The role of interleukin-2 during homeostasis and activation of the immune system

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Abstract | Interleukin-2 (IL-2) signals influence various lymphocyte subsets during differentiation, immune responses and homeostasis. As discussed in this Review, stimulation with IL-2 is crucial for the maintenance of regulatory T (T_{Reg}) cells and for the differentiation of $CD4^+$ T cells into defined effector T cell subsets following antigen-mediated activation. For $CD8^+$ T cells, IL-2 signals optimize both effector T cell generation and differentiation into memory cells. IL-2 is presented in soluble form or bound to dendritic cells and the extracellular matrix. Use of IL-2 — either alone or in complex with particular neutralizing IL-2-specific antibodies — can amplify $CD8^+$ T cell responses or induce the expansion of the T_{Reg} cell population, thus favouring either immune stimulation or suppression.

Central memory T cells
Antigen-experienced resting T cells that express cell-surface receptors that are required for homing to secondary lymphoid organs. These cells are generally long-lived and can serve as the precursors for effector T cells during recall responses.

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Interleukin-2 (IL-2) was discovered more than 30 years ago through its *in vitro* T cell-stimulatory capacity[1,2]. Subsequent studies using relevant animal models, as well as IL-2- or IL-2 receptor (IL-2R)-deficient mice, have highlighted the crucial role of IL-2 not only for protective immunity but especially for peripheral immune tolerance mediated by $CD4^+$ regulatory T (T_{Reg}) cells[3].

In this Review, we discuss recent findings on the role of IL-2 in T cell biology, focusing on the decisive influence of IL-2 on the differentiation and fate of $CD4^+$ and $CD8^+$ T cells engaged in an immune response, the distribution of IL-2R subunits on lymphoid and non-lymphoid cells and the significance of T cell contact with soluble versus cell-associated IL-2. We also summarize recent insights into the homeostasis of IL-2 and describe how modulation of IL-2 can be used to selectively target IL-2 to particular T cell subsets.

IL-2 and the IL-2 receptor system

During steady-state conditions, IL-2 is produced mainly by $CD4^+$ T helper (T_H) cells in secondary lymphoid organs (FIG. 1) and, to a lesser extent, by $CD8^+$ T cells, natural killer (NK) cells and natural killer T (NKT) cells[3,4]. Under certain conditions, IL-2 can also be synthesized in small amounts by activated dendritic cells (DCs) and mast cells[5,6]. IL-2 production by $CD4^+$ and $CD8^+$ T cells is strongly induced following activation by antigen, although IL-2 synthesis by $CD8^+$ T cells is comparatively weak and the responses of these cells often require help from $CD4^+$ T cells (see below). IL-2 production is regulated by several mechanisms, including silencing of the *Il2* gene by the transcription factor B lymphocyte-induced maturation protein 1 (BLIMP1; also known as PRDM1)[7]. BLIMP1 is activated by IL-2 and in turn represses IL-2 production, thus providing a negative feedback mechanism. Effector T cells that have differentiated into long-lived memory cells — especially central memory T cells, which can home to lymph nodes — express low levels of BLIMP1 and thus retain (or regain) the ability to produce IL-2 (REFS 8,9). However, prolonged exposure of T cells to antigen leads to the upregulation of BLIMP1 expression in these cells, which in turn progressively reduces the capacity of these cells to secrete IL-2 as they become terminally differentiated or exhausted[8–10]. Such persisting stimulation of T cells through their T cell receptor (TCR) by antigens (including self antigens), together with IL-2 signals, can also induce the expression of the death receptor FAS (also known as CD95) and FAS ligand (also known as CD95L) on the T cells, which can promote the apoptosis of these cells via activation-induced cell death[11,12].

IL-2 acts on cells expressing either the high-affinity trimeric IL-2R or the low-affinity dimeric IL-2R[13] (FIG. 2; TABLE 1). The dimeric IL-2R consists of CD122 (also known as IL-2Rβ) and the common cytokine receptor γ-chain (γ_c; also known as CD132); owing to its weak affinity for IL-2 (dissociation constant (K_d) ≈ 10^{-9} M), this receptor needs to be expressed at a relatively high level for IL-2 responsiveness. Expression of the dimeric IL-2R is almost undetectable on naive $CD4^+$ T cells, but

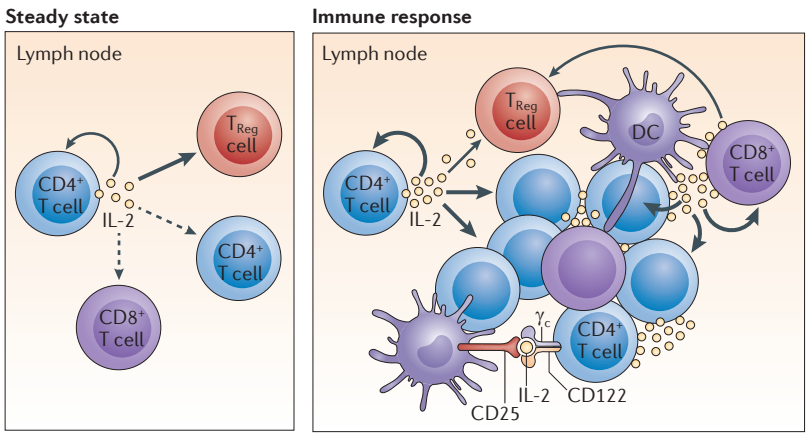


Figure 1 | **IL-2 homeostasis in steady-state conditions and during an immune response.** Under steady-state resting conditions, interleukin-2 (IL-2) is mainly produced by $CD4^+$ T cells that are activated by foreign- and self-peptide–MHC class II complexes on dendritic cells (DCs; not shown) in secondary lymphoid organs, such as the lymph nodes. The secreted IL-2 is then consumed at the same site by $CD25^+$ cells, notably regulatory T (T_{Reg}) cells, and also by adjacent activated $CD4^+$ and $CD8^+$ T cells. During an immune response, activated DCs home to the draining lymph nodes, where activated $CD4^+$ and $CD8^+$ T cells produce large amounts of IL-2. IL-2 is then consumed by $CD25^+$ effector T cells and T_{Reg} cells. Activated DCs express CD25 on their cell surface; such CD25 molecules might bind to either T cell- or DC-derived IL-2 for *trans*-presentation to neighbouring $CD25^{low}$ effector $CD4^+$ T cells (and perhaps also $CD8^+$ T cells) early during T cell activation, before the T cells express high levels of CD25. γ_c, common cytokine receptor γ-chain.

this receptor is present at low but significant levels on naive $CD8^+$ T cells and memory $CD4^+$ T cells and at high levels on memory $CD8^+$ T cells and NK cells. Cells expressing high levels of the dimeric IL-2R are sensitive to exogenously administered IL-2, but these cells are presumably unresponsive to the low physiological levels of IL-2 found *in vivo*[14].

CD25 (also known as IL-2Rα) is the third chain of the trimeric IL-2R; it does not appear to participate in signalling but, instead, functions to increase the affinity of the IL-2R for its ligand by 10–100-fold ($K_d \approx 10^{-11}$ M)[13]. High levels of the trimeric IL-2R are transiently expressed by $CD4^+$ and $CD8^+$ T cells following TCR activation. Unlike other T cell subsets, T_{Reg} cells constitutively express high levels of CD25, with intermediate levels of CD122 and γ_c. The expression of CD25 by T cells is regulated by TCR stimulation and also by contact with IL-2, the latter providing a positive feedback loop that involves the binding of signal transducer and activator of transcription 5 (STAT5) to the *Cd25* gene locus[3]. Interestingly, recent data on the structure of human IL-2 complexed with the trimeric IL-2R suggested that IL-2 initially binds to CD25 ($K_d \approx 10^{-8}$ M), resulting in a structural change in IL-2, which is followed by the recruitment of CD122 and finally γ_c[15]. However, even in the absence of CD25, IL-2 can bind with low but significant affinity to the dimeric IL-2R and lead to signal transduction, provided that the responding cell expresses high levels of the dimeric IL-2R[14]. Following receptor binding, the quaternary IL-2–IL-2R complex is rapidly internalized, and IL-2, CD122 and γ_c are subsequently degraded; by contrast, CD25 can be recycled to the cell surface[3].

