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Optimising IL-2 for Cancer Immunotherapy

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Abbreviations
APC, Ag-presenting cells; BCG, Bacillus Calmette-Guérin; DC, dendritic cells; hIL-2, human IL-2; ICB, immune checkpoint blockade; IL-2R, IL-2 receptor; IL-2v, IL-2 variant; ko, knockout; LCMV, lymphocytic choriomeningitis virus; LIP, lymphopenia-induced proliferation; mAb, monoclonal Ab; MP, memory phenotype; MPECs, memory-

ABSTRACT

The key role of T cells in cancer immunotherapy is well established and is highlighted by the remarkable capacity of Ab-mediated checkpoint blockade to overcome T-cell exhaustion and amplify anti-tumor responses. However, total or partial tumor remission following checkpoint blockade is still limited to only a few types of tumors. Hence, concerted attempts are being made to devise new methods for improving tumor immunity. Currently, much attention is being focused on therapy with IL-2. This cytokine is a powerful growth factor for T cells and optimises their effector functions. When used at therapeutic doses for cancer treatment, however, IL-2 is highly toxic. Nevertheless, recent work has shown that modifying the structure or presentation of IL-2 can reduce toxicity and lead to effective anti-tumor responses in synergy with checkpoint blockade. Here, we review the complex interaction of IL-2 with T cells: first during normal homeostasis, then during responses to pathogens, and finally in anti-tumor responses.

Keywords: IL-2; IL-2 therapy; Cancer immunotherapy; T cells

INTRODUCTION

The notion that stimulation of the immune system can lead to regression of tumor growth dates back more than a century to the studies of Coley. During his career as an orthopedic surgeon, Coley was intrigued that his patients with sarcoma occasionally showed remarkable improvement after an infection, especially erysipelas. In support of this idea, in 1891 he reported that injection of *Streptococcus pyogenes* caused tumors to disappear in some of his patients (1). Subsequent studies with various components of bacteria, Coley's toxins, gave erratic results and this approach gradually fell into disfavour. Nevertheless, the idea that the immune system has the potential to attack tumors caught on and led Ehrlich in 1909 to postulate that tumors are normally rare because of surveillance by "the organism's positive mechanisms" (2). Later in the 1950s, this view was refined by the suggestion of Thomas (3) and Burnet (4) that tumor cells are recognized by the immune system via expression of neoantigens. This concept of self/nonself discrimination by the immune system can be traced back to earlier studies on the "antigenic properties" of chemically-induced tumors (5).

The discovery of T and B cells in the 1960s (6,7) focussed attention on the cell types controlling tumor immunity (8). Initially, in contrast to normal mice transplantable tumours

precursor effector cells; PEG, polyethylene glycol; SLECs, short-lived effector cells; SPF, specific-pathogen-free; TCR, T cell receptor; TIL, tumor-infiltrating lymphocyte.

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were found to grow well in T-cell deficient nude mice, indicating an important anti-tumor role for T cells but not B cells. Notably, however, nude mice did not show an increase in the development of spontaneous or chemically-induced tumors, implying an important protective role for the innate immune system. Direct support for this idea came from the finding that preventing the appearance of chemically-induced tumors required the presence of NK cells and IFN-γ production by these cells (9,10). Notably, tumors appearing in mice lacking both T and NK cells showed increased immunogenicity. This finding has given rise to the concept of immunoediting whereby overtly immunogenic tumor cells are normally eliminated at an early stage by the combined function of T cells and NK cells, leaving only residual extensively-edited, poorly-immunogenic cells to survive and form macroscopic tumors (11,12).

Although mature tumor cells display a number of target epitopes for immune cell recognition, expression of MHC molecules is particularly important, MHC-I molecules for CD8 T cells and MHC-II for CD4 T cells. CD8 T cells and NK cells both have the capacity to react to MHC-I ligands on tumor cells and destroy the cells by perforin-dependent lysis (13). However, the mechanisms involved are fundamentally different. Thus, whereas CD8 T cells kill tumours via recognition of MHC-I-bound antigenic peptides, lysis by NK cells is generally directed selectively to cells that are MHC-I negative; such recognition of "missing-self" allows NK cells to recognize and destroy neoplastic cells but avoid killing normal cells (14). CD4 T cells seem to play a lesser role in immune surveillance but may be important for tumors that display MHC-II ligands; via cytokine release, CD4 T cells are also needed for optimal CD8 T cell function, as discussed below.

IMMUNOSTIMULATION OF T CELLS: THE ROAD TO IL-2 THERAPY

Spurred by the remarkable if inconsistent success of Coley's toxins, a wide array of bacterial products and other stimuli were tested for anti-tumor activity. Of the tumor types examined, melanoma received particular attention because on rare occasions these tumors mysteriously underwent spontaneous regression and completely disappeared. Sporadic cases of prominent tumor remission in melanoma patients were also seen following treatment with certain immune stimulants, notably Bacillus Calmette-Guérin (BCG), *Cryptosporidium parvum* or type I IFN (15,16). Though rare, these findings gave rise to the notion that melanomas are intrinsically more immunogenic than other tumors and that, if effective, stimulation of the immune system can lead to melanoma rejection. In support of this view, it is now well documented that melanomas display a high incidence of gene mutations, reflecting prolonged exposure to ultra-violet light (17). Through expression of neoantigens resulting from these mutations, melanomas are thus strongly immunogenic for T cells (18). The same applies to established tumor cell lines which, like melanomas, show prominent mutations and neoantigens. Nevertheless, in addition to melanoma only a few types of human tumors show a high mutation rate, including lung squamous cell carcinoma and adenocarcinoma in smokers (19). Hence, many tumors are poorly immunogenic and therefore resistant to immunotherapy.

