Cytometry
PART A
Journal of the International Society for Advancement of Cytometry

Ex Vivo Expansion of $CD4^+CD25^+$ T Regulatory Cells for Immunosuppressive Therapy

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Received 19 May 2008; Revision Received 26 August 2008; Accepted 14 September 2008

Grant sponsor: Ministry of Science and Higher Education, Poland; Grant number: 2P05A10030; Grant sponsor: Iceland, Liechtenstein, and Norway through the European Economic Area Financial Mechanism Office (HOMING program of the Foundation for Polish Science).

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Published online 14 October 2008 in Wiley InterScience (www.interscience.wiley.com)

DOI: 10.1002/cyto.a.20659

Abstract

Immunosuppressants are powerful drugs, capable of triggering severe adverse effects. Hence, there is tremendous interest in replacing them with less-toxic agents. Adoptive therapy with $CD25^+CD4^+$ T regulatory cells (Tregs) holds promise as an alternative to immunosuppressants. Tregs have been described as the most potent immunosuppressive cells in the human body. In a number of experimental models, they have been found to quench autoimmune diseases, maintain allogeneic transplants, and prevent allergic diseases. A major stumbling block in their clinical application is related to Treg phenotype and the very limited number of these cells in the periphery, not exceeding 1–5% of total $CD4^+$ T cells. Recent progress in multicolor flow cytometry and cell sorting as well as cellular immunology has found ways of overcoming these obstacles, and has opened the doors to the clinical application of Tregs. In the review, we describe Treg sorting and expansion techniques that have been developed in recent years. In the experience of our laboratory, as well as some published reports, Treg adoptive therapy is a promising tool in immunosuppressive therapy, and should be considered for clinical trials. © 2008 International Society for Advancement of Cytometry

Key terms

immunosuppression; $CD4^+CD25^+$ T regulatory cells; cellular therapy; adoptive therapy; humans

IMMUNE tolerance can be defined as the lack of an immune response towards a specific antigen without additional immunosuppression (1). It was first demonstrated in animal models in the 1950s (2,3). In the 1970s and 1980s, immune tolerance was linked to the function of lymphocytes called regulatory cells (4–7). The first detailed description of these cells was in 1995, when $CD4^+CD25^+$ T regulatory cells (Tregs) were shown to prevent multiple autoimmune diseases in mice (8). Since then, research regarding the phenotype and activity of Tregs as the master regulators of immune response has contributed tremendously to our understanding of autoimmune and allergic disease mechanisms and immune tolerance. Recently, the utility of Tregs in adoptive therapy (Tregs administered to the body as a drug) was investigated, and they were found to effectively quench autoimmune and allergic reactions and increase tolerance after allotransplantations. The development of multicolor flow cytometry and cell sorting made the expansion of Tregs possible. It is of special importance in human immunology as pure sorting of these cells requires multicolor staining with sequential gating that involves gates based on differences in the expression levels of Treg markers. Reasonable purity and viability of sorted Tregs for ex vivo expansion was achieved only recently, with the last generation of sorting equipment. With the technology available, and keeping in mind potential problems, we must now decide whether it is time to move "from bench to bedside." Are Tregs a safe alternative to routine immunosuppression?

Suppressive Activity of Tregs

There are two main subsets of Tregs in the body: naturally occurring (nTregs) and adaptive. Immune response suppression is the hallmark of all Treg types, but the

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targets and the way in which Tregs regulate immune suppression differ among the subsets. nTregs arise in the thymus, where they are anergized towards self-antigens. Mature nTregs emigrate from the thymus into the peripheral lymphoid system. Immune responses of any background (infectious, autoimmune) attract these cells to the site of inflammation, as well as to the local lymphoid tissue. The suppression mediated by nTregs occurs mainly as a result of cell-to-cell interactions with effector cells. Several receptors expressed on nTregs have been proposed to be active in this process. CTLA-4 (Cytotoxic T lymphocyte antigen 4, CD152) appears to be the most important receptor, because engagement of this receptor almost always triggers the suppressive activity of nTregs towards effectors (9,10). Apoptosis of effectors triggered by competition with nTregs for IL2 and other γ-chain family cytokines, along with inhibition by nTregs of IL2 production; is also regarded as an nTreg suppressive activity mechanism (11,12). Interestingly, nTregs secrete both perforin and granzymes, and were found to be cytotoxic towards effectors, which also suppress the immune response (13,14). Both $CD4^+$ and $CD8^+$ effector T cells can be suppressed by nTregs. In addition, there is increasing evidence that other subsets, such as NK cells, NKT cells, monocytes, dendritic cells, and granulocytes, can be also targets for nTregs (15–18). Importantly, the suppressive activity of nTregs can be directed not only towards self-reactive cells, but can also regulate alloresponses and xenoresponses (16,19,20). This is made possible-at least in part-by the effect of "linked suppression," in which Tregs inhibit the immune response towards a given alloantigen when it is presented together with previously tolerized antigens (21,22). Another possible explanation for the broad range of nTregs targets is that the selection of their TCRs is not limited only to those TCRs that recognize self antigens. Indeed, the TCR repertoire of $FoxP3^+$ nTregs is similar to that of T effector cells (23,24).

Adaptive Tregs differ from nTregs both in origin and in mode of action. They arise in the periphery during immune responses; mainly from naïve $CD4^+$ T cells (25). Because they are generated in this manner, adaptive Tregs are specific to the antigen that triggered the immune response and they are responsible for fine tuning of the response (25). Unlike nTregs, adaptive Tregs suppress the immune response by secreted cytokines. There are two main groups of adaptive Tregs. Tr1 cells mediate their action via secreted IL10, while Th3 cells work via secreted TGFβ (26,27).

Recently, novel mechanisms of suppression have emerged. Studies in mice suggest that IL35, a new member of the IL12 family, is an important factor that imposes immune tolerance when secreted by $FoxP3^+$ Tregs. The production of IL35 is strongly associated with the expression of FoxP3, and knockout mice lacking this cytokine have reduced regulatory abilities and suffer from autoimmune syndromes (28). Additionally, the addition of IL35 during suppression assays causes T cells to become anergic and stops their effector activities (29). The metabolism of extracellular nucleotides could constitute another important pathway of regulation via Tregs. Extracellular ATP is recognized as a "natural adjuvant," and Treg cells have been found to express ectonucleotidase CD39 (nucleoside triphosphate diphosphohydrolase-1) and CD73 (ecto-5′nucleotidase), which limit immune activity through degradation of ATP to AMP and adenosine, respectively. In addition, the products of these enzymatic reactions bind type 1 adenosine A2A receptors on T effector cells and diminish the activity of these cells (30,31). Tregs are also capable of storing suppressive cAMP, which can be then transferred to T effector cells via gap junctions formed during the immune response (32).