Activation-induced cell death
A process by which activated T cells undergo cell death through the engagement of death receptors (such as FAS or the TNF receptor) or the production of reactive oxygen species.

CD122 and γ_c are the signalling components of the IL-2R, and both of these subunits contain signalling motifs in their cytoplasmic tails. Signal transduction occurs via several intracellular pathways, including the Janus kinase (JAK)–STAT pathway, the phosphoinositide 3-kinase (PI3K)–AKT pathway and the mitogen-activated protein kinase (MAPK) pathway[3,13,16] (FIG. 2). γ_c confers responsiveness not only to IL-2, but also to IL-4, IL-7, IL-9, IL-15 and IL-21. Similarly to IL-2-mediated responses, responses to IL-15 are controlled by heterodimers of CD122 and γ_c. However, unlike most cytokines that signal through γ_c, IL-15 is normally presented in *trans* in cell-associated form by IL-15Rα expressed by DCs[16,17]. The *trans*-presentation of IL-2 is discussed below.

IL-2 optimizes $CD8^+$ T cell responses

Numerous studies have shown that IL-2 signals affect $CD8^+$ T cells during all stages of an immune response, including primary expansion, contraction, memory generation and secondary expansion[18] (FIG. 3). Following acute viral infection, the primary expansion of the $CD8^+$ T cell population is about threefold lower in *Il2*$^{-/-}$ mice than in IL-2-competent controls, and this results in less-efficient viral clearance by virus-specific cytotoxic T lymphocytes[19,20]. Similar results were obtained using TCR-transgenic *Cd25*$^{-/-}$ $CD8^+$ T cells activated by antigens encoded by recombinant bacteria or viruses[21,22]. The reduced response of *Cd25*$^{-/-}$ $CD8^+$ T cells is presumably a reflection of these cells receiving suboptimal IL-2 signals owing to their failure to express the trimeric IL-2R during primary expansion. Comparable findings were derived from bone marrow chimeric mice that contained a mixture of *Cd25*$^{-/-}$ and wild-type $CD8^+$ T cells; following an acute viral infection, the expansion of the antigen-specific $CD8^+$ T cell population in the chimeric mice was twofold to fivefold lower for cells of *Cd25*$^{-/-}$ origin than for their wild-type counterparts[23,24]. Using the same chimeric system, the secondary expansion of the antigen-specific *Cd25*$^{-/-}$ $CD8^+$ T cell population was even more compromised, leading to about a 15-fold reduction in cell counts compared with wild-type cells. Notably, this profound defect in the expansion of the antigen-specific *Cd25*$^{-/-}$ $CD8^+$ T cell population following secondary antigen challenge was shown to reflect the lack of optimal IL-2 signals during the primary expansion[23].

These studies raise the question of whether IL-2 has to be secreted by antigen-specific $CD8^+$ T cells or by other leukocytes. Although it is widely assumed that $CD8^+$ T cell responses are controlled by help from $CD4^+$ T cells, a recent study showed that antigen-specific $CD8^+$ T cells relied on autocrine IL-2 production rather than being dependent on paracrine IL-2 signals from $CD4^+$ T cells or DCs[25]. Thus, the IL-2-competent (but not *Il2*$^{-/-}$) antigen-specific $CD8^+$ T cell population underwent efficient primary and secondary expansion following activation by antigen-presenting DCs that had been 'licensed' by antigen-specific $CD4^+$ T cells via CD40–CD40 ligand interactions, even if the DCs or antigen-specific $CD4^+$ T cells were IL-2 deficient[25]. Nevertheless, these findings do not rule out a role for paracrine IL-2 produced by $CD4^+$ T cells under certain conditions, for example when $CD8^+$ T cell responses are too weak to elicit autocrine IL-2 production.

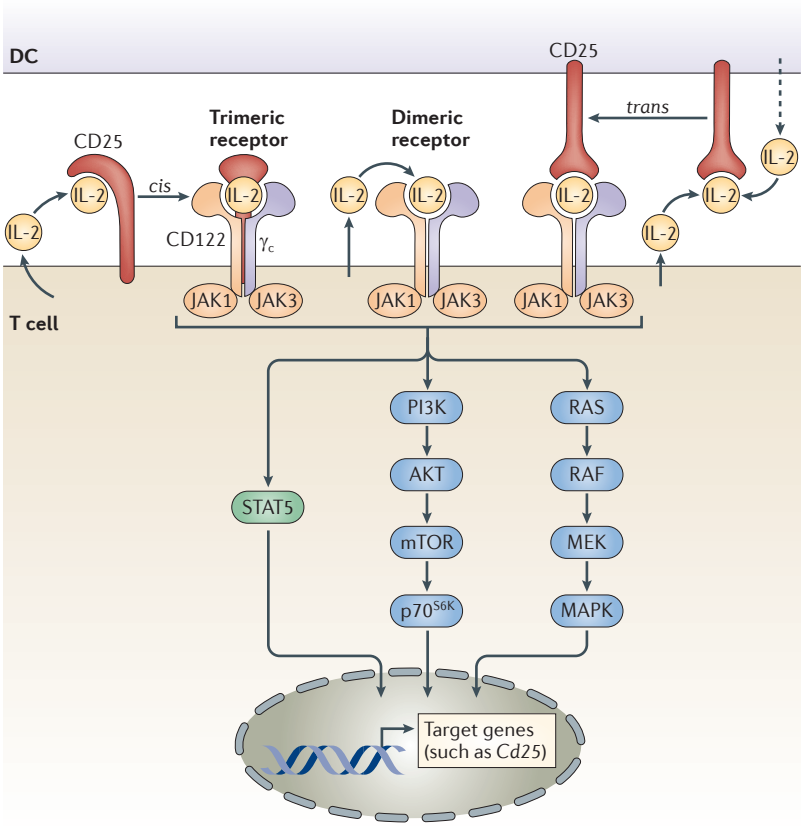


Figure 2 | **The IL-2 receptor system and IL-2 signalling.** Interleukin-2 (IL-2), a 15 kDa four-α-helix bundle cytokine, is secreted as a soluble molecule by activated T cells and to a lesser extent by activated dendritic cells (DCs). IL-2 can bind to the IL-2 receptor (IL-2R) subunit CD25 (also known as IL-2Rα) with a dissociation constant (K_d) of ~10^{-8} M, and this interaction induces a distinct conformational change in IL-2 that increases its affinity for the IL-2R subunit CD122 (also known as IL-2Rβ). Subsequently, the IL-2–CD25 dimer recruits CD122 followed by the common cytokine receptor γ-chain (γ_c), another subunit of the IL-2R. This quaternary IL-2–IL-2R complex has a K_d of ~10^{-11} M. In cells that lack expression of CD25, IL-2 can associate with the dimeric IL-2R directly (with a K_d of ~10^{-9} M). Alternatively, T cell- or DC-derived IL-2 can bind to CD25 molecules expressed by DCs, and this IL-2 can then be presented in *trans* to neighbouring T cells that express CD122 and γ_c. On binding to CD122 and γ_c, IL-2 induces the transcription of target genes (such as *Cd25*) through several signalling pathways, including the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway, the phosphoinositide 3-kinase (PI3K)–AKT pathway and the mitogen-activated protein kinase (MAPK) pathway. MEK, MAPK/ERK kinase; mTOR, mammalian target of rapamycin; $p70^{S6K}$, p70 S6 kinase.