Since neoantigens are recognized selectively by the adaptive immune system, attempts to amplify tumor immunity has focused largely on T cells rather than NK cells. Here, the emphasis is on developing and improving methods for expanding the numbers and function of tumor-specific T cells, especially CD8 T cells, both *in vitro* and *in vivo* (20). Since growth and survival of these cells is controlled by cytokines, much attention has been focussed on

the issue of which particular cytokines are best suited for stimulating CD8 T cells. Here, it is well established that normal CD8 and CD4 T cells are responsive to many different cytokines but are particularly sensitive to stimulation by members of the common γ chain (γc) family of cytokines (21); these cytokines are so called because each binds and signals to T cells via a cell-surface, two-chain receptor comprising a unique cytokine-specific chain complexed with the same invariant signalling chain, γc (also known as CD132). The point to emphasize is that the reactivity of T cells to γc cytokines depends upon whether the cells are in their normal resting state or are involved in responses to foreign Ags (including tumor neoantigens). So, to understand how best to use cytokines to expand tumor-specific T cells, it is first important to consider the role of cytokines in maintaining the homeostasis of normal resting T cells.

CYTOKINE CONTROL OF NORMAL T CELL HOMEOSTASIS

Based largely on studies in mice, it has been shown that the extended lifespan of normal CD8 and CD4 T cells requires contact with two cytokines, IL-7 and IL-15 (22-27). These cytokines are recognized by receptors composed of IL-7 receptor α chain linked to γc for recognition of IL-7, and IL-2 receptor β chain (also called CD122) attached to γc for IL-15 binding (**Fig. 1**). For typical naïve T cells, contact with these 2 cytokines keeps the cells alive in interphase for prolonged periods. However, such survival is dependent on the cells also receiving low-level T cell receptor (TCR) signals via contact with self-peptide/MHC molecules on the cell surface (27,28). Here, it should be pointed out that, because MHC molecules are highly polymorphic, "self" is determined by the particular MHC molecules encountered by T cells during their initial formation in the thymus. Constant TCR recognition of these self ligands by naïve T cells maintains their sensitivity to cytokines and is thus crucial for sustaining their viability.

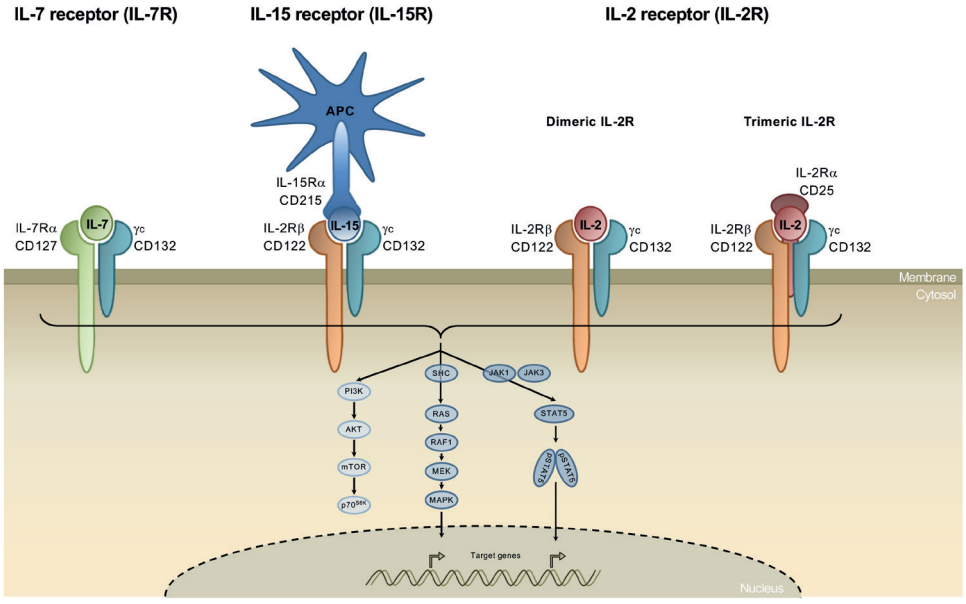

Figure 1. IL-7, IL-15 and IL-2 receptor composition and signalling. The IL-7R is made of a heterodimer of IL-7Rα (also known as CD127) and the common γ chain (γc, CD132) which controls signal transduction; IL-7 is produced in soluble form by mesenchymal and epithelial cells. The IL-15R consists of IL-15Rα (CD215), IL-2Rb (CD122) and γc. Notably, IL-15 is usually produced by APC and binds to IL-15Rα before being presented *in trans* to responding T cells and natural killer cells. The IL-2R consists of either a heterotrimer made of IL-2Rα (CD25), IL-2Rβ (CD122) and γc, or a heterodimer of CD122 and γc. For the trimeric IL-2R, soluble IL-2 initially binds with high-affinity *in cis* to CD25, an adjacent transmembrane receptor, which then focuses IL-2 to the dimeric IL-2R; IL-2 is synthesized either by the responding T cells (autocrine stimulation) or by adjacent T cells (paracrine stimulation). For each cytokine shown, binding-induced receptor activation via their γc-containing receptor and JAK3 tyrosine kinase triggers several intracellular signalling pathways, including the PI3K–AKT, MAPK, and JAK1/3-STAT5 pathway (40).

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Information on the relative importance of IL-7 and IL-15 for T cell survival came from studies on gene knockout (ko) mice deficient in IL-7 (24) or IL-15 (23,25). Examining T cells from these mice, or cells adoptively transferred to these mice, demonstrated that naïve CD4 T cells are solely dependent on IL-7, reflecting their complete absence of CD122 expression. Naïve CD8 T cells are also strongly dependent on IL-7 but show some reduction in IL-15 ko mice (23), correlating with their significant but low-level expression of CD122 (22). This scenario changes when naïve T cells are activated by Ag. Here, as considered later, responses of naïve T cells to Ag culminate in a small proportion of the proliferating cells eventually reverting to a resting state and forming memory cells. Notably, especially for CD8 T cells, memory CD8 T cells are no longer dependent upon contact with self-peptide/MHC molecules for their survival. Instead, these T cells become solely dependent on contact with IL-7 and IL-15. Selective interaction with IL-15 is particularly important and depriving memory T cells from contact with IL-15 leads to their rapid death (25). The strong dependency of memory CD8 T cells on IL-15 correlates with elevated expression of its receptor (CD122) on these cells relative to naïve cells, thereby substantially increasing sensitivity to IL-15 and causing memory CD8 T cells to be more metabolically active than naïve cells. However, the bulk of the cells are in interphase and only a small proportion (1%) are in active cell cycle, reflecting intermittent MHC-I-dependent transition of naïve CD8 T cells into resting memory cells (29). As discussed below, memory CD8 T cells also undergo occasional MHC-independent division through contact with cytokines, especially IL-15.

