REPORT

Can studies of tolerance ever lead to therapy?

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Studies in murine models have suggested that regulatory T (Treg) cells can be generated by any antigen and such antigen specific Treg cells not only prevent but also reverse unwanted immunity. Another key finding of these experimental studies is that Treg cells could possibly be used therapeutically to ameliorate disease because of their ability to interfere with the execution of effector programmes of fully differentiated T effector cells. However, more work is needed before these findings can be safely translated into better targeted therapy and prevention of unwanted immune responses in clinical settings.

The somewhat rhetorical question (im)posed by the organisers of this symposium can be answered by a simple “yes” because observations on tolerance after allogeneic T cell depleted bone marrow transplantation in the past have been successfully applied in the clinical setting of severe combined immunodeficiency. It was convincingly shown that T cell removal from murine bone marrow cells prior to bone marrow transplantation into semi-allogeneic hosts resulted in graft versus host disease-free reconstitution of the haemopoietic system of the heavily irradiated host and acquisition of specific tolerance to host antigens by the developing T cells.^{1,2} Similar procedures subsequently became successful in the therapy of children with severe combined immunodeficiency.^{3,4}

Admittedly these studies were carried out a long time ago and severe combined immunodeficiency is a relatively rare disease. Nevertheless, the principle of resetting the immune system by transfer of stem cells which is followed by acquisition of tolerance of the newly emerging immune system is still being successfully applied in severe autoimmune diseases such as lupus or multiple sclerosis.⁵

Recently, the concept of immunoregulation by a subset of T cells⁶ specifically selected for this purpose by self-antigens in the thymus^{7,8} has opened additional possibilities of exploiting this setting in prevention or even therapy of not only autoimmune disease but hopefully also in prevention of allergy or transplant rejection. These goals begged two major questions: can one generate regulatory T (Treg) cells by any antigen and can such antigen specific Treg cells not only prevent but also reverse unwanted immunity. Both goals have been achieved in mice but further studies are required to optimise procedures such that hopefully safe translation into clinical settings can be achieved.

HOW DO Treg CELLS WORK?

We have good information of how Foxp3 expressing Treg cells are selected in the thymus,^{9,10} but we know relatively little of how precisely they interact directly or indirectly with T effector cells to interfere with immunity. Nevertheless a few cues are worth considering here. Antigen specific Treg cells, just as naive T cells, home to and expand in antigen draining lymph nodes¹¹ where they exert their suppressive function on

neighbouring T cells. Thus even though the effector phase of suppression appears non-specific with regard to antigen, the specific recruitment of Treg cells and effector cells to the same anatomical site brings about an element of specificity.¹⁰ This is not restricted to antigen draining lymph nodes because Treg cells, like T effector cells, can extravasate and accumulate in inflamed tissue where they can further interact.¹² Depending on the local milieu, suppression may require secretion of cytokines such as interleukin (IL)-10 by Treg cells or some production of transforming growth factor β 1 (TGF β 1) either by Treg cells themselves or other cells in their neighbourhood.¹⁰ There is suspicion that cytokine production is not all that is required for effective immunosuppression,¹³ but unifying concepts have not emerged although possible molecular scenarios have been discussed.¹⁰ An important question is whether or not effector T cells can be suppressed at any stage of their differentiation or whether there is a point beyond which Treg cells can no longer interfere with T effector function. I will argue below on the basis of experiments involving the inhibition of cytolytic CD8+ T cells that Treg cells are not only suited to prevent the development of effector function but can interfere with the T cell receptor (TCR) dependent execution during the effector phase. This may explain why it is possible not only to prevent but to reverse type 1 diabetes^{14,15} at an early stage after β cell destruction has begun.