The strength and duration of IL-2 signals during a primary immune response have been shown to affect the differentiation of naive CD8$^+$ T cells into either short-lived effector T cells or long-lived memory T cells[22,26–28]. It has been shown that, following initial upregulation of CD25, a subset of CD8$^+$ T cells downregulates CD25 expression at an early stage of antigen-driven primary expansion[26]. These $CD25^{low}$CD8$^+$ T cells preferentially upregulated IL-7Rα (also known as CD127) and CD62L (also known as L-selectin), a phenotype that is characteristic of central memory T cells. Accordingly, most $CD25^{low}$ antigen-specific CD8$^+$ T cells survived the T cell contraction phase and became long-lived memory CD8$^+$ T cells that could efficiently proliferate following secondary antigen challenge[26]. Conversely, the $CD25^{hi}$ antigen-specific CD8$^+$ T cells sustained CD25 expression for an additional 1–2 days following antigen priming; these cells received strong IL-2 signals, which amplified their proliferation and drove the cells to differentiate into apoptosis-prone short-lived effector CD8$^+$ T cells, thus curtailing their differentiation into memory T cells[26,27].

Collectively, these data indicate that the strength and duration of the IL-2 signal controls both the primary and the secondary expansion of the antigen-specific CD8$^+$ T cell population (FIG. 3). Suboptimal IL-2 signals during priming lead to reduced primary expansion and severely impaired secondary expansion, whereas exposure to prolonged, strong IL-2 signals during priming generates short-lived terminally differentiated effector CD8$^+$ T cells. Notably, the formation of functional memory CD8$^+$ T cells requires brief, potent IL-2 signalling during antigen presentation by licensed DCs.

The mechanisms controlling CD25 upregulation and IL-2 production by CD8$^+$ T cells are complex and involve a range of intrinsic and extrinsic factors. These factors include: the TCR affinity for peptide–MHC class I complexes and the density of these complexes on DCs; the expression of co-stimulatory and co-inhibitory receptors on the responding T cells; the influence of cytokine and chemokine receptor signals; and contact with T_{Reg} cells[29–32]. T_{Reg} cells can fine-tune CD8$^+$ T cell responses by limiting IL-2 production, thereby reducing the generation of short-lived effector CD8$^+$ T cells and favouring memory T cell generation[22,31,32]. T_{Reg} cell-mediated inhibition of IL-2 production may involve several mechanisms, including IL-2 consumption[33] and the reduction of the co-stimulatory function of DCs via cytotoxic T lymphocyte antigen 4 (CTLA4)-induced inhibition of CD80 and CD86 expression or via the removal of these molecules through CTLA4-mediated trans-endocytosis[34,35].

Although IL-2 exerts its stimulatory effects mainly on cells expressing the high-affinity trimeric IL-2R, IL-2 can also stimulate resting naive CD8$^+$ T cells via the low-affinity dimeric IL-2R. Hence, exposure of naive CD8$^+$ T cells to high concentrations of IL-2 (or IL-15) leads to vigorous proliferation of these cells, both *in vitro* and *in vivo*[36]. Such proliferation is dependent on MHC class I molecules, and thus resembles IL-7-driven 'homeostatic' expansion of naive T cell populations under lymphopenic conditions (also termed lymphopenia-induced proliferation)[29]. Unlike homeostatic expansion, which is typically slow, IL-2-induced proliferation of naive CD8$^+$ T cells is rapid and leads to the generation of effector-like T cells[36]. The ability of IL-2 to stimulate naive CD8$^+$ T cells has been recently linked to the high densities of lipid rafts containing the ganglioside GM1 that are found on these cells; these lipid rafts are upregulated following TCR engagement and cluster with CD122 (REF. 37).

To summarize, autocrine and paracrine IL-2 signals act during the immune response to potentiate the expansion and differentiation of recently activated CD8$^+$ T cells and other cells expressing the trimeric IL-2R. At high levels, IL-2 can also stimulate cells expressing the dimeric IL-2R, notably naive CD8$^+$ T cells and, as mentioned above, resting memory CD8$^+$ T cells and NK cells.

Trans-endocytosis
The process by which a tightly associated receptor–ligand complex induces invagination of the plasma membrane and internalization of the complex into the receptor-bearing cell to form a membrane-limited transport vesicle.

Table 1 | **IL-2 receptor expression on different immune and non-immune cells**

Cell type	CD25	CD122	γ_c	Refs
Thymocyte*	–/+	–/+	+	3,114
Naive T cell‡	–	–/+	+	14,22,26,58,59
Effector T cell§	+++	++	+	14,22,26,58,59
Memory T cell‖	–	+/++	+	14,26,29,59,77,111
T_{FH} cell	–	?	+	58,59
T_{Reg} cell	+++	+	+	4
Immature B cell¶	+	–	+	3,46
Mature B cell	–	–	+	3,46
NK cell	–	++	+	77
NKT cell#	–/+	–/+	+	115,116
DC**	–/+	–	+	60,61,63,64,68–70
Langerhans cell	+	?	+	62
Endothelial cell	+	+	+	77,78
Fibroblast‡‡	+	+	–	75,76

–, background expression level; +, low expression level; ++, high expression level; +++, very high expression level; γ_c, common cytokine receptor γ-chain; DC, dendritic cell; IL-2, interleukin-2; NK, natural killer; NKT, natural killer T; T_{FH}, follicular helper T; T_{Reg}, regulatory T. *CD25 is expressed by double-negative 2 (DN2) and DN3 thymocytes. There exist controversial data regarding CD122 expression on DN thymocytes[3,114]. ‡CD122 expression is undetectable on naive $CD4^+$ T cells, whereas naive $CD8^+$ T cells have low but significant levels of CD122. §CD25 and CD122 are transiently upregulated on effector T cells. ‖CD122 expression levels are low on memory $CD4^+$ T cells but high on memory $CD8^+$ T cells. ¶Pre-B cells express significant levels of CD25 but no CD122. #NKT cells from the spleen express low to intermediate levels of CD25 (K. Webster and J.S., unpublished observations). **There exist controversial data regarding CD122 expression on human DCs[68,70]; in mice, mature DC do not express CD122, although CD122 may be present on DC precursors[63,117]. ‡‡Fibroblast cell lines have been reported to express CD25 and CD122, but not γ_c.

IL-2 signals controlling $CD4^+$ T cell subsets

Homeostasis of regulatory T cells. Mice deficient in IL-2, CD25 or CD122 develop systemic autoimmunity owing to the impaired development, survival and function of T_{Reg} cells[3]. These observations highlight the crucial role of IL-2 in T_{Reg} cell homeostasis. Naturally occurring T_{Reg} cells develop in the thymus through contact with self-peptide–MHC class II complexes and express the signature transcription factor forkhead box P3 (FOXP3). In addition, induced T_{Reg} cells can be generated from $FOXP3^-$ conventional $CD4^+$ T cells following stimulation by antigens in peripheral lymphoid organs or during culture with transforming growth factor-β (TGFβ) *in vitro*. Stimulation of naturally occurring T_{Reg} cells with normal background levels of IL-2 is important for their survival and homeostasis[38–40]. Moreover, IL-2 signals upregulate CD25 expression and amplify the suppressive capacity of T_{Reg} cells by maintaining high expression levels of FOXP3 (REFS 40,41) (FIG. 4). Thus, following acute ablation of IL-2 action by injection of a neutralizing IL-2-specific monoclonal antibody, the numbers of $FOXP3^+$ T_{Reg} cells decrease, and there is also a reduction of FOXP3 expression levels in these cells[42]. Similarly, recent *in vitro* and *in vivo* studies indicate that induced T_{Reg} cells are also highly dependent on IL-2 for their generation, survival and FOXP3 expression[43,44].

