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Sinks, suppressors and antigen presenters: how lymphodepletion enhances T cell-mediated tumor immunotherapy

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Abstract

Lymphodepletion followed by adoptive cell transfer (ACT) of autologous, tumor-reactive T cells boosts antitumor immunotherapeutic activity in mouse and in humans. In the most recent clinical trials, lymphodepletion together with ACT has an objective response rate of 50% in patients with solid metastatic tumors. The mechanisms underlying this recent advance in cancer immunotherapy are beginning to be elucidated and include: the elimination of cellular cytokine 'sinks' for homeostatic γ_C-cytokines, such as interleukin-7 (IL-7), IL-15 and possibly IL-21, which activate and expand tumor-reactive T cells; the impairment of $CD4^+CD25^+$ regulatory T (Treg) cells that suppress tumor-reactive T cells; and the induction of tumor apoptosis and necrosis in conjunction with antigen-presenting cell activation. Knowledge of these factors could be exploited therapeutically to improve the *in vivo* function of adoptively transferred, tumor-reactive T cells for the treatment of cancer.

Introduction

Adoptive cell transfer (ACT) of large numbers of autologous tumor-reactive T cells into a tumor-bearing host represents a promising therapy for the treatment of metastatic cancer in humans [1]. This exciting therapy uses the rapid *ex vivo* expansion of tumor-infiltrating or -reactive lymphocytes (TILs), which are subsequently transferred in conjunction with the administration of a high-dose of a stimulatory cytokine, in particular interleukin-2 (IL-2). Although other forms of immunotherapy, such as tumor-antigen (Ag) vaccination or the administration of immune-stimulating cytokines alone, are capable of raising tumor-reactive T cells *in vivo*, they do not reliably induce the regression of large established solid tumors (reviewed in Ref. [2]). ACT is capable of mediating tumor regression [3–6], however, these effects are even more pronounced in the absence of host lymphocytes [7,8]. This approach has resulted in the most consistent and dramatic clinical responses observed in the treatment of metastatic cancer [7] (Figure 1a).

These clinical responses are associated with autoimmune manifestations in sites that express shared melanocyte or melanoma Ags, such as the skin, in the form of vitiligo. With lymphodepletion, we have also observed melanocyte destruction at immune privileged sites, such as the eye (Figure 1b,c). Although skin-de-pigmentation has been previously associated

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with immunotherapies, inflammation of the anterior segment of the eye has not been previously observed, and might be indicative of a more potent activating stimulus. Fortunately, these eye manifestations are treatable with local steroids, which do not detract from the antitumor immune destruction.

The specific mechanisms that contribute to this enhanced state of immunity remain poorly understood. Recent insights in two rapidly expanding fields, the cytokine-mediated homeostasis of mature lymphocytes by γ_C-cytokines, such as IL-7, IL-15 and IL-21, and the control of autoreactive T cells by $CD4^+CD25^+$ regulatory T (Treg) cells, provide the foundation for what might be occurring after lymphodepletion. The removal of lymphocytes that compete for homeostatic cytokines or suppress tumor-reactive T cells might contribute to the enhancement and subsequent tumor destruction by the adoptively transferred T cells. The less described role of antigen-presenting cell (APC) activation by lymphodepletion might also have a role in T-cell activation. Knowledge of these factors presents the potential for therapeutic exploitation in the treatment of metastatic cancer in humans.