INDUCING AND EXPANDING Treg CELLS BY FOREIGN ANTIGEN

We do know that Treg cell development can be instructed by antigens expressed in the thymus,^{7,8} especially when expressed in thymic epithelial cells,⁸ but we have relatively little information on what the Treg cell repertoire encompasses and what it does not.¹⁶ Thus if one wants to make sure that Treg cells with specificity for a particular self-antigen or foreign antigen do exist one wants to exploit physiological mechanisms of Treg cell generation by introducing certain antigens. As that is difficult to do in the thymus, especially after the thymus involutes following sexual maturity, we have exploited the hypothesis that subimmunogenic delivery of antigen may induce Treg cells in peripheral lymphoid tissue. This approach was chosen since old experiments suggested that intravenous delivery of proteins in the absence of adjuvants may induce tolerance mediated by Treg cells.¹⁷ Also, more recent experiments have suggested that this mode could result in the accumulation of Treg cells even though the experiments did not clearly distinguish between de novo generation of Treg cells and accumulation of already existing Treg cells.^{18,19}

To provide rigorous proof for de novo generation²⁰ we transferred Foxp3 negative naive T cells with a transgenic receptor for peptide 111-119 of influenza haemagglutinin (HA) presented by E^d major histocompatibility complex (MHC) molecules into normal BALB/c mice. The donor cells

Abbreviations: TCR, T cell receptor; TGF, transforming growth factor; Treg, regulatory T (cells)

had to come from mice on the RAG^{-/-} background to make sure that the T cells expressed one α TCR only that would not encounter its (HA) agonist ligand during development and hence fail to generate Foxp3⁺ Treg cells. The Foxp3⁻ naive T cells from such mice were transferred in low numbers just sufficient to be detected by fluorescence activated cell scanning (FACS) analysis in lymphoid organs of the recipient mice. In this setting the relevant HA peptide was targeted to “steady state” dendritic cells by producing fusion DEC 205 antibodies that could bind to the endocytic receptor on dendritic cells (DEC 205) while containing the peptide in their tail. The Fc binding portion of such antibodies was mutated such that they could not bind to Fc receptors and thus target the DEC 205 receptor efficiently.²⁰ The targeting approach was chosen as the intravenous injection of peptides, especially without adjuvants, is extremely ineffective and peptides are eliminated from the blood in a matter of minutes. Before the use of fusion antibodies, peptide decay necessitated the use of osmotic pumps to deliver peptides continuously to achieve the conversion of naive T cells to Treg cells.²¹ In the case of fusion antibodies ~50 ng of the fusion protein was sufficient to see optimal conversion of a proportion (~20%) of Foxp3⁻ naive T cells into Foxp3⁺ Treg cells. A series of features of these de novo generated Treg cells are worth considering²⁰:

- When analysed for global gene expression on Affymetrix chips the de novo generated Treg cells were not different from Treg cells that are generated intrathymically under physiological conditions.
- The lifespan of these Treg cells did not differ from the lifespan of naive T cells—that is, even after the antigen that induced their formation was no longer present the cells survived in a so called “Treg cell memory pool”.
- Once formed, the Treg cells could be expanded much like naive T cells by applying antigen under more immunogenic conditions—that is, incomplete Freund’s adjuvant without losing their suppressive capacity.
- Higher doses of fusion antibody (1 μ g) or deliberate activation of DC (anti-CD40 antibodies) abolished the conversion process.
- Although TGF β 1 helped the conversion, IL-2 production by naive T cells was counterproductive. Pre-incubation of naive T cells with TGF β 1 and TCR ligands in vitro resulted in in vitro conversion in the absence of cell division. These results show that it is possible to induce Treg cells in the absence of an immune response, and experiments are under way to create in vivo conditions (general immunosuppression) which allow Treg cells generation without the danger of concomitant immune reaction.

These experiments employing the TCR-HA system²⁰ make it likely that in the future it will be possible to generate antigen specific Treg cells to a variety of antigens that include not only self-antigens but also allergens or foreign transplantation antigens. Thus de novo generation of antigen specific Treg cells is no longer an immunological illusion but can be achieved by delivering antigen under subimmunogenic conditions.

PREVENTION AND/OR THERAPY BY Treg CELLS?