The situation with memory CD4 T cells is somewhat different. Thus, although these cells resemble memory CD8 T cells in being dependent on IL-7 and IL-15 (27), most memory CD4 T cells appear to retain overt self-peptide/MHC-II reactivity and a substantial proportion of these cells (30%) are activated and in cell cycle (30). It should be noted that in humans and mice raised in a conventional environment, memory T cells are typically generated through contact with foreign Ags. However, this is not the case in laboratory mice. Thus, for these mice the proportion of T cells with a memory phenotype (MP), i.e., cells displaying the markers found on virus-induced memory cells, is about the same in Ag-free mice as in typical specific-pathogen-free (SPF) mice (31,32). Hence, the majority of these MP or "virtual" memory T cells appear to arise from self-peptide/MHC-reactive T cells rather than via contact with environmental Ags.

With regard to their turnover, naïve T cells remain in interphase for prolonged periods without division (33). However, reflecting their enhanced sensitivity to cytokines most memory T cells undergo intermittent cell division, largely by IL-15 for memory CD8 T cells (25); interestingly, such slow "homeostatic" turnover of memory T cells does not expand their total numbers, indicating that cell division is balanced by a comparable level of cell death. In mice, this resting pattern of T-cell homeostasis changes when purified subsets of cells are transferred to T-cell-depleted mice (27,34-38). In these lymphopenic hosts, donor-derived memory CD8 T cells proliferate more rapidly than in their original hosts. Likewise, injection of naïve CD8 T cells into lymphopenic mice causes a proportion of the donor cells to begin to proliferate and then differentiate into memory cells. For both naïve and memory T cells, this lymphopenia-induced proliferation (LIP) applies to CD4 T cells as well as CD8 T cells and reflects an increase in the relative concentration of IL-7 in the T-depleted hosts (the result of decreased IL-7 consumption). As for survival in interphase, LIP by naïve T cells is MHC dependent and is also significantly influenced by the relative avidity of TCR–self-peptide/MHC interaction. Thus, naïve T cells expressing high levels CD5 and CD45 — a presumed marker of strong self MHC avidity — give stronger LIP than cells with lower expression of these markers (37,39).

Collectively, these studies indicate that the survival and intermittent turnover of normal T cells is heavily dependent on just two cytokines, IL-7 and IL-15. This finding may seem surprising because T cells are also strongly reactive to IL-2, another γc family member closely related to IL-15 (40). Thus, IL-2 and IL-15 both share the capacity to bind to and stimulate T cells via the dimeric CD122-γc receptor (**Fig. 1**). Binding to this shared receptor is relatively weak, however, and in the case of IL-15 generally leads only to occasional division of T cells. However, the situation with IL-2 is different. When activated, T cells synthesize IL-2 receptor α (also called CD25), a unique molecule that does not itself induce signalling but selectively increases the binding of IL-2 to the dimeric CD122-γc receptor. Thus, CD25 associates with the intermediate-affinity CD122-γc dimeric IL-2 receptor (IL-2R) to form a trimeric IL-2R, IL-2Rαβγ, which has high affinity for IL-2 (**Fig. 1**). Under normal physiological conditions, constitutive expression of this trimeric IL-2R on T cells is restricted to Tregs, a specialized subset of CD4 T cells marked by expression of the transcription factor Foxp3; as documented elsewhere (41,42) and below, these cells play a vital role in maintaining self tolerance, and prominent autoimmunity develops in their absence. But the point to emphasize here is that, via CD25, Tregs are kept alive by their capacity to recognize and respond to the very low levels of IL-2 expressed constitutively in normal animals. For cells other than Tregs, however, the lack of CD25 on these cells means that normal T cells are nonresponsive to the background level of IL-2 that maintains Treg viability. It is for this reason that physiological concentrations of IL-2 play no direct role in normal T-cell homeostasis (40,43).

Nevertheless, by controlling the survival of Tregs, IL-2 does play a conspicuous indirect role in maintaining homeostasis. Thus, in IL-2 ko mice the absence of IL-2 precludes Treg formation, thereby preventing self tolerance of normal T cells and leading to overt T-cell autoreactivity and proliferation (44). This syndrome is even more prominent, and lethal, following conditional deletion of the *Foxp3* gene in adult mice (45); here, abrupt elimination of Tregs unleashes devastating autoreactivity, allowing T cells with the highest affinity for self-peptide/MHC molecules, namely $CD5^{hi}$ CD4 T cells, to proliferate and synthesize a plethora of effector cytokines (46,47). Hence the main, and perhaps sole, function of IL-2 in normal unstimulated animals is to keep Tregs alive. The source of IL-2 for Treg survival is unclear. Although a number of cell types including Ag-presenting cells (APC) can secret small amounts of IL-2, most IL-2 is thought to be produced by activated CD4 T cells. In the thymus, survival of newly-formed Tregs is reported to reflect local contact with IL-2 synthesized by thymocytes (48,49), perhaps by self-peptide/MHC-reactive T cells prior to their death *in situ* via negative selection. Hence, escape of some of these autoreactive T cells from the thymus might account for the low levels of IL-2 that maintain Treg survival in the periphery. Direct evidence on this issue is lacking.