A link between lymphodepletion and augmented immune function

It has long been observed that transfer of small numbers of T cells into lymphopenic hosts results in T-cell expansion, a process described as homeostatic proliferation [9–16]. As the T cells proliferate, they assume an Ag-experienced or memory phenotype, which is indicated by upregulation of CD44, Ly6C and CD122 (IL-2–IL-15Rβ) [12,15]. This T-cell expansion and acquisition of a memory phenotype is also associated with enhanced effector functions, determined by *ex vivo* analysis of interferon-γ (IFN-γ) release and cytolysis [12,14,15]. In a recent autoimmunity study, King *et al.* showed that the inherent lymphopenia in non-obese diabetic (NOD) mice and increased expression of IL-21 drives the homeostatic expansion of autoreactive cells, precipitating self-tissue destruction [17]. Furthermore, adoptive transfer of naïve lymphocytic choriomenigitis virus (LCMV)-specific $CD8^+$ T-cell receptor (TCR) transgenic splenocytes into LCMV-infected lymphodepleted, but not replete, hosts rapidly reduced viral titers to below detection limits [13]. Analogous results have also been observed in another model, based on the 2C T-cell receptor (TCR) transgenic model. 2C TCR transgenic T cells are specific for an L^d-restricted α-ketoglutarate dehydrogenase epitope or the SIYRYYGL peptide in the context MHC I H2-K^b. These cells expand homeostatically in $Rag^{-/-}$ mice and specifically treat tumors, at a comparable level to the T cells derived from Ag-specific vaccination [15]. Dummer *et al.* observed that adoptive transfer of a polyclonal population of T cells into a $Rag^{-/-}$ or sublethally irradiated mouse specifically inhibits tumor growth [18].

It is interesting to note that in these models, lymphopenic-induced activation and pathogen or tumor clearance are generated in the absence of Ag-specific vaccination. Ag-specific $CD8^+$ T cells are required for pathogen or tumor clearance, and the need for subsequent immunization might be negated [19,20]. These model Ags have given us great insight into homeostatic proliferation but focus primarily on Ags that are of a foreign origin. Perhaps using a self- or tumor-antigen might provide a setting that is more analogous to the human condition. A recently described tumor model, Pmel-1, which uses a transgenic $CD8^+$ T-cell specific for both the native and altered peptide ligand of the melanocyte and melanoma differentiation molecule gp100, might enable us to better model unmutated self-antigens in the clinical setting [5,21].

Evidence for the presence of homeostatic cellular cytokine ‘sinks’

The proliferation of adoptively transferred Tcells in lymphopenic hosts can be reduced in a dose-dependent manner, either by increasing the total number of Ag-specific cells transfused or by co-transferring an ‘irrelevant’ population of Tcells [9,15,16,22]. Host $CD8^+$ Tcells have a dominant role in modulating both donor $CD8^+$ and $CD4^+$ T-cell expansion in lymphopenic

hosts [16]. In the same study, host $CD4^+$ cells inhibited donor $CD4^+$, and to a lesser extent $CD8^+$ T-cell proliferation.

The ability of the host to inhibit the proliferation of adoptively transferred T cells could be the result of physical contact between T cells or competition between T cells for self-MHC–peptide complexes and supportive homeostatic cytokines (Figure 2). Naïve T-cell proliferation is a result of self-MHC–peptide interactions in a lymphopenic environment (reviewed in Ref. [23]), whereas memory T cells proliferate independently of these interactions [12,16]. This is suggestive of Ag-independent activation, a notion that has recently received a great deal of attention. Some studies have focused on the role of host-derived IL-7 and IL-15, and more recently IL-21, γ_C cytokines known to stimulate and induce the proliferation of mature T cells [24–26].

In transgenic mice that overexpress IL-7 or IL-15, there is a substantial increase in the absolute number of T cells [27,28]. This increase is driven largely by the expansion of $CD8^+CD44^{high}$ memory Tcells. Exogenous administration of IL-15 or induction of host expression of IL-15 in wildtype mice by reagents, such as poly I:C, lipopolysaccharide (LPS) and type I-IFNs, causes a selective increase in the proliferation of $CD8^+CD44^{high}$ T cells *in vivo* [29–31]. Exogenous administration of IL-7 to mice enhances T-cell number and function [32,33] and, in both intact and immunodeficient non-human primates, causes a considerable yet reversible increase in the circulating levels of naïve and Ag-experienced $CD4^+$ and $CD8^+$ T cells [34]. Although similar studies have yet to be conducted in humans, there is evidence to support an inverse correlation between serum levels of IL-7 and the severity of lymphopenia caused by conditions, such as AIDS and cytotoxic drug therapy (reviewed in Ref. [35]). Currently, there are no data available that evaluate the *in vivo* effects of IL-15 in primates or humans.