T_{Reg} cells are dependent on the production of IL-2 by other cells because, unlike activated effector $CD4^+$ T cells, T_{Reg} cells cannot produce significant amounts of IL-2 either *in vitro* or *in vivo*[42,45]. As T_{Reg} cells express high levels of CD25 on their surface but cannot synthesize IL-2, these cells can deplete proximal IL-2 concentrations, at least *in vitro*[33,45,46]. Hence, T_{Reg} cells may limit systemic IL-2 levels *in vivo*[47], perhaps aided by the uptake of IL-2 by $CD25^+$ non-immune cells[46] (see below).

IL-2 and $CD4^+$ T helper cell polarization. During antigen-driven $CD4^+$ T cell differentiation, IL-2 has an important role in the generation of T_H17 cells, which secrete IL-17 and are characterized by the expression of the transcription factor retinoic acid receptor-related orphan receptor-γt (RORγt). In the absence of IL-2 signals, the numbers of T_{Reg} cells decline substantially, whereas the numbers of T_H17 cells increase, leading to enhanced susceptibility to autoimmune disease and inflammatory disorders[48]. This observation has led to the concept that IL-2 signals are crucial for the reciprocal balance between T_H17 cells and $FOXP3^+$ T_{Reg} cells[48].

The molecular signals by which IL-2 controls T_H17 cell generation have recently been clarified (FIG. 4). Exposure of activated $CD4^+$ T cells to IL-2 leads to lower cell-surface expression levels of IL-6Rβ (also known as gp130), which together with IL-6Rα forms the IL-6R. Thus, IL-2 reduces IL-6-mediated STAT3 activation, which is required for the development of $RORγt^+$ T_H17 cells[49]. In another study, IL-2-activated STAT5 inhibited the binding of STAT3 to the *Il17* locus by competing for the same sites on that gene[50]; therefore, the balance between these two transcription factors — STAT3 and STAT5 — determined the extent of T_H17 cell generation[50]. These findings apply to non-polarized $CD4^+$ T cells. However, once T_H17 cell-polarizing conditions have been established, T_{Reg} cells can promote T_H17 cell survival and function (notably IL-17 and IL-22 production), perhaps through the consumption of IL-2 by T_{Reg} cells[51,52].

IL-2 signals also influence the differentiation of effector $CD4^+$ T cells into T_H1 or T_H2 cells. Polarization to the T_H1 cell phenotype depends on IL-12 signals, which lead to STAT4-mediated induction of the T_H1 cell signature transcription factor, T-bet[53]. T-bet then induces interferon-γ (IFNγ) production, which is enhanced by the co-production of IL-2 by the responding T cells. Recent data suggest that IL-2 promotes T_H1 cell differentiation via the induction of T-bet expression and the upregulation of the IL-12Rβ2 subunit[49] (FIG. 4).

Similarly to the generation of T_H1 cells, T_H2 cell differentiation appears to require stimulation by IL-2. T_H2 cells are known to express high levels of the transcription factor GATA-binding protein 3 (GATA3) and to produce IL-4, IL-5 and IL-13 in significant amounts[53]. IL-2 signalling in T_H2 cells induces early expression of IL-4Rα and maintains the *Il4* gene locus in an accessible configuration during the later stages of T_H2 cell differentiation[54,55]. Moreover, IL-2 also influences the accessibility of the *Il13* gene locus in T_H2 cells[55] (FIG. 4).

Lipid rafts
Structures that are proposed to arise from phase separation of different plasma membrane lipids as a result of the selective coalescence of certain lipids on the basis of their physical properties. This results in the formation of distinct and stable lipid domains in membranes that might provide a platform for membrane-associated protein organization.

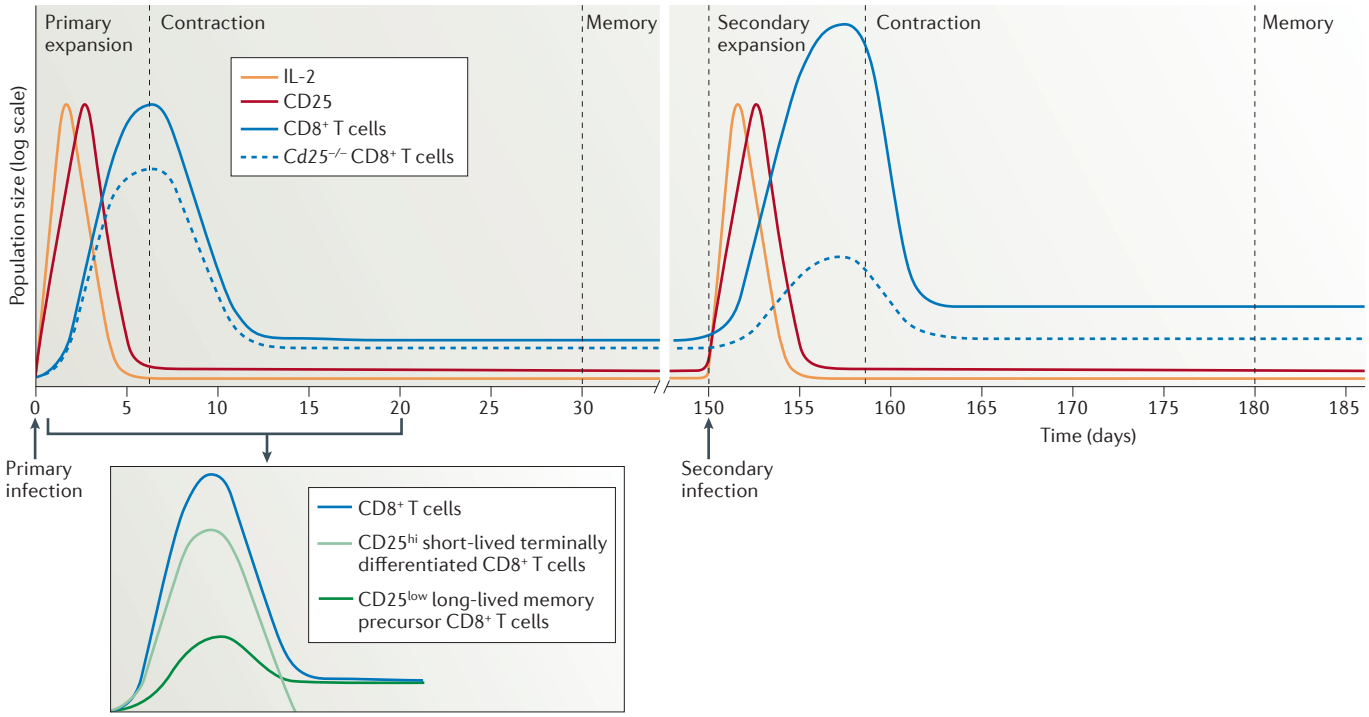


Figure 3 | **The role of IL-2 signalling in CD8⁺ T cell responses following acute infection by pathogens.** Following acute infection, interleukin-2 (IL-2) levels increase quickly in secondary lymphoid organs, and naive antigen-specific CD8⁺ T cells become activated and upregulate their expression of CD25. This is followed by the vigorous proliferation and primary expansion of this cell population. Following the clearance of the pathogen, most antigen-specific CD8⁺ T cells die by apoptosis (this is termed the contraction phase), and only a few cells survive as long-lived memory cells. Memory CD8⁺ T cells can mount a more pronounced immune response following secondary exposure to the same antigen. Antigen-specific CD8⁺ T cells that receive suboptimal IL-2 signals during priming (such as *Cd25*$^{-/-}$ CD8⁺ T cells; dashed blue line) show deficient primary and secondary expansions. Among antigen-specific CD8⁺ T cells that receive optimal IL-2 signals during priming, two effector T cell subsets can be discriminated based on their expression of CD25 (bottom panel): cells that express CD25 for extended periods (CD25^{hi} cells) are driven to form short-lived effector T cells that are destined to die by apoptosis; by contrast, cells that briefly upregulate and then rapidly downregulate CD25 (CD25^{low} cells) give rise to long-lived memory T cells.