Ex Vivo Expansion of $CD25^+CD4^+$ Tregs

Sorting Strategy

Although much has been learned regarding Tregs in recent years, there are still many problems that must be overcome before these cells can be effectively applied in adoptive therapies. The first problem is their phenotype. Although all $CD25^+CD4^+$ T cells are recognized as having regulatory functions in mice, only a small proportion of $CD4^+$ T cells with the highest expression of CD25 receptor appears to have regulatory properties in humans (33). Because of this, the best means of sorting human Tregs for further expansion is fluorescence-activated cell sorting (FACS). Another possibility is immunomagnetic isolation; recent reports of the expansion of immunomagnetically sorted Tregs are promising (34). However, the commonly held opinion is that the immunomagnetic technique does not guarantee the sorting of $CD25^{high}$ cells with reasonable purity. This does not mean that immunomagnetic isolation should not be used for research on human Tregs, because cells yielded via this method can be still used for phenotypic studies or for short-term functional assays. In addition, recent developments in clinical-grade immunomagnetic sorting have shown that the technique may yield high numbers of Treg-enriched $CD4^+$ T cells from leukapheresis; possibly, such Treg-enriched products could be administered to patients immediately after isolation (35). Many researchers raise the question of the biological safety of FACS, which is as non-GMP approved (non-good-manufacturing practice approved). However, FACS is still a better method than is immunomagnetic sorting, because of the unavoidable contamination with effectors that makes immunomagnetically enriched Tregs useless for long-term large-scale expansion. It is because Tregs are anergic, and their ability to proliferate is limited (36,37). On the other hand, effectors proliferate far more vigorously than do Tregs, and even a trace of contamination with effectors in the initial sorted population can be expanded to extremely high numbers. Hence, FACS remains the "gold standard" in the field.

Some data suggest that seemingly pure $CD25^{high}$ $CD4^+CD3^+$ T cells collected using FACS may be still contaminated with a small proportion of effectors, throwing into question the specificity of this phenotype (38). The most well-known marker of Tregs is the transcription factor FoxP3 (39). However, FoxP3 is intracellular, and the plasma membrane permeabilization procedure necessary to detect intracellular content kills the cells. Thus, FoxP3 cannot be used for FACS selection of viable cells for further expansion. Therefore, addi-

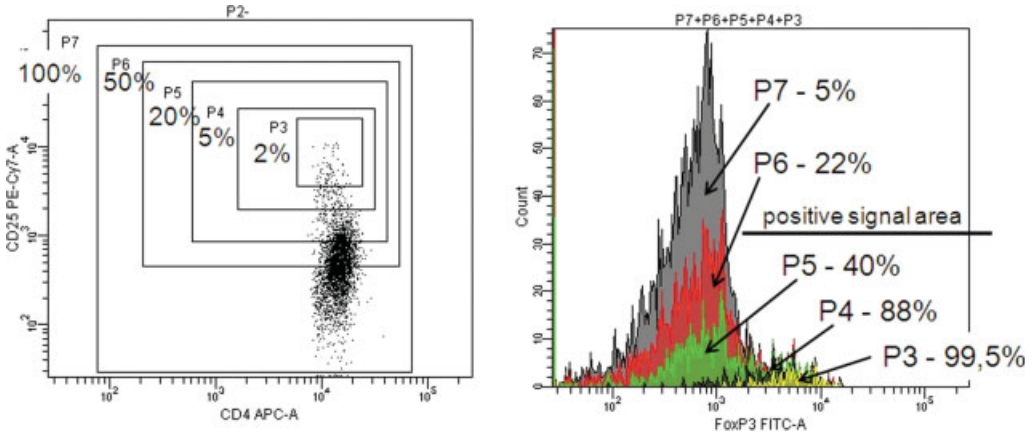

Figure 1. Gating of $CD25^{high}CD4^{+}$ Tregs for sorting. Example showing the procedure for gating human $CD25^{+}CD4^{+}CD3^{+}$ T cells. Dot plot on the left was generated from a $CD4^{+}$ T cell gate (entire $CD4^{+}$ T cell gating procedure is shown in Fig. 2). The events in the dot plot are then gated using the CD25 staining fluorescence intensity. P3 gates top 2%, P4 gates top 5%, P5 gates top 20%, P6 gates top 50%, and P7 gates 100% of $CD4^{+}$ T cells in the dot plot. The gates, from P3 to P7, are overlaid in the histogram on the right. The positive signal area line delimits the area for positive FoxP3 staining, which was established using the isotype control and FMO control for FoxP3 staining. Gating based on the brightness of CD25 staining in the dot plot shows that the top 2% of $CD25^{+}$ T cells (P3 gate) are almost entirely positive for FoxP3expression (see histogram, right). The P3 gate also contains mainly $CD4^{low}$ T cells. Widening of the range of gated events in the dot plot correlates with a decreasing percentage of $FoxP3^{+}$ cells in subsequent gates, as shown in the histogram on the right. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

tional strategies that will improve sorting are necessary. One simple means of improvement is associated with the expression of the CD4 receptor during FACS gating. Compared with $CD4^{+}$ T effectors, Tregs are characterized by lower expression of CD4 receptors, which may help to establish the most appropriate gate during sorting and analysis of these cells during flow cytometry (40,41). This supports an "upside down" gating strategy for efficient Treg sorting. Instead of routine isotype controls, the sorting gate would be established based on the fluorescence of around 2% of the brightest $CD25^{+}$ T cells. This gate would also contain $CD4^{low}$ T cells. If checked, the expression of FoxP3 in such $CD25^{high}CD4^{low}CD3^{+}$ T gate would be close to 100% (Fig. 1). A number of other surface proteins associated with the Treg function have been proposed for a panel of markers that can be used to separate pure Tregs for ex vivo expansion (Table 1). Additionally, some routinely used drugs have been found to affect the number and functioning of Tregs (Table 2).