Studies that examine mice deficient in IL-7 or IL-15 have demonstrated that these cytokines have a supportive role in the survival and/or proliferation of adoptively transferred T cells in lymphodepleted hosts [36–40]. The absence of IL-7 inhibits naïve $CD4^+$ and $CD8^+$ T-cell homeostatic proliferation and survival in a lymphopenic environment. The proliferation of donor cells in these *IL-7*$^{-/-}$ hosts is rescued by the administration of exogenous IL-7 [36,41]. IL-7 has a significant role in naïve T-cell homeostatic proliferation but has little impact on memory T-cell expansion and survival in a lymphopenic host [37,38]. In contrast to IL-7, IL-15 does not contribute to naïve T-cell homeostasis but does have a pivotal role in memory $CD8^+$ T-cell proliferation and durability [38]. The homeostatic expansion of memory $CD8^+$ T cells does not rely solely on IL-7, however, these cells do constitutively express high levels of both IL-7Rα (CD127) and the components of the IL-15–IL-2 heterodimeric complex, IL-2–IL-15Rβ(CD122) and γ_c (CD132), and thus might be sensitive to IL-7 [30,38,42].

In summary, it is clear that cytokines can have a profound impact in regulating the homeostasis of T cells in both the normal and lymphopenic settings. The expression of IL-7 and IL-15 receptors on memory T cells might indicate that the administration of these cytokines could enhance both the survival and proliferation of adoptively transferred, tumor-reactive T cells *in vivo*. In a recently described murine tumor model [5], it appears that the long-term tumor treatment efficacy of adoptively transferred, tumor-specific $CD8^+$ T cells is compromised in whole-body irradiated *IL-15*$^{-/-}$ but not wildtype hosts [43]. Endogenously produced IL-15 might contribute to a sustained antitumor treatment response by adoptively transferred $CD8^+$ T cells in a lymphopenic setting. It is likely that non-tumor-specific T cells and other immune cells, such as natural killer (NK) cells, consume IL-15 in the wildtype host. Thus, there could be competition for homeostatic cytokines in the replete host. It is also important to mention the newly discovered cytokine IL-21, which shares homology with IL-2 and IL-15 and binds their common γc receptor [44,45]. This cytokine might augment the function of adoptively

transferred tumor-specific $CD8^+$ T cells [17,46] and is the subject of ongoing investigations. Many unresolved questions regarding the use of γc receptor cytokines remain to be answered (Box 1).

Box 1. Unresolved questions regarding the use of γ_c cytokines

- Does the absence of endogenous IL-15 impair the early or late *in vivo* proliferation, functionality, and maintenance of adoptively transferred tumor-specific T cells, and thus their ability to mediate tumor destruction?
- What influence does host-derived IL-7 alone, or in combination with IL-15, have on tumor-reactive T cells and their function?
- What is the role of IL-21 in homeostatic proliferation, and does exogenous administration improve the ability of adoptive transferred tumor-specific T cells to kill tumor?

Role of regulatory cells in antitumor immunity

Naturally occurring and induced $CD4^+CD25^+$ Treg cells can potently suppress immune responses to self-Ags and foreign Ags in both humans and mice. The phenotype and function of Treg cells have been reviewed in detail elsewhere [47,48]. There are some features of Treg cells that might be of particular interest to tumor immunologists. $CD4^+CD25^+$ Treg cells express high levels of cell-surface molecules typically associated with activation; these include CD25 (IL-2Rα), glucocorticoid-induced tumor necrosis factor (TNF)-receptor (GITR) and cytotoxic T lymphocyte-associated Ag-4 (CTLA-4). In addition, they also express a unique intracellular protein, *Foxp3*. Treg cells are completely absent in mice that are deficient in the genes encoding IL-2, IL-2Rα, IL-2Rβ and Foxp3, suggesting a crucial role for both IL-2 signaling and Foxp3 expression in Treg-cell ontogeny and survival [49–51]. The role of Treg cells in maintaining immunological tolerance to self-Ags has been illuminated, in part, through adoptive transfer experiments. A spectrum of tissue-specific autoimmune diseases, including thyroiditis, oophoritis, gastritis and inflammatory bowel disease, occurs when immunodeficient mice are transfused with T cells depleted of $CD4^+CD25^+$ cells [52,53]. The concomitant transfer of Treg cells abrogates development of these autoimmune diseases. In some models, transfer of Treg cells after the onset of disease can even be curative [54]. The close correlation between autoimmunity and tumor immunity [5,7,55–59] suggests that Treg cells have a crucial role in T-cell tolerance to tumor [60–62].

