for clinical translation. We felt that T_{reg} hold the greatest promise and set out to translate these findings to the clinic. There are a number of challenges that must be overcome including that these are rare cell populations with a paucity of unique markers for their isolation. The MLR is a useful but limited functional assay since no information can be obtained about the ability of the T_{reg} to traffic to nodal sites can be obtained. Further a number of regulatory requirements must be satisfied that pose significant challenges to clinical translation. We and others have pursued magnetic bead based cell separation technologies. Using CD25 separation is a very effective first step resulting in ~50–70% FoxP3 expressing populations of cells. In these studies, recovery of ~60–70% can be readily obtained on a clinical scale and these cells have been used for clinical translation with limited toxicity. This approach has been applied in haploidentical transplantation with exciting initial results by the group from the University of Perugia. In their studies where rigorous T cell depletion is required in the absence of immunosuppressive medications, very low numbers of T cells of $>5 \times 10^4/kg$ are unacceptable due to the risks of GVHD¹³. The concept of mega CD34 cell utilization has also been extremely important and results in successful transplantation across these histocompatible barriers with high rates of engraftment and limited GVHD¹⁴. A major issue has been that these individuals are immunosuppressed and at risk for opportunistic infections and disease relapse. The addition of T_{con} has been impossible since even low numbers of cells result in GVHD. The setting of haploidentical transplantation is an ideal setting in which to test the concepts of T_{reg} biology since the number of T_{con} that is acceptable is known and low, no immunosuppressive medications are required that can have differential impact on T_{reg} function and there is an unmet clinical need. The initial results from the University of Perugia are highly encouraging where up to 2×10^6 were infused following T_{reg} with low levels of GVHD¹⁵. These patients appeared to develop more rapid immune recovery compared to their historical experience.

We sought to purify T_{reg} to the same level of homogeneity as achieved in the animal models. A frequent criticism of animal models is that they don't always translate to the clinic, however, it is also frequent that the clinical studies are not performed with the same rigor as the model systems for many technical, as well as reagent availability reasons. Nevertheless, we felt it was important to purify the T_{reg} for these translational studies to determine how likely they were to result in GVHD, especially across major histocompatibility barriers. High speed cell sorting has been utilized on a clinical scale to isolate hematopoietic stem cells with high purity. A similar strategy was utilized for the isolation of T_{reg} . The first approach utilized CD25 as the primary approach for T_{reg} isolation, however, this proved to be problematic in that there was significant variability amongst different individuals in the setting of the gates. In some individuals, the top 20%

brightest CD25⁺ cells resulted in uniformly FoxP3⁺ cells whereas in other individuals 40 or 50% of the CD25⁺ cell population was found to be FoxP3⁺. Further, a significant number of FoxP3⁺ cells were found within the negative cell population resulting in low yields. A key finding was that T_{reg} typically do not express the IL-7 receptor (CD127)¹⁶. Isolation of CD4⁺CD25⁺CD127^{lo/-} cell population resulted in a uniformly FoxP3⁺ population with high yields of the FoxP3⁺ cells. In these pre-clinical studies purity of 96% and a yield of $\sim 1 \times 10^8$ cells per apheresis product can be readily obtained. These cells can be cryopreserved, suppress the MLR and are stable for over 2 years once cryopreserved. We have developed the technologies and clinical trial to test these concepts which is currently enrolling patients.

In conclusion, murine studies have demonstrated that both T_{reg} and NK-T cells are capable of suppressing GVHD while maintaining GVT reactions. Both cell populations proliferate *in vivo* in allogeneic lymphopenic hosts. These studies set the stage for clinical translation that is on-going by a number of different groups. Initial studies are very encouraging and have demonstrated that T_{reg} based clinical studies are feasible, do not result in toxicity and appear to suppress GVHD risk of T_{con} while promoting enhanced immune reconstitution. These studies are not only important for allogeneic hematopoietic cell transplantation but could have major implications for the treatment of autoimmune disorders, as well as for induction of tolerance to solid organ transplantation.

Acknowledgments

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Table 1

Characteristics of T_{reg} and NK-T cells

Cell Type	Phenotype	Suppress GVHD	Expand in vitro	Expand in vivo	Suppress T _{con} proliferation
T _{reg}	CD4 ⁺ CD25 ⁺ FoxP3 ⁺	Yes	Yes	Yes	Yes
NK-T	αβTCR ⁺ CD4 ⁺ NK1.1 ⁺	Yes	?	Yes	No