An approach that has been pursued only recently is associated with the IL7 receptor on T cells (IL7R or CD127). IL7 is necessary factor for T cell survival and homeostatic proliferation and the great majority of T cells express the IL7R. Surprisingly, Tregs do not undergo homeostatic expansion on their own, and they do not express receptor (43,44,96). Hence, a reliable phenotype for Treg sorting to obtain a pure population for further expansion could be $CD127^{-}CD25^{high}$ $CD4^{+}$ (43) Indeed, such a strategy was found to be optimal in our lab (Fig. 2).

The main problem of sorting with so many markers is multicolor staining, which brings with it the problem of compensation. Modern flow cytometers are equipped with software that copes efficiently with multicolor compensation. However, some procedures should be followed, particularly in the beginning, when the sorting protocol is being developed. Although many authors suggest using beads, we found that it is useful to perform compensation using cells that are similar to those to be sorted. In addition to the fluorochromes being similar to those used during multicolor sorting, the intensity of fluorescence also counts toward accurate compensation. Hence, the protocol is improved when the antibodies for compensation are identical to those used during the actual sorting. Surprisingly, in contrary to a general view, we found that it is of special importance that those with low-intensity fluorescence should not be replaced with very bright antibodies in the compensation samples. In our hands, both using the cells and antibodies corresponding to those used in the actual sorting improved the purity of the sorted population. Particularly while the protocol is being developed, compensation should be checked with a "fluorescence minus one" (FMO) control. Each of the FMO control samples is stained with all but one antibody used in the final sorting. The missing antibody is different for each FMO control sample, and the appropriate isotype control for the omitted antibody is used instead in the staining cocktail. Thus, during the acquisition, at each time point, a different single channel contains no positive events. Comparing percentage results obtained with FMO control samples, it is possible to estimate the influence of particular fluorochromes/antibodies on compensation.

Poor viability of sorted cells can be also a problem. Dead cells are sticky due to the DNA that is expelled from their nuclei, and they form conglomerates that can clog the instrument capillaries. Dead cells or their aggregates may also contaminate sorted subsets if they stick to properly sorted cells. This problem can be minimized by filtering cell suspensions or by adding a small amount of deoxyribonuclease to the cells before sorting, and excluding doublets and 7-AAD-positive

Table 1. Molecules involved in the activity of Tregs that can be used for their identification and sorting.

	FUNCTION	LITERATURE
CD4 (low)	Human Tregs belong to $CD4^+$ T cells and express slightly lower number of CD4 receptors than their effector $CD4^+$ T counterparts	(40,41)
CD25 high	High affinity receptor for IL2 (IL2Rα), In humans Only $CD4^+$ T cells with high expression of CD25 receptor possess regulatory activity	(33)
CD152 (CTLA-4)	Cytotoxic T lymphocyte Antigen-4 (CTLA-4)—the most important surface receptor mediating cell-to-cell contact inhibition of effector cells in the immune synapse, fusion proteins CTLA-4Igs are currently used in clinical trials as immunosuppressive drugs	(42)
CD127 (negative)	Receptor for IL7 (IL7R); IL7 is believed to be involved in homeostatic proliferation of lymphocytes; as Tregs hardly proliferate in a homeostatic manner, they do not express IL7R	(43,44)
FoxP3	Transcriptional factor (scurfin); currently the most reliable marker of Tregs; not for use in sorting procedures due to its intracellular expression	(39)
CD62L	Selectin that allows lymphocytes trafficking through lymphoid organs; Treg subset expressing CD62Lis recognized as naive subset and was found to be more suppressive than CD62L negative Tregs	(45)
CCR7	Marker of naive and central memory T cells, Tregs expressing CCR7 are recognized as highly suppressive naive subset	(46)
CD45RA	Marker of naive lymphocytes; the expression of CD45RA on starting population of sorted Tregs was revealed to correlate with high suppressive ability of expanded Tregs	(47)
CD27	Both $CD27^+$ and $CD27^-$ Tregs are suppressive against naive and effector cells but $CD27^+$Tregs suppress also ongoing T cell responses	(48,49)
CD39	Ectonuclotidase expressed on Tregs; degrades immunostimulatory extracellular ATP to AMP, which may be subsequently catabolised to immunosuppressive adenosine	(30)
CD73	Ectonuclotidase expressed on Tregs; a product of the enzymatic activity, adenosine, suppress T effector cells	(31)
CD45RB (negative)	A subset of Treg negative for CD45RB was revealed as crucial in maintaining tolerance in mucosal surface of digestive tract; accumulation of Tregs without this marker was also described in peripheral blood Tregs in human ageing	(50,51)
CD45RC	CD45RC is expressed upon activation in some subsets of Tregs, notably on Tregs associated with mucosa of respiratory tract	(52,53)
CD45RO	Marker of memory/activated lymphocytes; the expression of CD45RO correlates with low suppressive ability of expanded Tregs	(47)
Adhesive molecule: ICAM-1 (CD54); Integrins: LFA-1 (CD11a/CD18), α4β7 (LPAM-1), αEβ7 (CD103) and α4β1 (CD49d/CD49)	High expression of adhesive molecules and integrins gives Tregs the advantage of strong cell-to-cell interaction with APC and/or effectors and efficient seeking for inflammation	(10,54,55)
CD86	$CD86^+$ Tregs were found during some parasite infections, Blockade of CD86 receptor on DCs during their cooperation with Tregs increases suppressive activity of the later ones.	(56,57)
CD95 (Fas)	Freshly isolated Tregs express Fas and are more susceptible to Fas-mediated cell death than effectors; Tregs express also FasL and are capable of triggering apoptosis of monocytes and B cells via Fas/FasL pathway	(58–60)
CD122 (IL-2Rβ)	Subunit of IL2 receptor (IL-2Rβ) necessary Tregs for induction of intracellular expression of FoxP3 during thymic development and at the periphery	(61)
CD134 (OX-40)	Expression of CD134 receptor on the surface of human Tregs abrogates their suppressive function in some settings (e.g. GvHD)	(62)
CD137 (4-1BB) positive	Negative regulator of Treg cell function, the expression of CD137 receptor on the surface of human Tregs abrogates their suppressive function	(63)

Table 1. Molecules involved in the activity of Tregs that can be used for their identification and sorting (continued)