Although the significance of Treg cells in human cancers has only recently begun to be explored, many of the findings in mice could be recapitulated in humans [61,63,64]. Wang *et al.* recently reported on the isolation of $CD4^+CD25^-$ TIL clones derived from a melanoma patient [65]. These clones displayed many of the phenotypic and functional properties associated with naturally occurring Treg cells and were antigen-specific. Another report has shown that Treg cells present in the circulation of patients vaccinated against melanoma Ags can suppress the proliferation of a polyclonal population of $CD4^+CD25^-$ T cells [66], however, the Ag-specificity of these cells was not determined. Treg cells capable of suppressing the *in vitro* function of tumor-reactive T cells in humans has also been found in other tumors besides melanoma [67,68]. In one instance, tumor deposits taken from patients with lung cancer reportedly contained large numbers of $CD4^+CD25^+$ Treg cells capable of suppressing the proliferation of autologous TILs [68]. In contrast to these murine studies, no conclusive data link the *in vivo* function of tumor-reactive Treg cells and the progression of cancer in humans, although some recent evidence suggests that Treg tumor infiltration might be associated with survival of cancer patients [69]. However, removal of these regulatory elements (Box 2),

perhaps in conjunction with cytokine sinks, could reverse or prevent the tolerance of adoptively transferred tumor-reactive T cells (Figure 3).

Box 2. Eradication of Tregs

Although Food and Drug Administration-approved drugs are available that can selectively deplete CD25-expressing cells in humans, including humanized anti-Tac (anti-CD25) and ONTAK™ (IL-2 conjugated to diphtheria toxin), it is currently unknown what effect these reagents might have on Treg cells as well as on activated tumor-reactive T cells.

Until a unique cell surface lineage marker capable of discriminating between Treg and activated T cells can be discovered, non-specific lymphodepletion might be the only practical approach to removing Treg cells from patients for the purpose of augmenting their *in vivo* immune reactivity to a tumor.

Effects of lymphodepleting radiation and chemotherapy on APC function

Lymphodepletion before ACT uses total body irradiation (TBI) or cytotoxic drugs. Although these modalities were initially intended to deplete the lymphoid compartment of recipients, they can also facilitate the presentation of tumor antigens by triggering tumor cell death and antigen release. Subsequently, these antigens can be taken up and presented by APCs to enhance the activation of the adoptively transferred tumor-reactive cells [70].

Indirect evidence indicates that there might be other beneficial effects of lymphodepletion in our current ACT model. Irradiation and/or chemotherapy leads to activation of host cells, resulting in the release of proinflammatory cytokines, such as TNF-α, IL-1 and IL-4 [71–74], and upregulation of co-stimulatory molecules, such as CD80 [75]. In a recent study, activation markers I-A^b (class II) and CD86 were upregulated on splenic DCs as early as 6 h after TBI. Furthermore, *ex vivo* IL-12 production was significantly higher from DCs isolated 6 h after irradiation than DCs from non-irradiated mice [76]. Consistent with this finding, serum levels of IL-12 were also increased in these mice. Elimination of competitive T cells through lymphodepletion might improve donor T-cell access to, and activation by, antigen-bearing APCs [77], although other workers have found that competition might not play an influential role [78].