Germinal centres
Lymphoid structures that arise in B cell follicles after immunization with, or exposure to, a T cell-dependent antigen. They are specialized for facilitating the development of high-affinity, long-lived plasma cells and memory B cells.

Class switching
A region-specific genetic recombination process that occurs in antigen-activated B cells. This recombination occurs between switch-region DNA sequences and results in a change in the class of antibody that is produced — from IgM to either IgG, IgA or IgE. This imparts flexibility to the humoral immune response and allows it to exploit the different capacities of these antibody classes to activate the appropriate downstream effector mechanisms.

Follicular helper T cells. Follicular helper T (T_{FH}) cells are a distinct subset of effector CD4⁺ T cells that reside in germinal centres and are specialized in providing help to antibody-producing B cells for antibody affinity maturation and class switching[56]. T_{FH} cells are known to produce and use IL-21, and they are characterized by high levels of expression of inducible T cell co-stimulator (ICOS), CXC-chemokine receptor 5 (CXCR5) and the transcriptional repressor B cell lymphoma 6 (BCL-6)[56,57]. Two recent studies found that strong IL-2 signals can inhibit the generation of T_{FH} cells[58,59]. Paralleling the influence of IL-2 on effector CD8⁺ T cells (see above), the relative expression levels of CD25 on CD4⁺ T cells responding to antigen were shown to correlate with the proportion of cells that differentiated into effector CD4⁺ T cells rather than T_{FH} cells. Following acute infection, most antigen-specific effector CD4⁺ T cells were shown to upregulate CD25 to high levels and to receive strong IL-2 signals, which resulted in high levels of BLIMP1 expression in these cells and their differentiation into short-lived effector CD4⁺ T cells. Conversely, a CD25^{low} subset of antigen-specific CD4⁺ T cells underwent ICOS-dependent differentiation into BCL-6^{hi}CXCR5^{hi} T_{FH} cells, presumably because these cells avoided strong IL-2 signalling[58,59] (FIG. 5). Moreover, some CD25^{low} antigen-specific effector CD4⁺ T cells became CXCR5^{hi}CCR7^{hi}T-bet^{low} precursors of central memory CD4⁺ T cells via a BCL-6- and ICOS-dependent pathway[59]. The factors that determined why some CD25^{low} antigen-specific effector CD4⁺ T cells became T_{FH} cells whereas others gave rise to central memory T cells remain unclear, although strong stimulation through the TCR combined with early ICOS signals seemed to favour the generation of T_{FH} cells[59].

Taken together, these data indicate that strong IL-2 signals drive both CD4⁺ and CD8⁺ T cells to become terminally differentiated effector T cells that are destined for rapid death following the clearance of the pathogen concerned. Conversely, low-level IL-2 signals allow responding CD4⁺ T cells to differentiate into T_{FH} cells or central memory T cells, and CD8⁺ T cells to survive as long-lived memory T cells.

IL-2 and antigen-presenting cells

In addition to its prominent expression by T_{Reg} cells and activated T cells, CD25 is expressed at low levels by DCs and Langerhans cells in mice and by human DCs[60–64]. Moreover, mature DCs produce low but significant amounts of IL-2 *in vitro* and *in vivo* following stimulation

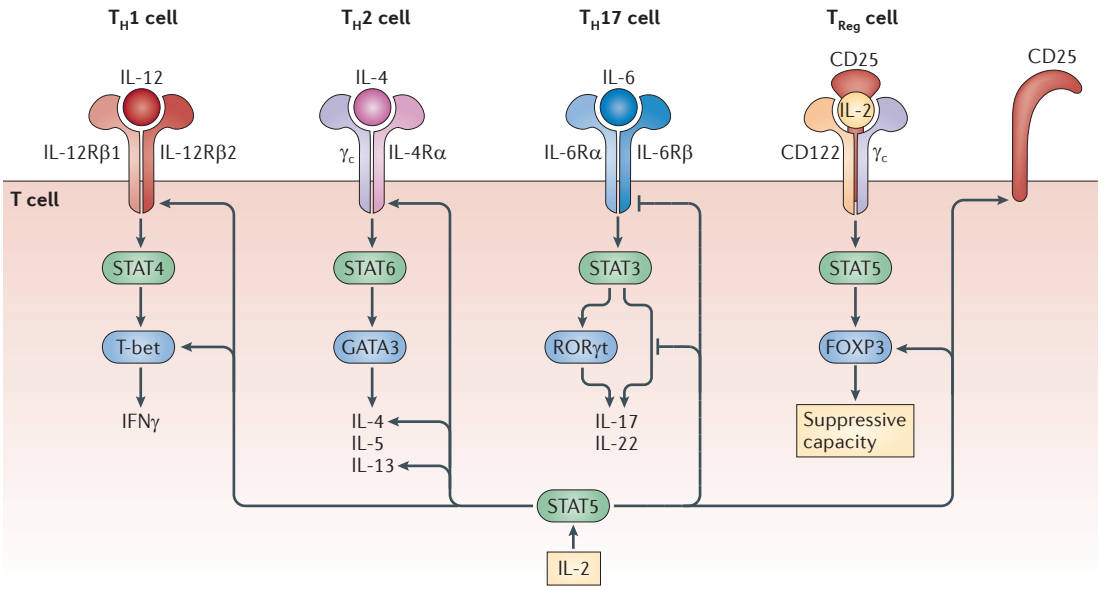


Figure 4 | **The role of IL-2 signals in the differentiation and survival of CD4⁺ T helper cell subsets and T_{Reg} cells.** Interleukin-2 (IL-2) signals act via signal transducer and activator of transcription 5 (STAT5) and influence the differentiation of T helper (T_H) cell subsets (including T_H1, T_H2 and T_H17 cells) as well as the homeostasis of regulatory T (T_{Reg}) cells. Contact with IL-2 promotes T_H1 cell generation by increasing the levels of the IL-12 receptor β2 subunit (IL-12Rβ2) and of T-bet, and this results in enhanced interferon-γ (IFNγ) production. For T_H2 cells, IL-2 signals induce the upregulation of IL-4Rα by these cells and enhance the production of typical T_H2-type cytokines, notably IL-4, IL-5 and IL-13. The generation of T_H17 cells depends on IL-6 and transforming growth factor-β signals. Unlike the formation of T_H1 and T_H2 cells, T_H17 cell differentiation is inhibited by IL-2 signals. Indeed, IL-2-mediated activation of STAT5 limits the responsiveness of these cells to IL-6 by downregulating the expression of IL-6Rβ; STAT5 also competes with STAT3 for binding to the *Il17* gene locus. T_{Reg} cells depend on IL-2 signals for their generation and homeostasis. Moreover, contact with IL-2 maintains high levels of CD25 and forkhead box P3 (FOXP3) expression by T_{Reg} cells and enhances their suppressive capacity. γ_c, common cytokine receptor γ-chain; GATA3, GATA-binding protein 3; RORγt, retinoic acid receptor-related orphan receptor-γt.

by pathogens or microbial products[5,65–67]. This ability applies to CD8α⁺ DCs, CD8α⁻ DCs and Langerhans cells from mice, as well as to human monocyte-derived and plasmacytoid DCs. As discussed below, IL-2 synthesis by these DC subsets might serve as a source of help for T cells[5].