	FUNCTION	LITERATURE
GITR (Glucocorticoid-induced TNF Receptor)	Negative regulator of Treg cell function, agonist anti-GITR antibodies abrogate suppressive function of human Tregs	(64)
CD154 (CD40L) negative negative	Expression of the marker was found to be necessary for induction of tolerance in some settings; agonist anti-CD154 antibodies are currently used in clinical trials as immunosuppressive drugs	(65,66)
CD223 (LAG3)	Important during Treg-dependent suppression of NK cells via membrane-bound form TGF-β, not expressed on resting Tregs	(67)
CD28	Necessary for the development and homeostasis of Tregs	(68,69)
PDL1	Expression of PDL1 on Tregs is associated with induction of foetomaternal tolerance	(70)
IL-10	One of the most potent suppressive cytokines – secreted mainly by Tr1 cells but also Tregs	(27)
TGF-β	One of the most potent suppressive cytokines – secreted mainly by Th3 cells but also Tregs, Membrane-bound form of the cytokine on Tregs is involved in the suppression of NK cells	(26,67)
Toll-like receptors	TLR4, TLR5, TLR8 in humans; signals through those receptors decrease suppressive abilities of Tregs but stimulate proliferation of Tregs	(71)
neopterin	$CD4^+CD25^+FoxP3^+$ Tregs were found to express high levels of neopterin	(72)
CCR4, (to the lesser extent also CCR8)	Ligands for: CCL22, CCL17, CCL1, and vMIP-I preferentially expressed on Tregs, the ligands are important for Tregs trafficking through lymphoid organs and inflammed tissues, e.g. tumour sites	(73,74)

Table 2. Pharmacologic agents known to affect activity of Tregs

	FUNCTION	LITERATURE
Kalcitriol (Vitamin D3)	Increases suppressive abilities of Tregs, Induces oral tolerance via induction of Th3 cells	(26,75)
Rapamycin	Immunosuppressive drug found to select for Tregs in vivo and in vitro	(76-78)
Calcineurin inhibitors	Group of immunosuppressive drugs that decrease strongly the number of Tregs in vivo and in vitro and inhibit their function through the inhibition of IL2	(79,80)
Glucocorticosteroids	Treatment with glucocorticosteroids increases the number of Tregs in the peripheral blood	(81,82)
Anti-CD25 antibodies	Treatment with the antibody does not change the number and activity of Tregs in the periphery	(83)
Alemtuzumab (anti-CD52 antibody)	Mixed reports, with some that revealed the increase in the level of Tregs and other which found no changes in the level of Tregs after the treatment	(77, 84,85)
Anti-thymocyte antibodies	Treatment with rabbit, but not horse, form of the antibody was found to increase the level of Tregs	(86,87)
Anti-TNFα antibodies	TNFα decreases suppressive abilities of Tregs, anti- TNFα antibodies were found to suppress via enhanced activity of Tregs	(88,89)
IL2	Abrogates suppressive function of Tregs, necessary during Treg development and proliferation	(90)
Cyclophosphamide	Anticancer drug that reduces the number of Tregs in the body	(91)
Fludarabine	Anticancer drug that reduces the number of Tregs in the body	(92)
Estrogens	Estrogens induce increased levels of $FoxP3^+$ Tregs, which is related to immunosuppression in pregnancy as well as in the lutheal phase of menstrual cycle; The effect of estrogens is associated with upregulation of PD-1	(93,94)
Retinoic acid	Stimulates growth and gut homing of Tregs	(95)