In addition to enhanced APC function and availability, the preconditioning regimen can damage the integrity of mucosal barriers through radiation-induced apoptosis of the cells lining these organs. Damage inflicted on the intestinal tract might permit the translocation of bacterial products, such as LPS, into the systemic circulation[79]. LPS activates T cells *in vivo* [31] and might enhance the antitumor response. Thus, proinflammatory cytokines and microbial products provide crucial ‘danger signals’ for the activation and maturation of DCs, thus enhancing T cell-mediated tumor treatment.

Although initially beneficial, lymphodepletion can depress the absolute number of host APCs. As reported in several murine studies, the total number of monocytes, macrophages and DCs are only slightly reduced at 6 and 24 h but are significantly reduced 5 days after TBI [71,76]. This could be detrimental in the case of the current tumor therapy model, which requires an active vaccination for successful tumor therapy[5]. Interestingly, late clearance did not hinder the activation of the donor T cells[76]. This corroborates with several reports that demonstrate antigenic stimulation, before APC clearance, is all that is required to initiate activation and proliferation of T cells [80,81].

Therapeutic implications and future directions

It is clear that lymphodepletion before adoptive transfer of tumor-reactive T cells into animals and humans with cancer augments *in vivo* function of the transferred cells and the therapeutic outcome. Increased access to the homeostatic cytokines, such as IL-7 and IL-15, through elimination of cytokine sinks, eradication of the suppressive influence of Treg cells and enhancement of APC activation and availability appear to be the underlying mechanisms involved in this paradigm (Figure 4). The mechanisms are complex, however, elucidation through the use of targeted cytokine administration, add-back of sink or suppressor elements and selective deletion or addition of APCs might explain how these mechanisms interact.

Other modalities that mimic or enhance the positive effects of lymphodepletion could have great therapeutic potential. The exogenous administration of supportive cytokines, such as IL-2, IL-7, IL-15 or IL-21, either alone or in concert, could stimulate the adoptively transferred T cells and enhance their tumor-killing ability. Ways to selectively deplete or inhibit $CD4^+CD25^+$ Treg cells with the use of antibodies against CD25 or GITR remain the pursuit of many workers, although clear effects have been less then forthcoming. Modulation of adoptively transferred cells either through overproduction of cytokines, such as IL-7, IL-15 [43] or IL-21, or cytokine receptors, such as IL-7Rα and IL-15Rα, are currently being explored as possible means to enhance T-cell function *in vivo*. Reagents that stimulate host expression of IL-15 and IL-15Rα [30,31,82], including type I IFNs, CpG DNA, LPS, dsRNA and dsRNA-imitators, such as polyI:C, might enhance the functionality of adoptively transferred cells.

Finally, although IL-2 has been the preferred cytokine for the *ex vivo* [83] and *in vivo* [7,8] activation and expansion of tumor-reactive T cells, it also drives Treg proliferation and activation [84] and triggers apoptosis in activated T cells [85]. Exploration of other cytokines that can drive tumor-reactive T cells, such as IL-7, IL-15 and IL-21, might prove to be more efficacious in the activation of adoptively transferred tumor-reactive cells.