Although the constitutive background level of IL-2 is insufficient to stimulate normal T cells, exposure to exogenous IL-2 either *in vitro* or *in vivo* does lead to rapid CD8 T-cell proliferation, both for naïve and memory cells (40,43,50). Paradoxically, this pattern of rapid proliferation also occurs following injection of mice with particular anti-IL-2 monoclonal Abs (mAb) (43). Here, binding of anti-IL-2 mAb to endogenous IL-2 augments IL-2 function and redirects IL-2 from Tregs to CD8 T cells, thereby causing overt stimulation of CD8 T cells via their dimeric CD122-γc receptor, especially $CD122^{hi}$ memory CD8 T cells. Interestingly, a similar pattern of rapid proliferation occurs when naïve (i.e., $CD122^{lo}$) CD8 T cells are transferred to CD25 ko hosts, i.e., mice in which the lack of CD25 impairs Treg formation and IL-2 consumption, thereby causing a sharp increase in IL-2 levels (51). Naïve CD8 T cell proliferation is even more marked with transfer to CD122 ko hosts, i.e., mice where Treg dysfunction plus lack of IL-2 and IL-15 consumption causes both IL-2 and IL-15 to rise to high levels; here, donor

cell expansion also applies to naïve CD4 T cells, though at a lower level than for naïve CD8 T cells. Notably, production of IL-2 can also occur when T cells are transferred to lymphopenic mice. In this situation, gut bacteria in the immunodeficient hosts become immunogenic for the injected T cells, causing donor CD4 T cells to respond to these bacteria and synthesize IL-2; adjacent CD8 T cells, especially memory cells, then respond to this IL-2 and rapidly proliferate (52-54). This process of non-Ag-specific "bystander" proliferation fails to occur in germ-free mice (52), but is prominent in adult mice during virus infection (55). Here, proliferation is driven by IL-15 rather than IL-2 and reflects that viruses induce strong production of type I IFN which then elicits IL-15 synthesis by APC (55,56).

For IL-2, it should be noted that intense proliferation of naïve T cells by high concentrations of IL-2 reflects TCR-dependent upregulation of CD25 expression, thereby substantially increasing sensitivity to IL-2 by formation of the high-affinity trimeric IL-2R (40). The situation with IL-15 is more complex because, unlike IL-2, IL-15 is usually not presented as a soluble molecule. Instead, IL-15 is synthesized by APC and then presented on the surface of these cells tightly bound to a unique IL-15-specific α-chain, IL-15Rα (also known as CD215) (**Fig. 1**) (56). T cells then recognize and respond to cell-bound IL-15 *in trans* via the dimeric CD122-γc receptor; this situation is analogous to recognition of IL-2 *in cis* by the trimeric IL-2R. As with IL-2, formation of a trimeric receptor for IL-15 recognition by T cells leads to high-affinity interaction and strong intracellular signalling. It should be noted that, under *in vivo* conditions, nearly all IL-15 is cell associated. Nevertheless, T cells are readily responsive to injection of synthetic soluble IL-15, especially when presented as IL-15/IL-15Rα complexes (57,58). These laboratory-prepared constructs bind well to dimeric CD122-γc receptors and are strongly stimulatory for CD8 T cells *in vivo*; their use in cancer immunotherapy is receiving close attention (see below).

In all of the above situations, homeostasis of normal T cells is influenced by three factors: 1) the relative TCR affinity of T cells for self-peptide/MHC molecules to control sensitivity to cytokines, 2) the concentrations of γc cytokines, especially IL-7, IL-15, IL-2, and 3) the relative expression on T cells of the receptors for these cytokines, especially CD122 and CD25. In general, CD8 T cells are more sensitive to γc cytokines than CD4 T cells, partly because of their higher density of CD122 but also because CD8 T cells show higher expression of lipid rafts, which amplify signalling via dimeric CD122-γc and other cytokine receptors (59). Hence, exposing CD8 T cells to abnormally high concentrations of IL-2 or IL-15, e.g., in CD25 ko or CD122 ko hosts (see above), makes these cells proliferate at a conspicuously high rate, equal to that seen when T cells respond to a foreign Ag. Such proliferation can contribute significantly to the immune response. Thus, both for CD8 and CD4 T cells, strong proliferative responses to γc cytokines are associated with differentiation into effector cells capable of providing substantial non-specific protection against pathogens, thus enabling MP T cells to function as innate immune cells (51,54,60,61). In addition, it should be noted, like naïve cells, MP T cells have a broad TCR repertoire, thereby allowing MP T cells to also mount Ag-specific responses to pathogens (60).

In extrapolating to the human immune system, it should be pointed out that much of the above evidence has come from studies on a single strain of laboratory mice, C57BL/6. This is a significant concern because other strains of mice can show subtle differences in their pattern of T cell homeostasis. For example, instead of dependence on IL-15, memory CD8 T cells in BALB/c mice are largely dependent on a different γc cytokine, IL-4 (60); C57BL/6 T cells are also sensitive to IL-4 but the levels of IL-4 in this strain are too low to affect T cell homeostasis.

Another concern is that laboratory mice are maintained in a clean (SPF) environment and hence have fewer memory T cells than humans (62). Nevertheless, the responsiveness of human and mouse T cells to cytokines is broadly quite similar. Also, the notion that MP T cells in mice arise via self-peptide/MHC reactivity is supported by the presence of MP T cells in human fetal tissues, though these cells are not present in cord blood at birth (60).

ROLE OF IL-2 DURING THE IMMUNE RESPONSE

It tends to be forgotten that lymphocytes in organs such as the spleen and lymph nodes are not resident there but are in constant motion. Thus, typical mature T (and B) cells reside within the recirculating lymphocyte pool and migrate continuously between blood and lymph through the lymphoid tissues (63). As they pass through these tissues, T cells come into close contact with specialized APC, namely dendritic cells (DC), and screen these cells for MHC-bound peptides. Normally T cells encounter only self-peptide/MHC molecules on DC; such recognition delivers a low-level TCR survival signal to T cells but does not impede their migration. During an infection, however, self-peptides on DC are replaced by immunogenic foreign peptides, which causes T cells reactive to these epitopes to bind tightly to DC and become activated. Hence, the first sign of an immune response is that Ag-reactive T cells are withdrawn from the circulation and become sequestered at the site of Ag localization (64).