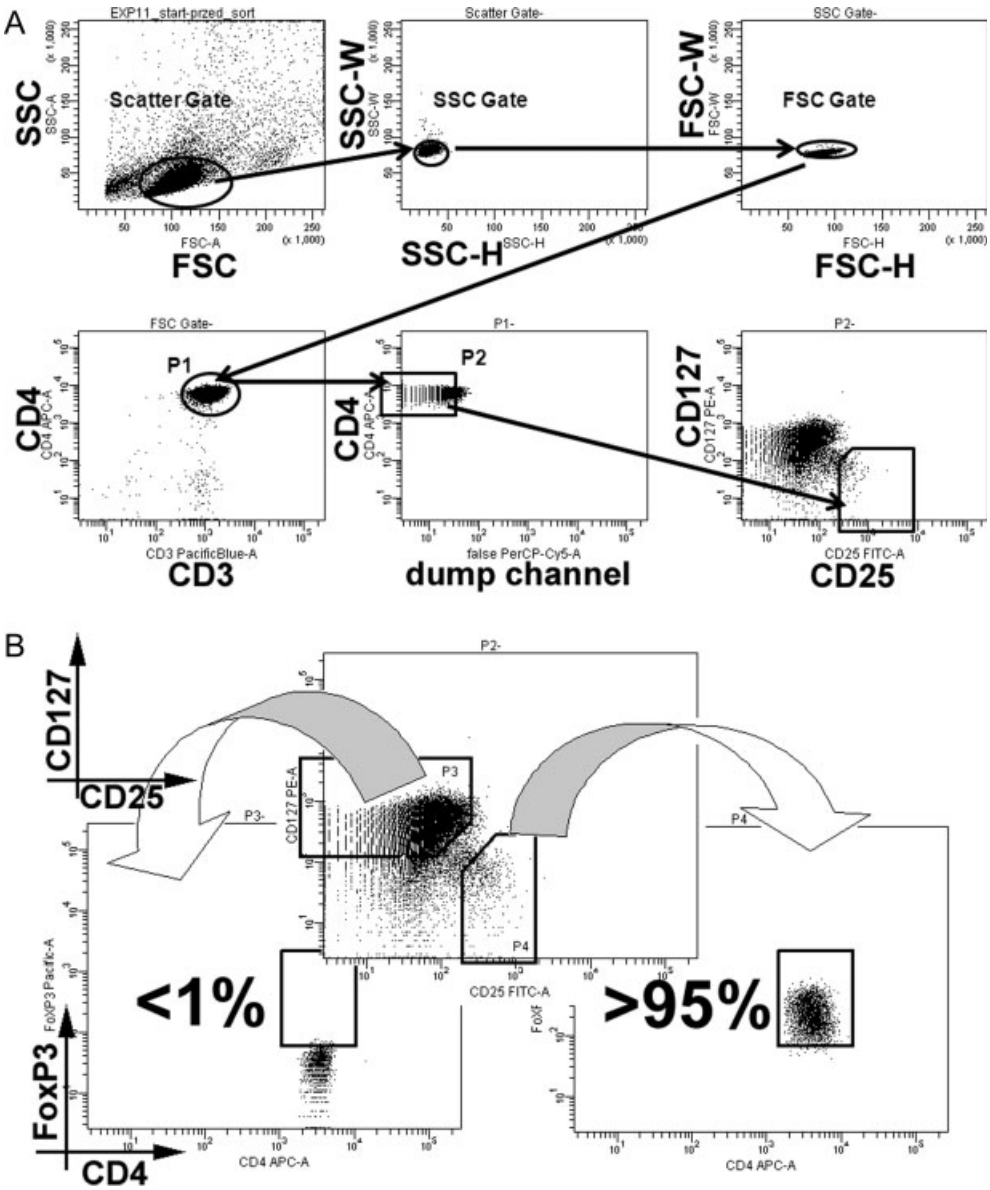

Figure 2. Gating strategy for Treg sorting in the Laboratory of Experimental Transplantology, Medical University of Gdańsk, Poland. (**A**) Cells are presorted with a $CD4^+$ T negative immunomagnetic sorting kit (StemCell Technology, Canada), and then sorted by FACS. Lymphocytes are identified using a routine scatter gate procedure. First, the doublets (cell conglomerates stuck to specifically stained cells and responsible for poor sort purity) are eliminated. This is accomplished using laser light signal characteristics (width and height) with two subsequent gates: the side scatter gate and the forward scatter gate (SSC-W vs. SSC-H and FSC-W vs. FSC-H dot plots, respectively). Events from the later gate are then transposed to the CD3 vs. CD4 dot plot (P1) to obtain $CD4^+$ T cells only. Before the final gating, potential dead cells and non-CD4 remnants from the P1 gate are excluded using a dump channel (P2 gate). In addition to specific staining, the cells are stained with 7-AAD (7-amino-actinomycine, BDBiosciences, Poland) and CD8, CD19, CD16, and CD14 PerCP-conjugated antibodies. Because all of the fluorescence from the cells stained with dump channel antibodies is detect by the same PMT sensor, it is possible to gate out the remnants and dead cells in a single step. It is unlikely that this debris would make it as far as this step, because it should have been sorted out with the immunomagnetic antibodies cocktail, the contents of which are similar to those of the dump channel cocktail. However, in this procedure, purity is an absolute priority, and the second round of exclusion of unwanted cells improves it, as we have confirmed in our lab. To maintain our rigorous criteria, we usually gate off around 10% of the cells from the top of dump channel, even if the entire population appears to be alive and unstained with the dump channel antibody cocktail. Finally, the cells from P2 gate are transposed to the CD127 vs. CD25 dot plot and the top 2% of the $CD25^+$ T cells, which are $CD127^-$, are sorted. The following anti-human monoclonal antibodies are used in this procedure (fluorochrome, class, and clone given in the parentheses): anti-CD3 (PacificBlue or PE-Cy7, IgG1, UCHT1), anti-CD4 (APC, IgG1, RPA-T4), anti-CD8 (PerCP IgG1, SK1), anti-CD19 (PerCP, IgG1, 4G7), CD14 (PerCP, IgG2b, MφP9), anti-CD16 (PerCP-Cy5.5, IgG1, 3G8), anti-CD25 (FITC, IgG1, M-A251), and anti-CD127 (PE, IgG1, hIL-7R-M21). All of the antibodies were purchased from BDBiosciences, Poland. Gates shown are slightly wider than those used during routine sorting to show the position and shape of the gated subsets. (**B**) The upper dot plot is generated from human $CD4^+CD3^+$ T cells during the sorting procedure. Anti-CD127 staining during phenotyping shows that the $CD4^+$ T cells with the highest CD25 receptor expression are devoid of CD127 expression. Moreover, reanalysis of sorted subsets, shown in the bottom dot plots, shows that more than 95% of the sorted $CD127^{negative}CD25^{high}CD4^+CD3^+$ T cells (P4 gate, right bottom dot plot) express FoxP3, while the majority of $CD127^+CD25^{low/negative}$ $CD4^+CD3^+$ T cells are negative for FoxP3. The anti-FoxP3 antibody (PacificBlue or FITC, IgG1, clone 236A/E7) used in our protocols comes from eBioscience, USA.

events during sorting (Fig. 2). An obvious disadvantage of so many gates is a substantial loss of Tregs during sorting. In our hands, sorting from half a liter of peripheral blood taken from a healthy blood bank donor can yield as low as 5×10^5 of $CD25^{high}CD4^+CD127^-$ Tregs that are $FoxP3^+$, as checked after the sorting (e.g., mean from 10 sorts in our lab – 1.5×10^6 Tregs, minimum – 5×10^5 Tregs, maximum – 3.0×10^6 Tregs). Another viability problem is related to the buffers used during FACS. The high pressure of the fluids during sorting increases the partial pressure of CO_2 in these solutions, which, if the capacity of the buffers is inadequate, may affect the pH (97). A more acidic pH due to the generated H_2CO_3 decreases the viability of sorted cells, and some proportion (from 5% to as high as 50%) will die within 12 h of sorting. This problem can be minimized by carefully checking the quality of the buffers. This is of special importance during aseptic sorting, in which new sets of buffers must be used for each sort, and buffers are often sterilized by increased pressure before the sorting. We and others have found that using HEPES buffer and keeping the collecting tubes at $+4^\circ C$ improves viability (97). It might also be possible to shorten the FACS time by starting from a negative presort to $CD4^+$ T cells with an immunomagnetic kit. Yet another maneuver to increase viability is to sort Tregs directly to the culture medium. Viability also has an impact on the suppressive activity of Tregs at a per-cell level, which is visible when the number is corrected for dead cells during quality-check tests. In our experience, viability below 70% after the sort is a strong indicator of poor suppressive abilities of the sorted Tregs, and therefore such cells are not routinely expanded.

Expansion Technique

While the purity of sorting is a necessary step towards immunosuppression based on the adoptive transfer of Tregs, it is only the beginning. Because the number of available Tregs is so low, there are not enough to sort and then transfer directly to the patient. Tregs comprise no more than 5% of total $CD4^+$ T cells (92,98,99). As we know from animal studies, such a low number of Tregs in relation to effectors will not efficiently suppress immune responses (68). Hence, the Treg yield must be increased prior to administration to the patient. An obvious obstacle achieving this goal is the terminal differentiation of Tregs, which makes them difficult to proliferate (37). Fortunately, a few efficient strategies for the large-scale expansion of Tregs have been developed recently.