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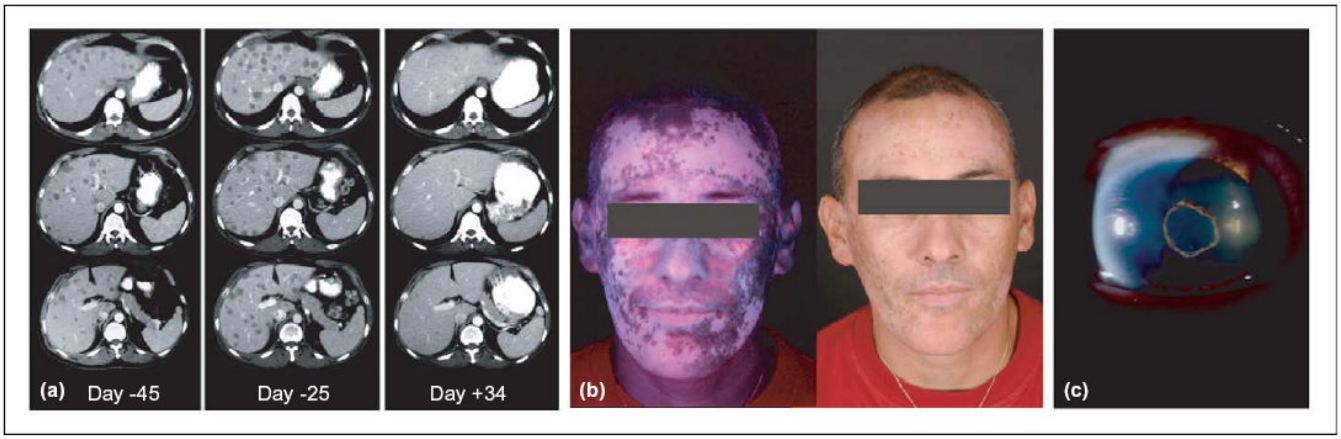

Figure 1.

Potentiation of immune response to non-mutated shared self-antigens and tumor antigens by lymphodepletion. Following adoptive cell transfer therapy with tumor-infiltrating lymphocytes (TILs), in the setting of chemotherapy-mediated lymphoablation: (**a**) Computed tomography (CT) scan of liver metastases in a melanoma patient. Metastatic lesions progressed rapidly before the treatment (from day −45 to day −25) then regressed dramatically after adoptive T-cell transfer (day +34). (**b**) Extensive destruction of normal melanocytes (vitiligo) in a patient who experienced a remarkable clinical response. (**c**) Anterior uveitis (inflammation of the eye) in a patient who exhibited >99% tumor reduction Photographs courtesy of S.A. Rosenberg.

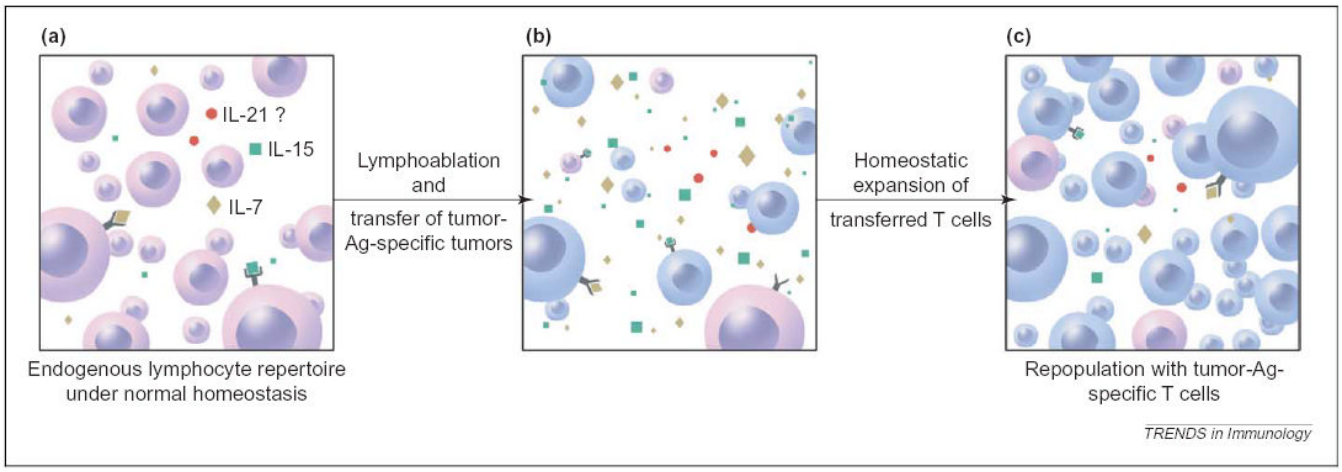

Figure 2.