Although the significance of CD25 expression by DCs is controversial[63], a recent study has suggested that, analogously to the *trans*-presentation of IL-15 by IL-15Rα molecules on DCs[16,17], CD25 expression on DCs can serve to present IL-2 in *trans* to antigen-specific T cells[68] (FIG. 2). Using an *in vitro* system, antigen-driven proliferation of human T cells was found to depend on CD25 expression by antigen-presenting DCs. Thus, pre-incubation of DCs with a blocking CD25-specific monoclonal antibody abrogated T cell proliferation. Moreover, the proliferation of T cells from a CD25-deficient patient in response to an antigen presented by CD25-competent DCs was inhibited by a CD25-specific antibody[68]. In this study, DCs were shown to produce IL-2 and to express CD25, especially at the DC–T cell interface. Other groups have reported that human monocyte-derived DCs upregulate their expression of CD25 when cultured with pro-inflammatory cytokines or lipopolysaccharide, and this upregulation correlates with improved DC-mediated stimulation of allogeneic CD4⁺ T cell proliferation[69,70]. By contrast, whether human DCs express CD122 is controversial[68,70].

The above findings support a model in which activated DCs express CD25 on their cell surface to bind to either T cell- or DC-derived IL-2, which they then *trans*-present to adjacent T cells (FIG. 2). As T cells produce higher amounts of IL-2 than DCs, the expression of CD25 by DCs might simply serve to trap T cell-derived IL-2 within the T cell–DC synapse, thus making a *cis* interaction with the dimeric IL-2R more likely. Alternatively, DC-derived IL-2 might be *trans*-presented through direct binding to CD25 expressed by DCs, although a problem with this model pertains to the low affinity of CD25 for IL-2 ($K_d \approx 10^{-8}$ M). Both mechanisms are possible and presumably operate very early during T cell stimulation, before the responding T cells start to express CD25. Indeed, one can envisage that the expression of CD25 by T cells might preclude the recognition of CD25-bound IL-2 on DCs. Moreover, effective *trans*-presentation of IL-2 may depend on direct cell–cell contact between T cells and DCs.

As already mentioned, a similar mechanism of *trans*-presentation also applies to IL-15. However, unlike IL-2 *trans*-presentation, the high-affinity association ($K_d \approx 10^{-11}$ M) of IL-15 with IL-15Rα occurs inside the DC that produces these molecules, and the IL-15–IL-15Rα complexes are then shuttled to the DC surface to stimulate $CD122^{hi}\gamma_c^+$ cells — such as CD8⁺ T cells and NK cells — in *trans*[16,17]. For IL-2, it should be noted that the presentation of IL-2 in a cell-bound form does not preclude other mechanisms of presentation. Indeed, IL-2

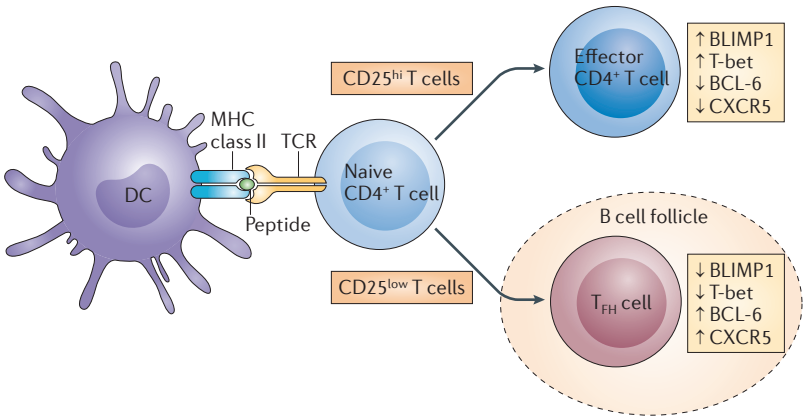


Figure 5 | **The impact of IL-2 signals on the differentiation of effector and follicular helper CD4⁺ T cells.** After making contact in secondary lymphoid organs with dendritic cells (DCs) that express peptide–MHC class II complexes together with co-stimulatory molecules (not shown), naive CD4⁺ T cells become activated and differentiate into either $CD25^{hi}$ effector CD4⁺ T cells or $CD25^{low}CD4^+$ follicular helper T (T_{FH}) cells and $CD25^{low}$ precursors of central memory CD4⁺ T cells (not shown). Effector CD4⁺ T cells express high levels of the transcription factors B lymphocyte-induced maturation protein 1 (BLIMP1) and T-bet, whereas the levels of CXC-chemokine receptor 5 (CXCR5) and the transcriptional repressor B cell lymphoma 6 (BCL-6) are low in these cells. T_{FH} cells have high levels of BCL-6 and CXCR5 expression but, in these cells, the levels of BLIMP1 and T-bet are low. Notably, expression of CXCR5 retains T_{FH} cells in B cell follicles. TCR, T cell receptor.

is readily released from activated T cells in soluble form, and there is also evidence that IL-2 can become bound to the extracellular matrix via heparan sulphate moieties[71].

IL-2 homeostasis

IL-2 is thought to be produced mainly by activated T cells in secondary lymphoid organs, where it is consumed by these and other CD25⁺ cells, including T_{Reg} cells[3,4,46]. Accordingly, the influence of IL-2 on T cell homeostasis is dependent on a combination of the rate of IL-2 production and the rate of IL-2 consumption in the steady state. IL-2 production by cells from unmanipulated mice varies considerably: the highest levels are produced by CD4⁺ T cells expressing intermediate levels of CD25, followed by $CD25^{low}CD4^+$ T cells, $CD25^{hi}CD4^+$ T cells, NKT cells, NK cells and CD8⁺ T cells[3,4,72]. Notably, both TCRαβ⁺ and TCRγδ⁺ T cells produce high levels of IL-2 (REF. 72). However, during an immune response, IL-2 production by antigen-activated CD4⁺ and CD8⁺ T cells rapidly increases within hours and remains elevated for a few days in secondary lymphoid organs[26]. Most IL-2 secreted during an immune response is confined to secondary lymphoid organs, where it is consumed locally by activated CD4⁺ and CD8⁺ T cells, as well as by CD25⁺ T_{Reg} cells (FIG. 1). Thus, interfering with IL-2 uptake by these cells through injection of a depleting CD25-specific monoclonal antibody into animals, or the use of mice in which T_{Reg} cells can be inducibly depleted, leads to elevated serum levels of IL-2 following T cell stimulation[73]. High serum concentrations of IL-2 are also observed in CD25-deficient animals[36,47,74], and transferring wild-type CD25⁺ T_{Reg} cells to *Cd25*$^{-/-}$ mice reduces serum IL-2 to normal levels[47]. These findings further highlight the importance of T_{Reg} cells for maintaining systemic IL-2 homeostasis.

Extravasation
A cellular process in which circulating leukocytes bind to and migrate through the endothelium into the underlying tissue.

It has been known for several years that, in addition to lymphoid cells, non-lymphoid cells can express components of the IL-2R, including CD25. Indeed, several human fibroblast cell lines were shown to express CD25 and CD122 at both the mRNA and protein levels[75,76]. Interestingly, these fibroblast cell lines did not express detectable levels of γ_c, but nevertheless had functional heterodimers of CD25 and CD122, as assessed by the secretion of CC-chemokine ligand 2 (CCL2) and the upregulation of intercellular adhesion molecule 1 (ICAM1) and ICAM2 following culture with IL-2 (REFS 75,76). More recently, mouse pulmonary non-immune cells were shown to express CD25, CD122 and γ_c at both the mRNA and protein levels[77]. These cells were negative for typical immune-cell lineage markers and the leukocyte marker CD45, but they expressed CD31, thus identifying them as pulmonary endothelial cells. The function of the trimeric IL-2R on these pulmonary endothelial cells was demonstrated by the finding that stimulating the cells with IL-2 led to an increase in the levels of phosphorylated STAT5 and the production of high levels of nitrite (NO_2^-)[77]. In line with these *in vivo* data, several *in vitro* studies have reported that IL-2 binds directly to bovine and human endothelial cells[78–80] in a CD25-dependent manner[78].