Large-scale Treg expansion strategies center around artificial antigen presenting cells (APC), which are actually plastic beads coated with agonistic antibodies that will link to the stimulatory receptors expressed on Tregs. The most commonly targeted receptors are CD3 and CD28 (100). It is possible to use soluble antibodies or culture plates coated with immobilized antibodies as an alternative to bead-immobilized antibodies (101). There are also reports regarding the use of CD28 superagonists in the expansion procedure (102,103). However, this approach ended in deadlock after the catastrophe that occurred during the first clinical trial with the CD28 superagonist antibody "TeGenero" (TGN 1412) (104). Some less-common antibody cocktails have also been suggested in the literature. Anti-CD3 together with anti-4C8 (a monoclonal antibody that inhibits the migration of T cells through human endothelium) was found to generate Tregs with a contact-dependent mode of suppression activity (105). The 4-1BB receptor is another interesting target. Although 4-1BB has been shown to be a negative regulator of Tregs (106), recent studies found that ligands binding to this receptor are efficient stimulators of ex vivo Treg expansion, when used together with anti-CD3 or IL2 (107,108).

Another factor inevitable for Treg growth and proliferation is IL2. The cytokine is administered to cultures at extremely high doses, equal to or higher than 1,000 UI/ml (41). The crucial and nonredundant role of IL2 in the homeostasis of Tregs has been highlighted by many authors (90,109–112). In addition to being effective as a culture media supplement, this cytokine take a part in the generation of Tregs when it is administered to patients (113,114). Another IL2 family member, IL15, has also been found to promote the proliferation of Tregs ex vivo. Importantly, stimulation with IL15 allows for manipulation of the antigenic specificity of expanded Tregs (115,116). Altogether, stimulation via cell surface receptors and high doses of IL2, IL15, or both can cause as high as the 1,000-fold amplification of low numbers of Tregs yielded immediately after sorting (Figs. 3B and 3C). At least in some circumstances, this excessive proliferation does not appear to significantly affect Treg telomere lengths, which is of great importance when a high number of cells is a priority (117). However, problems do occur during expansion. As we mentioned previously, even a small amount of contamination with effectors can result in preferential growth of these cells. Recent evidence suggests that the addition to the culture of the immunsuppressive drug rapamycin results in selective ex vivo expansion of Tregs, which could be a solution (118,119).

However, even pure sorted Tregs can be problematic. It is now known that $CD25^{high}CD4^+$ Tregs are heterogeneous, and that only some of them are efficient suppressors after expansion. Several groups have found that Tregs in the subset characterized by the expression of markers of naïve cells, such as CD45RA or CD62L, are superior to their memory counterparts for expansion purposes (45–47). In addition to their less-differentiated phenotype, naïve Tregs have another advantage. At least some of the receptors expressed on naïve cells only are responsible for trafficking to the peripheral lymphoid tissue. Some authors have suggested that the suppressive activity of Tregs is the highest in the environment of the peripheral lymphoid tissue, particularly the lymph nodes (120,121). Sorting of T naïve cells also has the advantage of eliminating effector/memory cells. This is important, because memory cells are known to trigger robust immune responses and undergo homeostatic proliferation (122).

Another obstacle hampering ex vivo expansion of Tregs is contamination with endotoxins transferred with culture media or other reagents used during expansion. This type of contamination results in high levels of proinflammatory TNFα and IL6 cytokines in the culture, which seriously inhibit Treg function (88,123). Moreover, Tregs themselves, in the presence of

IL6 and autocrine TGFβ, can differentiate into highly proinflammatory Th17 cells without any suppressive activity (124). Thus, the use of GMP products, careful surveillance of the cultures, and repeated testing of the cells' suppressive abilities during expansion is required to ensure that the cells maintain their suppressive activity and can be potentially administered as a drug. This part of the expansion procedure may, at least in part, utilize flow cytometry (125). Repetitive checking for the expression of intracellular FoxP3 in samples of expanded cells is probably the simplest test of suppressive quality (Fig. 2B). However, activated T effectors may also transiently express FoxP3 (126). Furthermore, ex vivo expansion of Tregs poses the threat of methylation of the *foxp3* gene locus, which halts transcription and abrogates the suppressive activity of these cells (127). In animal models, high expression of FoxP3 and preserved Treg suppressive abilities depend on the demethylation of CpG motifs in the evolutionarily conserved region within the *foxp3* gene locus upstream of exon-1, the so-called "Treg-specific demethylated region" (TSDR) (128). Importantly, nTregs are characterized by complete demethylation, while adaptive Tregs and T effector cells keep this region partially or completely methylated, respectively (129). Because the current view is that the stable lineage of nTregs has a constitutively demethylated locus, we believe that routine measurement of the level of TSDR methylation in expanded cells would be superior to simply assessing FoxP3 expression. Importantly, TGFβ can demethylate the TSDR region, which results in elevated expression of FoxP3. This may explain the beneficial effects of TGFβ in culture on the suppressive activity of Tregs during expansion (130–132). Unfortunately, demethylation in the presence of exogenous TGFβ is incomplete and reverts when the cytokine is no longer present in the culture (129). Hence, functional tests, such as mixed lymphocyte reactions with autologous responders in the presence of expanded Tregs, so-called suppression assay, remain "gold standard" quality checks for expanded Tregs (Fig. 3C) (39,100).

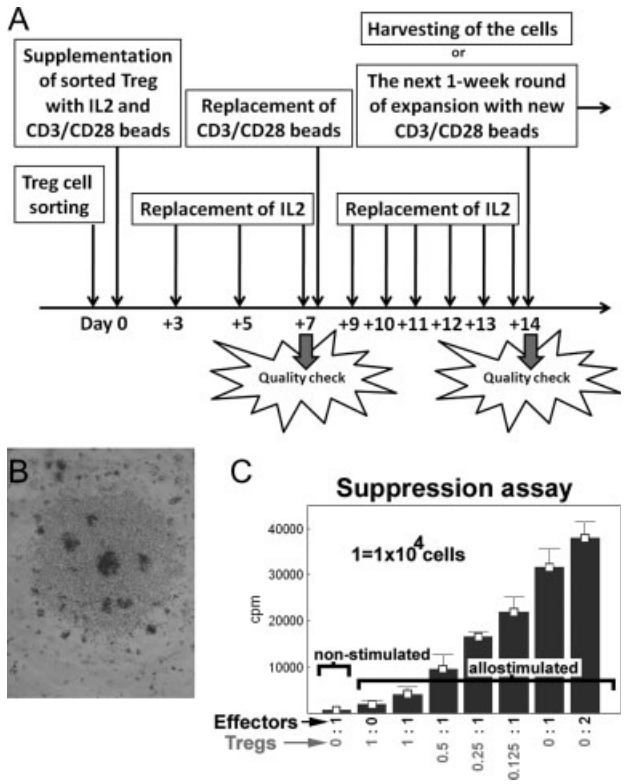

Figure 3.