In several murine models of type 1 diabetes, it has been shown that Treg cells can prevent the development of disease when supplied in sufficient numbers.^{15–22} It has also been shown that a deficiency of Treg cells can be associated with severe autoimmune disease.²³ In two independent studies it was shown that Treg cells not only have the capacity to prevent disease but can also be employed to revert already established disease.^{14–15} In one particular study,¹⁴ it was

shown that Treg cells could be generated in vitro by expanding CD4⁺ T cells with specificity for antigens expressed by islet cells in vitro and subsequently converting them into Treg cells by retroviral Foxp3 transduction. Such transduced CD4⁺ T cells became anergic 24 hours after Foxp3 expression and functioned as potent suppressor cells in the standard in vitro assay. Similarly CD4⁺ T cells from NOD mice could be expanded in vitro and became potent Treg cells after retroviral Foxp3 transduction. However, after transfer of 10⁵ Treg cells into diabetic NOD mice whose blood glucose levels were indicative of just beginning β cell destruction in pancreatic islets, only the Treg cells specific for islet antigen were able to revert the diabetes for at least 100 days. Even a 10-fold higher dose of polyclonal Treg cells, which were effective suppressors in vitro, were unable to cause any amelioration of disease. In contrast, these animals became severely diabetic and died.¹⁴ These experiments in NOD mice, a murine model of human disease, show two important facts. Firstly, it is clear that antigen specificity is a major factor in amelioration of disease mediated by Treg cells because it permits the Treg cells to be co-recruited with effector T cells to antigen draining lymph nodes and inflamed tissue such that effective suppression of neighbouring T cells ensues. Secondly, the results suggest that somehow Treg cells can interfere with already established effector function of disease causing T cells.

CAN Treg CELLS INTERFERE WITH THE FUNCTION OF FULLY DIFFERENTIATED CD8⁺ T EFFECTOR CELLS?

The above scenario suggests that there is perhaps no stage during an immune response at which T effector cells are beyond a point in differentiation at which they can be suppressed by Treg cells. It is known that the effector functions such as cytokine secretion and specific killing are dependent on continuing TCR signals and perhaps Treg cells have the capacity to interfere with these. We analysed the possibility in close collaboration with the von Andrian lab at Harvard Medical School by employing two-photon microscopy, pioneered by that lab, to monitor the impact of Treg cells on the development and function of CD8⁺ cytolytic T cells in antigen draining lymph nodes. The results can be summarised as follows: when CD8⁺ T cells and CD4⁺ Treg cells with specificity for different peptides from the same protein are present in an antigen draining lymph node at a ratio of 1:1 and an initial low frequency (<1:1000) both subsets of T cells will exponentially expand during an eight day period without measurably interfering with each other’s proliferation.

Nevertheless, the Treg cells completely prevent the rejection of tumour cells bearing the relevant antigen recognised by the CD8⁺ T cells. When the differentiation of CD8⁺ T cells into CD8⁺ effector cells was analysed, it was noted that the Treg cells had no impact on the effector phenotype: the lysosomal structures and content of perforin, granzyme B, and all other cytolytic molecules such as Fas were the same in suppressed and non-suppressed CD8⁺ T cells after the eight day differentiation period. However, the Treg cells were considerably slowing down the release of cytolytic vesicles from the differentiated CD8⁺ T cells without measurably inhibiting the conjugate formation of CD8⁺ T cells with antigen expressing target cells. This effective in vivo inhibition of cytolytic activity required susceptibility of CD8⁺ T cells to signals initiated by TGF β but in vitro analysis revealed that TGF β alone could not inhibit cytolytic activity.¹³ Thus there are likely additional functions of Treg cells that can result in reversible interference with the function of CD8⁺ cytolytic effector cells¹³ perhaps by interfering directly or indirectly with TCR signalling. We presently favour the second

possibility since no remarkable longer term interaction of Treg with CD8+ effector cells could be observed in vivo. Thus although these experiments do not yet provide a molecular explanation of the interaction between Treg cells and fully differentiated T effector cells, they show that such interactions do exist and result in the interference with the final execution of effector programmes.

CONCLUSION

Studies with antigen specific Treg cells in murine models have come up with three remarkable findings. Firstly, the physiological process of Treg cells generation can be exploited such that Treg cells with specificity for any desired antigen can be generated de novo by applying the antigen in vivo under subimmunogenic conditions. Secondly, thus generated Treg cells can be exploited to specifically prevent or even ameliorate already established autoimmunity. Thirdly, the possibility to use Treg cells therapeutically stems from their ability to interfere with the execution of effector programmes of fully differentiated T effector cells. The described properties of Treg cells make it likely and desirable that they will replace antigen non-specific immunosuppression that is accompanied by serious side effects, and thus hopefully afford better targeted therapy and prevention of unwanted immune responses than hitherto possible.

Competing interests: none declared

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