Why non-immune cells, including endothelial cells, express CD25 molecules remains elusive. One possibility is that the expression of CD25 on non-lymphoid cells contributes to the control of IL-2 homeostasis in the lungs and other non-lymphoid organs. On this point, injecting mice with IL-2 leads to increased CD25 expression by lung endothelial cells[77]. Hence, one can envisage that enhanced expression of CD25 by non-lymphoid cells may serve to reduce local IL-2 levels *in vivo*, thereby preventing 'bystander' stimulation of effector T cells and NK cells by IL-2 in vital organs, such as the lungs[46,77]. Alternatively, the *trans*-presentation of IL-2 bound to endothelial cells in inflamed blood vessels might provide survival signals to activated effector T cells that are extravasating to the site of an ongoing immune response. In support of this idea, biologically active IL-2 is present in human blood vessels bound to heparan sulphate on endothelial and smooth muscle cells[71].

Elevated levels of CD25 can be observed in autoimmune and inflammatory diseases, and are also present during transplant rejection and in B and T cell neoplasias; here, the use of CD25-specific monoclonal antibodies has shown some promise in the treatment of multiple sclerosis, uveitis and renal transplant rejection[16]. In addition to its usual cell-bound form, CD25 can be shed by CD25⁺ cells via proteolytic cleavage[81] as a 45 kDa soluble molecule, which is smaller than the 55 kDa protein found on the cell surface. Elevated concentrations of soluble CD25 (sCD25) molecules are found in the serum of humans and animals with the above-mentioned pathological conditions associated with increased CD25 expression[82]. Activated T cells, T_{Reg} cells and mature DCs have been reported to release sCD25 molecules *in vitro*[64,83]. Hence, under *in vivo* conditions, measurable levels of sCD25 in the blood are thought to reflect sustained immune activation

or, in patients with cancer, the rapid turnover of $CD25^+$ malignant cells[82]. With regard to biological significance, sCD25 competes *in vitro* with activated T cells for IL-2 binding, thereby inhibiting T cell proliferation[64,83,84]; whether sCD25 also impairs IL-2 responses *in vivo* is unclear. Recently, sCD25 molecules were shown to present IL-2 *in vitro* to $CD4^+$ T cells and to cause the differentiation of these cells into induced $FOXP3^+$ T_{Reg} cells that were able to suppress $CD8^+$ T cells[85]. Hence, the inhibitory effects of sCD25 *in vivo* may reflect a combination of depriving effector T cells of IL-2 and inducing the generation of T_{Reg} cells. On this point, it is notable that high serum levels of sCD25 in patients with B and T cell malignancies correlate with a poor prognosis[82,83,85].

Implications for IL-2 immunotherapy

Because of its potent ability to stimulate cytotoxic T cells and NK cells, IL-2 is an attractive candidate for immunotherapy of metastatic cancer. Through its ability to expand and activate antitumour lymphocyte populations (formerly termed lymphokine-activated killer cells), high-dose IL-2 immunotherapy has resulted in considerable long-term survival in some patients with metastatic melanoma or renal cell carcinoma[86–88]. However, the therapeutic use of IL-2 for cancer has been limited by IL-2-induced toxicity (see below). For chronic viral infections, IL-2 infusion has been used to boost $CD4^+$ T cell numbers in patients with HIV infection, although the clinical benefit of this treatment was not significant[89].

Despite its potential, IL-2 immunotherapy is hampered by several problems, including the short half-life of IL-2 and the serious adverse side effects of IL-2 administration[90]. In addition, the ability of IL-2 to stimulate T_{Reg} cells diminishes the beneficial effects of stimulating tumour- or virus-specific T cell responses[90]. Irrespective of the route of injection, IL-2 is rapidly removed from the circulation via renal clearance with a half-life measured in minutes[91]. This problem can be overcome to some extent by increasing the half-life of IL-2, for example by using IL-2 coupled to carrier proteins such as IgG antibodies, or by repeated administration of soluble IL-2 (REF. 90).

However, this does not solve the additional problem of IL-2 toxicity. In high doses, IL-2 administration causes vascular leak syndrome (VLS; also known as capillary leak syndrome), which is associated with increased vascular permeability, hypotension, pulmonary oedema, liver cell damage and renal failure[90,92]. The prevailing view is that VLS results from IL-2-mediated stimulation of $CD122^{hi}$ NK cells, leading to the release of pro-inflammatory cytokines (such as tumour necrosis factor) and the production of vasoactive mediators[90]. However, recent data suggest that VLS is primarily mediated by the direct binding of IL-2 to $CD25^+$ endothelial cells rather than to NK cells or other $CD25^-CD122^+$ cells[77]. This finding implies that selectively blocking IL-2 contact with CD25 while allowing interaction with CD122 would decrease the induction of VLS. As discussed below, this can be achieved by conjugating IL-2 to certain IL-2-specific monoclonal antibodies, thus generating IL-2–monoclonal antibody complexes.

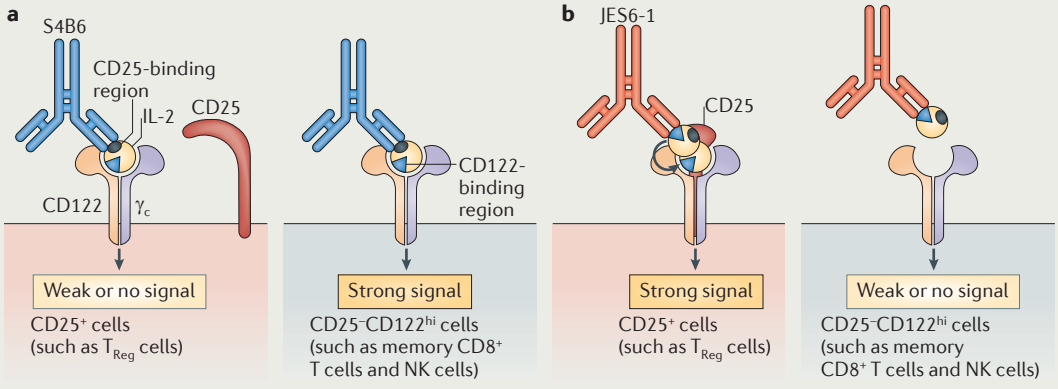
It has long been known that the association of IL-2 with non-neutralizing IL-2-specific monoclonal antibodies can enhance the half-life of IL-2 *in vivo*, in a similar manner to coupling IL-2 to other carrier proteins[90,93,94]. However, recent studies using the neutralizing IL-2-specific monoclonal antibody S4B6 showed that the injection of mice with IL-2–S4B6 complexes led to strong stimulation and expansion of memory $CD8^+$ T cell and NK cell populations (that is, $CD25^-CD122^{hi}$ cells), but with little or no stimulation of $CD25^+$ cells (most notably $CD4^+$ T_{Reg} cells)[14] (BOX 1). The marked potency of IL-2–S4B6 complexes for stimulating $CD8^+$ T cells and NK cells was due primarily to the decreased binding and uptake of this form of IL-2 by $CD25^+$ cells (including T_{Reg} cells and other $CD25^+$ cells), as this considerably increased the biological availability of IL-2 for $CD25^-$ cells[95]. Significantly, IL-2–S4B6 complexes caused a much lower incidence of VLS than soluble IL-2 following administration to mice[77]. This finding, together with the failure of these complexes to stimulate T_{Reg} cells, makes IL-2–S4B6 complexes a potentially useful tool for cancer immunotherapy and also for the treatment of chronic viral infections[90]. Indeed, several recent studies have provided support for this notion using different tumour models[77,96–98] as well as acute and chronic infection models in mice[99,100]. Selective specificity for $CD8^+$ T cells and NK cells was also observed following the injection of mice with human IL-2 in complex with the human IL-2-specific monoclonal antibody MAB602 (REFS 14,77). Whether these complexes have a comparable function in humans is still unclear.