Figure 3. Ex vivo expansion of Tregs in the Laboratory of Experimental Transplantology, Medical University of Gdańsk, Poland. (**A**) In our hands, the protocol proposed for the first time by Hoffmann et al. (41), with small modifications, yields high numbers of Tregs with great efficiency. The flowchart outlines the procedure, starting from sorting of the Tregs (detailed sorting procedure described in Figure 2). Sorted $CD127^{negative}CD25^{high}CD4^{+}CD3^{+}$ Tregs are cultured in 96-well U-bottom plates in RPMI 1640 medium supplemented with 10% inactivated fetal calf serum (FCS) (Invitrogen-Gibco, USA) and a high dose of IL2 (1,000 IU/ml, Proleukin, Chiron, USA). Tregs (1×10^5 per well) are placed with anti-CD3/CD28 beads (T Cell Expander, Invitrogen-Dynal, USA) in 1:2 ratio. One-third of the medium is replaced every two days with new medium during the first week of expansion, and the cells are passaged to new wells on the plate as they grow, to maintain their number at around 1×10^5 cells per well. On day +7, the beads are replaced with a fresh set (two beads per single cell) and the second round of expansion begins. A small number of the expanded cells can be used for a quality check of the culture. Because Treg proliferation during the second round is more intense than during the first, the medium must be replaced every day. The number of cells increases exponentially, and therefore they should be placed in 24- or 12-well plates (around 1×10^6 cells per ml). The second round ends on day +14 with replacement of the beads, and subsequent rounds can be performed this way every 7 days. In our lab, up to six rounds are performed. A quality check of the cells must be done before every round of expansion, because Tregs tend to lose their suppressive abilities with senescence of the culture. (**B**) Photograph of sorted $CD127^{negaive}CD25^{high}CD4^{+}CD3^{+}$ T cells during expansion. The cells are cultured in the presence of a high concentration of IL2 and plastic beads coated with anti-CD3 and anti-CD28 antibodies, known as artificial APCs (T Cell Expander, Invitrogen-Dynal, USA). Beads are visible, mixed with proliferating cells, as dark plaques in the center of the photo. (**C**) Example of a Treg expansion quality check. The suppressive ability of Tregs during expansion can be checked in the suppression assay. This is a modified mixed lymphocyte reaction in which PBMC or $CD4^{+}$ T cells autologous to Tregs are used as "effectors." The effectors are mixed in different ratios with expanded Tregs, as shown in *x*-axis labels, and all but the first co-culture are stimulated with irradiated allogeneic PBMCs, which serve as stimulators. The effectors without stimulators in the first culture on the left and Tregs only (not shown) are important controls for the quiescence of these cells. This is very important because of the high dose of IL2 that is used during expansion. Contamination with traces of IL2 may abolish the suppressive activity of Tregs and activate effectors. The Tregs used for the suppression assays are washed a few times with PBS and placed in RPMI 1640 medium with 10% FCS and without IL2 for at least 2 days before the suppression assay commences. The suppression assay is incubated at 37°C in 5% CO_2 for five days. 3H-thymidine is added for the last 16 h of the assay. The radioactivity of the harvested cells is then measured using a liquid scintillation counter, and the quality of the Tregs is measured as the suppression of autologous effector proliferation. The assay must be performed with serial dilutions of expanded cells. Achieving suppression of proliferation that correlates with the number of Tregs in particular wells is proof that the expanded cells have kept their suppressive abilities during expansion.

Finally, sorted Tregs require serum in the culture medium. Although highly unlikely, this could lead to problems, because administering expanded cells grown in the presence of allogeneic or xenogeneic serum increases the likelihood of infectious disease transmission. It also presents a threat to the patient, because exposure to serum allo- or xenoantigens could cause an anaphylactic reaction during the first, or, more likely, subsequent administrations of expanded Tregs. A possible solution to this problem is the use of autologous serum from the patient during the entire expansion, although many patients have elevated levels of proinflammatory cytokines in their serum, which could theoretically switch Tregs to Th17 cells (133).

Ex vivo expansion provides the advantage of selective expansion of antigen-specific Tregs, a "Holy Grail" of immunosuppression therapy. The efficiency of antigen-directed Tregs appears to be superior to polyclonal expansions in animal models (96,134). Some authors generated specific Tregs by in vivo challenge with specific antigens, which selected Tregs expressing specific TCRs (135). This could also be achieved by pulsing Tregs with antigen immediately before adoptive transfer; however, in this instance, selective expansion occurs via bystander regulation rather than antigen-specific suppression (22). Alternatively, the antigen to be tolerated can be presented on MHC receptors fixed to the beads used for Tregs expansion (38). There are other variations on antigen delivery to Tregs in animal models. The general conclusion of these studies is that antigen-specific Tregs are more suppressive than are polyclonal Tregs (136,137). Some advances in this area were achieved in human studies. In these studies, expanded Tregs directed against specific antigens were obtained when these cells were incubated with the antigens (138), or antigen presentation was performed using live allo-APCs with or without suppressive cytokines, such as IL10 or TGFβ (34,38,139). However, we believe that the optimal means of achieving Treg antigen specificity is to mimic nature and apply professional presenters, such as dendritic cells (DCs).

DCs in Immunosuppressive Therapy

DCs are a recently introduced "laboratory tool" in the expansion of Tregs for therapeutic purposes (140). They are effective because they can be tolerogenic themselves, are able to induce tolerogenic Tregs (both naturally occurring and adaptive), and can be used to direct tolerance towards specific antigens.

DCs have long been regarded as the pivotal inducers of strong immune responses. However, in some circumstances, DCs may promote suppression rather than inflammation. Several reports document experimental conditions in which murine DCs either suppress immunity or promote tolerance through the inhibition of T cell responses to tissue auto- and alloantigens (141). Inflammation at mucosal surfaces, such as the gastrointestinal tract and the maternal-fetal interface, may be also associated with the suppressive-like regulation of T cell responses to foreign antigens (142). The maturation level of DCs at the time of antigen presentation to T cells is important. Immature DCs, which express low levels of MHC and costimulatory molecules, are capable of inducting the apoptosis or anergy of effector T cells in animals and humans (143–148). Immature tolerogenic DCs present antigens to antigen-specific T cells, but fail to deliver adequate costimulatory signals for T cell effector activation. In addition, at least in humans, immature DCs promote the expansion of various subsets of Tregs (147,149–151).