Removal of consumptive cytokine ‘sink’ by lymphoablation. (**a**) Under normal homeostasis, a smaller quantity of cytokines [IL-7 (yellow), IL-15 (green) and possibly IL-21 (red)] are available owing to consumption by the endogenous lymphocyte population. (**b**) After lymphodepletion preconditioning by chemotherapy or irradiation, the consumptive cellular cytokine ‘sinks’ are removed, enabling the adoptively transferred T cells a less competitive environment and greater access to IL-7, IL-15 and IL-21. (**c**) This is followed by preferential homeostatic expansion and re-population of the peripheral lymphoid compartments with the adoptively transferred, tumor-reactive T cells.

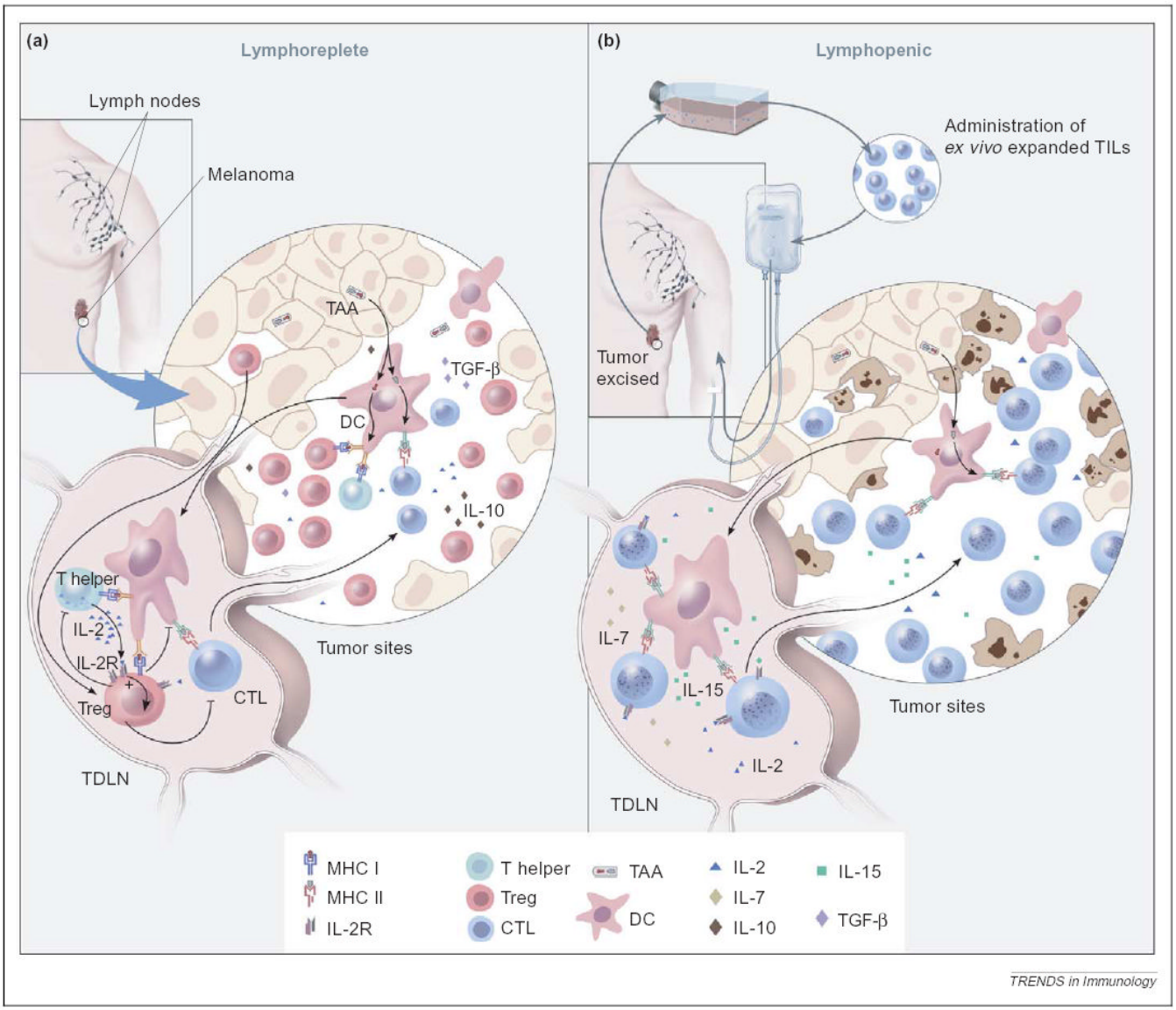

Figure 3.