Basic information on the initiation of the immune response came largely from studies in mice on the response to viruses and other pathogens and has since been broadly confirmed and extended to humans (40,65-73). Although the precursor frequency of naive T cells for Ag is very low, DC presentation of Ag derived from pathogens is strongly immunogenic and causes the responding T cells to proliferate extensively and undergo massive expansion in their numbers. As detailed in any basic textbook of immunology, stimulation by Ag is strictly dependent upon the responding T cells receiving costimulation via the interaction of CD28 molecules on T cells with B7 (CD80, CD86) ligands expressed on DC. B7 expression on DC is normally kept at a low level by Tregs and, to be immunogenic, presentation of Ag by DC has to be accompanied by upregulation of B7 via the "adjuvant" activity of the pathogen. Thus, during infection with viruses and bacteria, evolutionarily-conserved structures on the pathogen interact with "pattern-recognition" receptors on DC and cause these APC to become activated and upregulate B7 levels. Via joint recognition of peptide/MHC molecules (Signal 1) and elevated levels of B7 (Signal 2) on DC, the reactive T cells are then activated and induced to proliferate; initiation of proliferation also depends on synthesis of cytokines, especially IL-12 and type I IFN, released from the activated DC (Signal 3).

For CD8 T cells, optimal immune responses to pathogens are known to be IL-2 dependent. Much of the information on this topic has come from studies on viruses that induce acute infection and are then rapidly eliminated, such as lymphocytic choriomeningitis virus (LCMV) (65-67). Here, CD8 T cell activation by DC causes T cells to rapidly synthesize IL-2 and express CD25. With their upregulated high-affinity IL-2R, the T cells then undergo strong signalling by IL-2, leading the cells to mount an efficient primary immune response. This response of T cells has 3 main components, namely: 1) extensive expansion and generation of effector cells leading to rapid elimination of the virus (in less than a week), 2) subsequent death of most of the now-redundant effector cells, and 3) differentiation of the remaining cells into long-lived resting memory cells, i.e., cells that mount an enhanced response when the host re-encounters the virus concerned. Notably, in terms of cell expansion, the bulk of

this response is IL-2 independent. Thus, studies with CD25 ko CD8 T cells have shown that these cells can mount a near-normal primary response to virus with only mild impairment of effector and memory cell formation (67). Although CD25-deficient CD8 T cells can receive low level IL-2 signals via dimeric CD122-γc receptors, essentially similar findings are seen with CD8 T cells lacking CD122 (74) or γc (75), implying that contact with IL-2 or other γc cytokines is largely unnecessary for the primary response. Indeed, T cell expansion is thought to be driven largely by TCR signalling *per se*, induced by T-DC interaction and aided by contact with IL-12 and type I IFN. Nevertheless, IL-2 does play a decisive role in the programming and function of memory CD8 T cells. Thus, despite effective primary responses, secondary responses by CD25-deficient CD8 T cells are poor (67).

The main conclusion from these studies on LCMV and other acute viral infections is that strong signalling of CD8 T cells via IL-2 is crucial only for the optimal function of memory T cells. In normal mice, the virus-specific CD8 T cells found at the height of the primary response comprise a mixture of short-lived effector cells (SLECs) and memory-precursor effector cells (MPECs). These lineages are formed early in the response and reflect, among others, the intensity of initial IL-2 signalling (73,76,77). Typical SLECs arise from precursors that receive strong initial TCR/CD28 signals which elicit a sequence of high CD25-dependent IL-2 production and signalling followed by rapid proliferation and differentiation into effector cells; these cells eliminate the virus and then die. By contrast, MPECs receive a lower-level IL-2 signal that drives slower but more extended proliferation followed by restricted effector formation but efficient differentiation into long-lived memory cells. Why some of the early responding T cells receive stronger IL-2 signals than others is unclear. One possibility is that MPECs arise from late-comer T cells that migrate to the site of infection from distal sites in the body (78-80). An alternative idea is that MPECs are formed as the result of asymmetric cell division, perhaps leading to unequal distribution of cytokine receptors in the daughter cells (77). Whatever the explanation, it has to be emphasized that the division of the responding T cells into SLECs and MPECs is by no means clearcut. In particular, the proportion of effector T cells in MPECs is only slightly less than in SLECs; moreover, in terms of cytolytic function, the efficacy of the effectors in the two populations is very similar. A key difference, however, is that MPECs have a greater capacity for self-renewal than SLECs. As considered later, this property of "stemness" for MPECs appears to be important for effective cancer immunotherapy.

The immune response of CD4 T cells closely parallels the response of CD8 T cells and also involves production and consumption of IL-2 (81). This topic is mentioned only in passing, however, because, unlike CD8 T cells, CD4 T cells seem to play only a subsidiary role in anti-tumor responses. Moreover, CD4 T cells have no discernible influence in the above model of acute LCMV infection (65). Nevertheless, in certain situations CD4 T cells do play an important role as T "helper" cells for CD8 T cells, i.e. by providing IL-2 for those CD8 T cells that fail to synthesize IL-2. Most of these "helpless" CD8 T cells have low affinity for Ag and/or display exhaustion, as discussed below.

The above data refer to acute viral responses. Since cancer is a chronic disease, it is obviously relevant to consider the immune response to persistent infections. Here, much useful information has come from studies on T cells responding to an LCMV variant, clone 13 (LCMV-Cl13), with a higher replicative rate (82-84). With this virus the immune response is protracted and leads to elimination of virus only after about 2 months. As now well documented in other chronic infections — and also in anti-tumor responses — the reactivity of both CD8 and CD4 T cells during LCMV-Cl13 infection is severely impeded by the cell-

surface expression of a series of inhibitory receptors, notably PD-1, which lead to a process of T-cell "exhaustion." As PD-1 upregulation is driven by TCR triggering, PD-1 is also expressed on T cells during acute LCMV infection but is rapidly down-regulated when the virus is cleared. For LCMV-Cl13, the persistent high viral load prolongs expression of PD-1 and other inhibitory markers and blocks T-cell division, thereby limiting expansion of the responding CD8 T cells to substantially less (<10%) than the numbers generated during acute infection.