Some DCs may deploy specific molecular mechanisms that allow them to limit T cell proliferation and/or modify T cell differentiation independent of their APC maturation status (152). Research regarding tolerogenic DCs has been performed mainly with splenic DCs in mice. The interactions of surface CD80/86 receptors on splenic DCs subsets ($CD8^+$/$CD8^-$) with CTLA-4, which is expressed on T cells, induce the expression of functional indoleamine 2,3-dioxygenase (IDO) in DCs. Even small numbers of IDO^+ DCs are capable of suppressing T cell responses in vitro, including dominant inhibition of T cell effectors activated by antigens presented by other nonsuppressive APCs (153). In vivo, pharmacologic activation of the IDO pathway can completely inhibit clonal expansion of large numbers of alloreactive T cells (142). IDO catalyzes synthesis of kynurenines from tryptophan. Hence, its action results in the depletion of tryptophan, an essential amino acid, which subsequently leads to the inhibition of T cell proliferation. This mechanism was initially called "suppression by starvation." However, kynurenines generated as a result of IDO enzymatic activity should also be taken into account in this mechanism. These tryptophan-derived toxic metabolites have been found to promote T cell apoptosis. It is possible to modulate the activity of IDO, because the synthesis of this enzyme by DCs requires IFNγ and may be inhibited by IL6 secretion (153,154). Interestingly, Tregs have been found to secrete small amounts of IFNγ, most probably during crosstalk with DCs, with the purpose of inducing IDO expression in the DCs (155). Importantly, IDO^+ DCs directly activate resting $CD4^+CD25^+Foxp3^+$ Tregs, and regulate the transition of naïve T cells to adaptive subsets of Tregs. In vivo, Tregs isolated from tumor-draining lymph nodes (TDLNs) are constitutively activated and capable of immediate suppression of antigen-specific effector T cells ex vivo. In vitro, IDO^+ DCs from TDLNs rapidly activate resting Tregs from nontumor-bearing hosts without the need for mitogens or exogenous anti-CD3 cross linking. The activation of Tregs by IDO^+ DCs is MHC restricted and can be prevented by CTLA4 blockade (156). Emerging evidence suggests that human DCs and macrophages also exert suppressive activity via IDO and the kynurenine pathway (157–159). Both myeloid and plasmacytoid subsets of human DCs have been found to take part in Treg expansion (160,161). Studies on primates have revealed that DCs are superior to monocytes in the generation of Tregs (162). Interestingly, human Tregs also influence DCs, mainly myeloid DCs, inhibiting their maturation through secreted IL10 and TGFβ (163,164).

Direct proof that tolerogenic DCs interact with Tregs in antigen-specific way was provided by experiments investigating antibody-mediated targeting of autoantigens to steady-

state DCs. For this antigen delivery technique, hen egg lysozyme (HEL) or ovalbumin (OVA) was covalently linked to anti-DEC-205 antibody and injected into mice. This resulted in loading of the lymph node DCs with the antigens and their presentation to T cells; surprisingly, this triggered not inflammation, but tolerance towards the presented antigens (165). Further analysis revealed that anti-DEC-treated animals were characterized by increased numbers of fully active Tregs (166). Anti-DEC-antigen conjugates were tested in animal models of contact hypersensitivity, diabetes, and multiple sclerosis, and were found to be effective at relieving the symptoms of these diseases (167). In human studies, when added to the expansion culture, autologous DCs pulsed with antigens were capable of selecting Tregs with indirect allospecificity (168). This is particularly relevant in some clinical settings, notably after allotransplantations, when the long-term outcome depends mainly on the indirect pathway of antigen presentation (169–171). However, the expansion of Tregs with antigen-primed DCs has some disadvantages. If the antigen dose during Treg expansion is too high and the DCs are mature and activated, these Tregs pose a threat, because of the possibility that some effector T cells might have escaped suppression (172).

Other Expansion Approaches

There are a few approaches to large-scale Treg sorting and expansion in addition to those presented above. For example, alternative sources of Tregs have been suggested; cord blood and bone marrow as the most enriched with Tregs (42,173,174). However, the most interesting studies have focused on the conversion of $CD25^{-}CD4^{+}$ T cells to Tregs. This possibility was suggested for the first time by Waldmann and coworkers, who found that suppressive capabilities can be transferred from Tregs to naive T cells in vivo (“infectious tolerance”) (175). The most popular means of achieving the conversion ex vivo utilizes exogenous TGFβ, as already mentioned (130–132). Recent evidence suggests that TGFβ requires an active Notch-dependent pathway in naïve T cells for conversion to occur (176–178). Human $CD25^{-}CD4^{+}$ T cells can be also converted to Tregs by IL10 (179), immature subsets of DCs (180,181), and calcitriol (vitamin D) (182). The presence of mesenchymal stem cells in cultures of immune cells also appears to result in increased generation of allo-specific Tregs (183–185). Animal studies have revealed that tolerogenic T cells can also be induced from $CD25^{-}CD4^{+}$ T cells after stimulation with staphylococcal enterotoxins (186). Interestingly, the herbal compounds triptolide and (5R)-5-hydroxytriptolide, obtained from *Tripterygium wilfordii Hook*, have been reported to expand Tregs. Mice injected with these compounds after bone marrow transplantation have increased levels of Tregs (187,188).

Perspectives

Despite doubts that have arisen regarding the application, efficiency, and sometimes even the existence of suppression mediated by Tregs in clinical protocols (189), Treg-based adoptive therapies will likely be an important branch of clinical medicine in the foreseeable future. The need for clinically administered expanded Tregs, rather than agents that trigger the activity of endogenous Tregs, is even greater in light of the notorious consequences of the clinical trial of TeGenero’s antibody-based drug (TGN 1412) (190). Currently, several centers are preparing to administer expanded Tregs in clinical settings (35,139,191). Our laboratory’s ongoing program treating graft-versus-host disease with ex vivo-expanded Tregs has demonstrated that the procedures presented in this article are feasible. In our team’s hands, Treg expansion appears to be safe; we did not observe any negative effects when expanded Tregs were transferred back to healthy volunteers. The participants received a single transfusion of a small quantity (5×10^6 cells/transfusion) of autologous Tregs that were expanded ex vivo. Neither the transfusion nor the post-transfusion follow-up has revealed any negative effects resulting from the procedure. This result led to the next step, administering Tregs to GVHD patients, which is currently under way.

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