Removal of the inhibitory effect of Treg cells and activation of DCs by lymphoablation. (**a**) Under normal homeostasis, Treg cells (red) in the tumor draining lymph node (TDLN), might exert inhibitory effects on the transferred tumor-specific T cells (blue). In addition, Treg cells might prevent $CD4^+$ Th cells (green) from providing supportive cytokines, such as IL-2 (blue). DCs (pink) in the tumor, which cross-present tumor-associated antigens (TAAs), might induce tolerance in the absence of inflammation and in the presence of immunosuppressive cytokines, such as IL-10 (brown) and TGF-β (purple). The net outcome of these inhibitory factors could abrogate an effective antitumor response. (**b**) After the induction of lymphopenia, *ex vivo* expanded tumor-reactive TILs are adoptively transferred (blue). This preconditioning before cell transfer effectively eliminates Treg cells and other cytokine-competing cells, thus removing inhibition and freeing supportive cytokines, such as IL-2, IL-7 (yellow) and IL-15 (green). The ablative conditioning might also activate DCs that are capable of cross-presenting TAAs in the presence of danger signals. The effect of ablation is a ‘permissive’ and activating environment that augments tumor-specific T cell-induced tumor destruction.

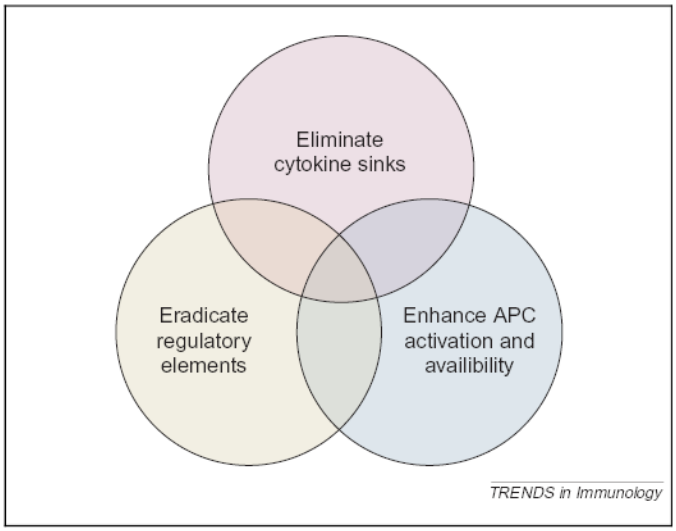

Figure 4.

An interactive model for the mechanisms underlying the impact of lymphodepletion on adoptively transferred T cells. (i) Elimination of cytokine sinks: lymphoablation, either by chemotherapy or irradiation, reduces the consumptive cytokine ‘sinks’ created by the endogenous lymphocyte repertoire, thus enabling the transferred T cells a less competitive access to homeostatic cytokines, such as IL-7 and IL-15. (ii) Eradication of regulatory elements: Treg cells, such as the $CD4^{+}CD25^{+}$ cells, that would otherwise exert an inhibitory effect on the transferred T cells are diminished in number and function after lymphoablation. (iii) Enhancement of APC activation and availability: lymphoablation, either by irradiation or chemotherapy, can induce tumor apoptosis and necrosis, resulting in uptake and presentation of tumor antigens by APCs. Furthermore, there is some evidence that suggests that after lymphodepletion, APCs become activated, thus enhancing stimulation of the adoptively transferred T cells. The transferred T cells also have less competition with irrelevant T cells for APCs, which might improve activation. These mechanisms can act independently or in synergy to augment the function of the tumor-reactive T cells.