As with other chronic infections, there is much interest in devising methods to rejuvenate the exhausted T cells in LCMV-Cl13 infection. Here, early studies showed that, in marked contrast to acute infection, the protracted response to virus in chronic LCMV infection is heavily dependent on CD4 Th function (85). This finding raised the question whether infusion of IL-2 would overcome immune exhaustion and hasten viral clearance. Indeed, this was found to be the case, although reducing virus levels by IL-2 injection was most effective when combined with mAb treatment to counter the inhibition mediated by PD-1 expression. These and other findings led to the conclusion that the immune inhibition seen during chronic infection is largely the result of impaired IL-2 production, affecting both CD8 effector T cells and CD4 Th cells, and that infusion of exogenous IL-2 can overcome this deficit (82-84). Nevertheless, endogenous IL-2 production during chronic infection, though low, is clearly significant. Thus, during LCMV-Cl13 infection the residual IL-2 synthesis by responding CD8 T cells is able to sustain survival of a subset of stem-like PD-1^{lo} precursors that continuously spawn PD-1^{hi} effector CD8 T cells until the virus is eventually eliminated (84).

It should be noted that IL-2 treatment for chronic viral infections is associated with significant side effects. Thus, many studies in mice demonstrated that IL-2 injection is harmful in high doses and leads to severe pulmonary oedema and systemic signs of toxicity (86). Likewise, treatment with anti-PD-1 mAb (checkpoint blockade) during chronic LCMV infection had to be delayed because early treatment proved lethal, a reflection of the intense inflammation caused by an unregulated immune response. As discussed below, however, the beneficial results of treating chronic viral infections in mice with a combination of checkpoint blockade and IL-2 infusion are of obvious clinical relevance and this approach is receiving increasing attention as a potential method for boosting T-cell anti-tumour responses.

THE LEAP TO CANCER IMMUNOTHERAPY

Although the knowledge gained from studying immunity to pathogens has been invaluable, it has to be stressed that T-cell reactivity to foreign microorganisms is far removed from their response to autologous tumor cells. Thus, unlike the overtly strong immune response to pathogens, T-cell responses to tumor Ags are generally very weak, being directed to the neoantigens created by mutations or other perturbations of self proteins (87); normal self components are generally ignored because of stringent self tolerance induction by thymic selection and suppression by Tregs (88). Also, as mentioned earlier, tumours are constantly evolving to evade the immune system by reducing their immunogenicity (11). In addition, there is the problem that anti-tumor responses occur in the absence of the powerful adjuvants that amplify responses to pathogens. On this point, death of tumor cells, e.g. as the result of chemotherapy, does lead to the release of various self adjuvants, including mitochondrial DNA (89,90). However, the efficacy of these autologous adjuvants appears to be quite weak. A final issue is that presentation of tumor Ags to T cells is intrinsically inefficient. Thus, whereas Ag loading of APC by pathogens results from direct infection of these cells, presentation of tumor

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Ags involves a complex process of ingestion of tumor cell debris by APC followed by cross-presentation of tumor-derived peptides onto MHC molecules (87).

For the above reasons, unmanipulated immune responses to macroscopic cancers are generally poor and often barely detectable. Hence, enormous efforts have been made over the last 100 years to boost the efficacy of anti-tumor responses. As alluded to earlier, the major breakthrough came when it was discovered that markers such as PD-1 expressed on exhausted T cells in chronic virus infections and tumor infiltrates have a direct inhibitory function. Here, as in viral infections, the key finding was that infusion of mAb specific for these molecules could alleviate exhaustion and lead to efficient anti-tumor responses (91). However, despite spectacular initial success, immune checkpoint blockade (ICB) has proved to be effective in only about 20% of cancer patients, with the best outcomes largely limited to patients with overtly immunogenic malignancies, such as melanoma and lung cancer (92). For this reason, concerted attempts are now being made to further improve anti-tumor responses by combining ICB with other approaches to alleviate T cell exhaustion. Since T cells are especially sensitive to γc cytokines, a number of these cytokines, including IL-2, IL-7, IL-15, and IL-21, are being studied for their capacity to augment the anti-tumor efficacy of ICB (93,94). As discussed below, however, IL-2 is receiving particular attention.

ANTI-TUMOR IMMUNITY: THE TANTALISING PROMISE OF IL-2 THERAPY

Since the initial discovery of IL-2 as a T-cell growth factor (95), it has long been recognized that IL-2 has great potential for augmenting anti-tumor responses. Indeed, early results of IL-2 therapy were impressive and led to dramatic tumor remission in a small proportion of patients, followed by approval by the U.S. Food and Drug Administration of recombinant human IL-2 (hIL-2; aldesleukin) for treatment of metastatic melanoma and renal cell carcinoma (93,96). The major problem, however, was that as in animal models IL-2 treatment of cancer patients is highly toxic. Largely for this reason, the use of high-dose IL-2 for cancer treatment has been discontinued; currently, treatment with IL-2 is limited to use of low doses of IL-2 to expand Treg numbers for treatment of autoimmune diseases.

Reservation about IL-2 therapy for cancer treatment was compounded by the discovery that IL-2 is an obligatory growth factor for Tregs. Hence, it was feared that, in addition to expanding effector T cells, IL-2 treatment may promote an influx of Tregs into tumors and thus counter the local anti-tumor function of effector T cells. However, interest in IL-2 therapy was reawakened by the discovery in mice that interaction of IL-2 with a particular anti-IL-2 mAb, S4B6, inhibited the capacity of IL-2 to stimulate Tregs (43). As mentioned earlier, injecting mice with this anti-IL-2 mAb led to formation of complexes with endogenous IL-2, thus redirecting IL-2 away from Tregs to effector T cells; these IL-2/mAb complexes then became stimulatory for effector T cells, notably IL-2-sensitive ($CD122^{hi}$) memory CD8 T cells. Similarly, stimulation and proliferation of memory CD8 T cells occurred following injection of mice with preformed complexes of S4B6 mAb bound to exogenous IL-2 (43). The surprising finding, however, was that T-cell stimulation by these complexes was largely selective for $CD122^{hi}$ cells, i.e. memory CD8 T cells and also NK cells, and was only weakly stimulatory for $CD25^{hi}$ cells, namely Tregs. This observation posed the question whether S4B6 mAb blocked the site on IL-2 that interacts with CD25. In fact, direct support for this idea came from blocking studies with CD25 mAb (97), later confirmed by

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X-ray crystallography (98). Another key observation was that injection of IL-2 in the form of IL-2/mAb complexes was much less toxic than with injection of normal IL-2, especially for pulmonary oedema; this observation was explained by the demonstration of CD25 expression on lung endothelial cells (86).