The IL-2 dependency of T_{Reg} cells has focused attention on the potential use of IL-2 for expanding T_{Reg} cell numbers to induce immunosuppression and establish tolerance, especially after organ transplantation. Hence, there is considerable interest in using IL-2, together with other stimuli, to expand the T_{Reg} cell population in tissue culture before transferring these cells to transplant recipients[101]. The alternative approach is to induce the expansion of the T_{Reg} cell population *in vivo*. As mentioned earlier, the problem here is that injection of soluble IL-2 stimulates both cytotoxic T cells and T_{Reg} cells. However, this problem can be overcome by injecting IL-2 in complex with the neutralizing IL-2-specific monoclonal antibody JES6-1A12 (known as JES6-1)[14]. In marked contrast to S4B6 and related monoclonal antibodies, JES6-1 focuses the activity of IL-2 selectively on $CD25^+$ cells (BOX 1). Thus, injecting mice with IL-2–JES6-1 complexes had little effect on $CD25^-$ cells (such as $CD8^+$ T cells and NK cells) but caused marked expansion of $CD25^+$ cells, most of which were typical $FOXP3^+$ T_{Reg} cells[14,95,102]. Similar effects were seen using human IL-2 coupled with the human IL-2-specific monoclonal antibody 5344 (REFS 77,95). Injecting mice with IL-2–JES6-1 complexes to increase T_{Reg} cell numbers was shown to prevent the rejection of allogeneic pancreatic islets[102] and to impair the development of several autoimmune diseases, including type 1 diabetes in non-obese diabetic mice (NOD mice)[103], experimental autoimmune encephalomyelitis (EAE)[102] and experimental myasthenia[104]. T_{Reg} cell function was improved in EAE by the administration of IL-2–JES6-1 complexes together with the drug

Non-obese diabetic mice (NOD mice). NOD mice spontaneously develop type 1 diabetes mellitus as a result of the destruction of pancreatic β-islet cells by autoreactive T cells.

Box 1 | **IL-2–monoclonal antibody complexes mediate selective stimulation of lymphocyte subsets**

Depending on the type of neutralizing interleukin-2 (IL-2)-specific monoclonal antibody used, IL-2 can be preferentially directed either to $CD25^+$ cells (see the figure, left panel of part **b**) that express high levels of CD25 (also known as IL-2Rα) along with intermediate levels of CD122 (also known as IL-2Rβ) and the common cytokine receptor γ-chain (γ_c), or to $CD25^-CD122^{hi}$ cells (see the figure, right panel of part **a**), which express high levels of the dimeric IL-2 receptor (IL-2R). The basis for such selective targeting of IL-2 to different T cell subsets is thought to depend on the precise sites on IL-2 that are bound by particular neutralizing IL-2-specific monoclonal antibodies. The antibody S4B6 binds to the region of IL-2 that interacts with CD25 (this site is represented by a black dot in IL-2), whereas JES6-1 covers up the region of IL-2 that makes contact with (one of) the other two IL-2R subunits, most likely CD122 (this region is depicted as a blue triangle in IL-2)[14,90,95]. Complexes of IL-2 with the S4B6 monoclonal antibody (see the figure, part **a**) lead to vigorous stimulation of $CD122^{hi}$ cells, such as memory $CD8^+$ T cells and natural killer (NK) cells, but to negligible activation of cells expressing low to intermediate levels of CD122. Similar antibodies are JES6-5H4 for mouse IL-2 and MAB602 for human IL-2. By contrast, IL-2 bound to the JES6-1 monoclonal antibody (see the figure, part **b**) selectively stimulates $CD25^+$ cells, such as regulatory T (T_{Reg}) cells. Whereas JES6-1 is specific for mouse IL-2, the JES6-1-like antibody 5344 is specific for human IL-2.



rapamycin (also known as sirolimus)[102]. Rapamycin is an inhibitor of mammalian target of rapamycin (mTOR), which is activated via the IL-2–PI3K–AKT pathway in effector T cells but not in T_{Reg} cells or long-lived memory $CD8^+$ T cells[3,28] (see FIG. 2). Thus, co-administration of rapamycin prevented the activation of effector T cells by the IL-2 treatment without affecting the T_{Reg} cell response. The injection of IL-2–JES6-1 complexes also suppressed the development of allergic airway disease[105] and ameliorated type 2 diabetes by increasing T_{Reg} cell numbers in adipose tissue[106].

Collectively, the above studies in mice indicate that injection of IL-2 bound to particular neutralizing IL-2-specific monoclonal antibodies can be used to target IL-2 to distinct T cell subsets, thereby either boosting or suppressing the immune response.

Perspectives

The ability of IL-2 to activate both T_{Reg} cells and cytotoxic lymphocytes clearly hampers its use as an immunotherapeutic agent. One approach to circumvent this problem is to utilize the different expression patterns of the IL-2R on these two cell types. Thus, for T_{Reg} cells, the high levels of CD25 (along with intermediate expression levels of CD122 and γ_c) allow these cells to respond readily to low concentrations of IL-2 *in vivo*. Hence, IL-2 at low doses might be used to expand T_{Reg} cell numbers in conditions of relative T_{Reg} cell paucity, such as autoimmunity and chronic inflammatory conditions. Indeed, injection of low-dose IL-2, either alone or together with rapamycin, has proved beneficial in the treatment of NOD mice[103,107] and is currently being tested in patients with type 1 diabetes[108]. Moreover, in two recent early-phase clinical trials using low-dose IL-2, patients with chronic graft-versus-host disease or with hepatitis C virus-induced vasculitis showed substantial clinical improvement, which correlated with increased T_{Reg} cell numbers in these patients[109,110].

By contrast, the results of low-dose IL-2 regimens for the treatment of cancer have been disappointing, presumably because of the combined effects of the expansion of the $CD25^+$ T_{Reg} cell population and the poor stimulation of $CD25^-$ antitumour T cells[90]. However, high-dose IL-2 administration (either alone or together with tumour vaccines) to patients with metastatic melanoma or metastatic renal cell carcinoma has led to significant therapeutic responses in approximately 13–20% of cases and to long-term survival beyond 10 years in approximately 10% of cases[86–88].

An alternative approach for IL-2 immunotherapy would be to use improved IL-2 formulations. As discussed earlier, administration of IL-2 bound to particular IL-2-specific monoclonal antibodies could be used clinically to target IL-2 selectively to either cytotoxic immune cells or T_{Reg} cells. For S4B6-like monoclonal antibodies, it should be noted that these antibodies might also be effective without combination with exogenous IL-2. Indeed, the injection of mice with the S4B6 monoclonal antibody alone leads to strong expansion of $CD122^{hi}CD8^+$ T cells without stimulating $CD25^+$ T_{Reg} cells, apparently by targeting the activity of endogenous IL-2 *in vivo*[14,111]. Similar selectivity might be achieved by the generation of IL-2 muteins with increased affinity for either CD25 or CD122 binding[112,113]. Future studies and clinical trials will show whether these reagents have clinical relevance.

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Competing interests statement
The authors declare competing financial interests: see Web version for details.

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