The major clinically-relevant finding in the above studies was that treatment with IL-2/S4B6 mAb complexes proved to be highly effective at impairing the growth of several different tumors in mice (86). This observation led to the development of anti-hIL-2 mAbs, such as NARA1 (99) and TCB2 (100), capable of selectively stimulating CD8 T cells and NK cells over Tregs and achieving potent anti-tumor effects in several mouse cancer models (99-102). Like S4B6, these mAbs block the IL-2-binding site of IL-2 that interacts with CD25. On this point, it should be noted than some IL-2 mAbs, notably JES6-1, bind to other sites on IL-2 and can be used to form IL-2/JES6-1 mAb complexes that preferentially stimulate Tregs rather than CD8 T cells (43). Thus, injection of IL-2/JES6-1 mAb complexes can be used to expand Treg numbers *in vivo* and thereby provide a treatment for autoimmune disease and also to prevent allograft rejection (103-106).

For cancer immunotherapy, the promising preclinical results observed with IL-2/mAb complexes stimulated widespread interest in developing other methods for modifying IL-2 to selectively stimulate CD8 T cells rather than Tregs. Here, a number of groups have used engineering to alter the structure of IL-2 so as to block interaction with CD25 (107,108), or enhance IL-2 binding to CD122 (109), or both (110). Like IL-2/mAb complexes, in mice these engineered muteins lead to preferential stimulation of CD8 T cells *in vivo* and efficient anti-tumor activity, especially when supplemented with ICB. In some cases, the short half-life of these IL-2 variants has been increased by attachment to a larger molecule, albumin or polyethylene glycol (PEG), before injection (110). Similar enhanced IL-2 half-life and efficient anti-tumor activity in preclinical models has been seen with other enlarged IL-2 variants, namely NARA1leukin, a fused complex of hIL-2 and NARA1 mAb (111); ALKS 4230, a fused complex of hIL-2 and CD25 (112); and NKTR-214, where hIL-2 is PEGylated (113).

Despite many ongoing clinical trials, none of the above modified IL-2 products has yet gained approval for clinical use. In addition, in 2022, phase 3 trials of NKTR-214 plus ICB with anti-PD-1 mAb (nivolumab) for treatment of metastatic melanoma gave negative results and has led to discontinuation of NKTR-214 development. Also, the toxicity of NKTR-214 proved to be as prominent as with unmodified hIL-2 (aldesleukin) (114). These unexpected clinical findings have cast a shadow over the field of IL-2 therapy and led to re-examination of the results obtained in preclinical models. However, these negative results might be inherent to NKTR-214 because of imprecise PEGylation of IL-2 (115,116). Nevertheless, as outlined below the central assumption that IL-2 therapy hinges on nullifying Treg activity is now being challenged.

In the above studies the prevailing view is that successful IL-2 therapy depends upon sub-opimal IL-2 signalling, i.e., CD25-independent signalling delivered via the CD122-γc dimeric IL-2R, thereby allowing CD8 T cell expansion but preventing both toxicity and Treg expansion. Surprisingly, however, recent work on chronic LCMV infection in mice has questioned this viewpoint (117). Thus, investigating the capacity of anti-PD-1 mAb plus IL-2 combination therapy to augment virus elimination demonstrated that the effector CD8 T cells had to receive strong IL-2 signals to be effective. Here, the striking finding was that synergy between the effects of anti-PD-1 mAb and IL-2 occurred only with normal IL-2 and not with an IL-2 variant (IL-2v) that failed to bind CD25, indicating that IL-2 signalling via the CD122-

γc dimeric IL-2R was ineffective. The implication therefore is that IL-2 therapy should aim to target strong IL-2 signalling of CD8 T effector cells yet avoid parallel stimulation of Tregs and lung endothelial cells. An ingenious solution to achieving this requirement has come from physically linking anti-PD-1 mAb to an IL-2v that lacks CD25-binding ability (118). Here, the PD-1 molecule itself acts as a CD25 surrogate and focusses the mAb-bound IL-2 directly onto CD122-γc dimeric IL-2R *in cis*, thereby eliciting strong IL-2 signalling exclusively in the exhausted T cells. When used at a low dose, the PD1-IL2v construct is thus Ag specific and avoids stimulation of bystander CD8 T cells, the latter being an unwanted side effect of other forms of IL-2 therapy. Notably, when supplemented with a mAb specific for PD-L1, the ligand for PD-1, the PD1-IL2v construct led to regression of an ICB-resistant pancreatic tumor in mice (119). Comparable success has been seen with another similar PD1-IL2v construct (120); here, the attached IL-2v has reduced binding affinity for both CD25 and CD122.

In light of these recent findings, interest in IL-2 therapy is now shifting to methods that mildly reduce, rather than abolish, strong signalling of effector CD8 T cells, i.e., by preserving IL-2 binding to CD25 but reducing IL-2 interaction with CD122 (121) or γc (122). As expected, treatment with these CD25-biased IL-2 muteins does lead to an increase in Treg numbers. Surprisingly, however, the increase in Tregs seen with injection of CD25-biased IL-2 mutein was much less within tumors than in blood and was associated with an enhanced influx of effector CD8 T cells into tumors, a decrease in the Treg/CD8 T-cell ratio of tumor-infiltrating lymphocytes (TILs), and stronger anti-tumor activity, relative to a control mutein with no CD25 binding activity (121). Strikingly, toxicity with the CD25-biased IL-2 mutein was markedly less than with the control mutein.

CONCLUDING COMMENTS

As summarised above, and reviewed elsewhere (115,123,124), much progress has been made in optimizing IL-2 therapy for cancer treatment. The disappointment, however, is that currently the data are limited almost entirely to preclinical models. Also, although there is general agreement that IL-2 has to be modified for successful clinical use, opinion is split on how best to do this. Until recently, the goal has been to reduce the toxic effects of IL-2 by directing signalling to the intermediate-affinity CD122-γc dimeric IL-2R, thereby selectively stimulating effector CD8 T cells and NK cells but not Tregs. As mentioned above, this has been achieved by reducing IL-2 interaction with CD25 by various approaches, including complexing with certain mAbs, fusion to soluble CD25, selective PEGylation, and preparation of muteins with no or minimal CD25 binding activity. As a whole, these approaches have been a success in mouse models, leading to good selective expansion of effector CD8 T cells with low Treg stimulation, effective anti-tumor responses, and limited toxicity. Whether this approach of impairing strong CD25-mediated IL-2 signalling will work clinically, however, is still unclear and the failure of the PEGylated IL-2 product, NKTR-214, in a large, well-controlled clinical trial was clearly a major disappointment. Nevertheless, it should be cautioned that the sites of PEGylation of IL-2 on NKTR-214 were approximate rather than strictly confined to the CD25 binding region. Hence, there is still room for optimism that better clinical success will be seen with other, more site-specific IL-2 products.

Despite dangerous toxicity, high-dose treatment with unmodified IL-2 has the great advantage of eliciting potent effector CD8 T cell responses. Hence, it was surprising that in mice even better immune responses occurred with a partial IL-2 agonist, i.e., a CD25-biased

IL-2 mutein with reduced binding to γc but unaltered binding to CD25 (122). Moreover, relative to unmodified IL-2 the improved anti-tumor activity seen with this weaker IL-2 mutein was associated with preservation of stemness in the responding CD8 T cells followed by memory cell generation after tumor rejection. This finding paved the way for developing other similar muteins (121). Here, it was reassuring that toxicity with the CD25-biased IL-2 mutein was minimal. The surprising finding, however, was that treatment with a control IL-2 mutein that had no discernible CD25 binding and failed to stimulate Tregs was highly toxic and caused severe pulmonary oedema in mice (121). This unexpected recent observation clearly focuses attention on the pathogenesis of IL-2 toxicity. As mentioned earlier, studies in mice demonstrated that CD25 is expressed on lung endothelium and that IL-2 from activated T cells binds to IL-2R on endothelial cells and elicits capillary leak syndrome (86); hence, toxicity after IL-2 injection is attributed largely to direct action of IL-2 on lung endothelium. However, there is also evidence that the inflammation elicited by IL-2 injection can cause Treg dysfunction, thereby leading to generalized T cell activation and bystander damage to the gut (125). In this latter study, the toxic effects of IL-2 were enhanced by Treg depletion and lessened by treatment with a drug to improve Treg function; notably, the experiments were conducted on mice with a humanized immune system.

In light of these findings, one can make the case that avoiding toxicity during IL-2 therapy may hinge on preserving significant Treg function. On this point, IL-2 muteins and IL-2/mAb complexes with reduced CD25-binding activity generally have some capacity to expand Tregs, in addition to effector CD8 T cells. Hence, it will be of interest to see whether the IL-2 products with residual Treg-stimulating function show only limited toxicity in clinical trials. Making firm predictions on this topic is difficult, however, because unfortunately the role of Tregs in IL-2 toxicity in mice is still far from clear. Thus, the severe toxicity seen above with an IL-2 mutein devoid of CD25 binding activity (121) was not seen in two other studies (108,110), indicating that protection against IL-2 toxicity does not necessarily depend on Treg expansion. Moreover, toxicity occurred in mice after injection of high doses of IL-2/JES6-1 mAb complexes, i.e. a situation where Treg numbers are increased (86). Clearly, obtaining clearcut clinical data on this important issue is essential.

Leaving aside the issue of toxicity, it is intriguing (and confusing) that, in the various pre-clinical models described above, impressive T-cell anti-tumor responses have been seen irrespective of whether IL-2 interaction with CD25 is intact, partially inhibited or completely blocked. This finding is perhaps not surprising when it is borne in mind that CD25 expression on T cells varies according to their stage of differentiation, being maximal on effector cells but low on exhausted cells and also memory cells. Here, it is of interest that the capacity of IL-2/S4B6 complexes to expand effector CD8 T cells is quite poor soon after vaccination, i.e, at a stage when the cells are $CD25^{hi}$, but improves at later stages when the cells downregulate CD25 along with upregulation of CD122 (126). This finding raises the question whether $CD25^{+}$ effector CD8 T cells can be expanded with IL-2/JES6-1 complexes, i.e., in parallel with Tregs. Under defined conditions, treatment with IL-2/JES6-1 complexes can indeed promote anti-tumor responses (127). This approach is problematic, however, because the concomitant Treg expansion may absorb IL-2 and thereby impede effector T cell expansion. Because of this complexity, for any given tumor the efficacy of the IL-2 therapeutic agent used for treatment may hinge upon many different factors, including tumor size, the cell types and differentiation state of TILs, and the timing of IL-2 injection relative to ICB. Clearly, much more investigation will be needed to clarify this important issue.

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Currently, the new approach of preparing PD1-IL2v fusion products for IL-2 therapy has great appeal, in particular because it targets IL-2 directly and selectively to exhausted CD8 effector T cells (116,118-120). By focussing stimulation exclusively to Ag-specific T cells, this method could also be used to potentiate the anti-tumor activity of other γc cytokines, notably IL-15; indeed, impressive results have recently been reported for fusion proteins consisting of anti-PD-1 mAb and IL-15 (128,129). For tumor therapy, a final key advantage of tethering cytokines to T cells via anti-PD-1 mAb is that the cytokines might be effective in very low concentrations, thereby avoiding toxicity. In fact, in low doses a fusion product with unmodified normal IL-2 (PD1-IL2) might be even more effective than PD1-IL2v?

Despite the paucity of clinical data, recent progress with IL-2 therapy to augment checkpoint blockade is most impressive, and many clinical trials are in progress (116). The toxicity of IL-2 therapy is still a problem but the development of new techniques to focus IL-2 selectively to tumor-specific T cells may solve this issue. If these new IL-2 products can reliably display potent anti-tumor activity at low concentrations, eventual clinical success seems highly likely.

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