Potent antitumour activity of interleukin-2-Fc fusion proteins requires Fc-mediated depletion of regulatory T-cells

Rodrigo Vazquez-Lombardi1,2, Claudia Loetsch1,2, Daniela Zinkl1, Jennifer Jackson1, Peter Schofield1, Elissa K. Deenick1,2, Cecile King1,2, Tri Giang Phan1,2, Kylie E. Webster1,2, Jonathan Sprent1,2 & Daniel Christ1,2

Interleukin-2 (IL-2) is an established therapeutic agent used for cancer immunotherapy. Since treatment efficacy is mediated by CD8⁺ and NK cell activity at the tumour site, considerable efforts have focused on generating variants that expand these subsets systemically, as exemplified by IL-2/antibody complexes and ‘superkines’. Here we describe a novel determinant of antitumour activity using fusion proteins consisting of IL-2 and the antibody fragment crystallizable (Fc) region. Generation of long-lived IL-2-Fc variants in which CD25 binding is abolished through mutation effectively prevents unwanted activation of CD25⁺ regulatory T-cells (Tregs) and results in strong expansion of CD25⁻ cytotoxic subsets. Surprisingly, however, such variants are less effective than wild-type IL-2-Fc in mediating tumour rejection. Instead, we report that efficacy is crucially dependent on depletion of Tregs through Fc-mediated immune effector functions. Our results underpin an unexpected mechanism of action and provide important guidance for the development of next generation IL-2 therapeutics.

1 Immunology Division, Garvan Institute of Medical Research, Sydney, New South Wales 2010, Australia. 2 St Vincent’s Clinical School, University of New South Wales, Sydney, New South Wales 2010, Australia. Correspondence and requests for materials should be addressed to J.S. (email: j.sprent@garvan.org.au) or to D.C. (email: d.christ@garvan.org.au).
Intrleukin-2 (IL-2) is a pleiotropic cytokine essential for the development, activation and homoeostasis of multiple lymphocyte subsets. Initially identified as a potent T-cell proliferating factor present in mixed leukocyte cultures, IL-2 was first cloned and synthesized in *Escherichia coli* in 1983 (refs 3,4) and underwent initial clinical evaluation for cancer indications in 1985 (ref. 5). Despite severe toxicity, the potent antitumour activity observed in a subset of patients led to the regulatory approval of a high-dose recombinant IL-2 formulation for cancer immunotherapy of metastatic renal cancer in 1992 and for metastatic melanoma in 1998 (ref. 6).

IL-2 is a member of the common gamma-chain (\(\gamma_c\)) cytokine family and shares the \(\gamma_c\) receptor subunit with IL-4, IL-7, IL-9, IL-15 and IL-21. Importantly, immune cells express dimeric or trimeric IL-2 receptors (IL-2R), with the former composed of IL-2R\(\beta\) (CD122) and \(\gamma_c\) (IL-2R\(\beta\)\(\gamma_c\) intermediate-affinity receptor, \(K_D \sim 1\) nM), and the latter composed of IL-2R\(\alpha\) (CD25), IL-2R\(\beta\) and \(\gamma_c\) (IL-2R\(\alpha\)R\(\beta\)\(\gamma_c\) high-affinity receptor, \(K_D \sim 10\) pM)\(^{(1,8)}\). Dimeric receptors are expressed on cytotoxic CD8\(^+\) T-cells and natural killer (NK) cells, while trimeric receptors are predominantly displayed on activated lymphocytes and CD4\(^+\)CD25\(^-\)FoxP3\(^+\) regulatory T-cells (Tregs)\(^{(3)}\). Due to its increased affinity for trimeric receptors, IL-2 induces preferential stimulation of Tregs, which are crucial for maintaining immune tolerance and display pro-tumorigenic activity\(^{(4)}\). In addition to the undesired promotion of Treg activity\(^9\), IL-2 interferes with binding to IL-2 receptor, namely binding to components of the IL-2 receptor, namely binding to 2R\(\alpha\) (refs 15–17), 2R\(\beta\) (refs 16,18,19) and \(\gamma_c\) (refs 16,19).

Although cytokine-antibody complexes and variants are considerably more potent than unmodified cytokines\(^{12,18,20,21}\), development into validated human therapeutics has so far not been demonstrated. While this is likely a reflection of their relative recent discovery, the need for humanization of the antibody component and the requirement for formulating multiple proteins complicate development and regulatory approval of complexes, whereas IL-2 superkines suffer from short serum half-lives, due to their low molecular mass and absence of half-life extension. By contrast, Fc-fusion proteins generated through the genetic linkage of antibody Fc regions with an effector moiety (such as a cytokine or cytokine receptor) have a well-established track record as human therapeutics, as exemplified by the TNFR2-Fc-fusion protein etanercept (Enbrel)\(^22\). Indeed, a large proportion of new biologic drugs contain antibody Fc regions due to the commercial requirement for half-life extension\(^23\), further highlighting the relevance of this format for drug development applications. As such, a number of IL-2-Fc-fusion proteins with therapeutic potential for induction of transplantation tolerance\(^{19,24–26}\) and prevention of autoimmunity\(^{27,28}\) have been reported. Furthermore, recent studies highlight the synergistic nature of combination therapy strategies consisting of antitumour antigen antibodies and IL-2-IgG fusions in models of malignancy\(^{29,30}\). Here we apply the Fc-fusion protein concept to the IL-2 system and systematically investigate the contribution of cytokine and Fc components to antitumour activity.

## Results

### Abolition of CD25 binding enhances IL-2-Fc selectivity.

We first generated genetic fusions, by linking human IL-2 with the Fc region of murine IgG2c by means of a short glycine-serine linker (see Methods). The fusion proteins display a molecular weight of \(\sim 80\) kDa (Supplementary Fig. 1A) compared to about \(15\) kDa for human IL-2. As observed for IL-2/mAb complexes, the presence of the Fc component results in a molecular mass well above the glomerular filtration cut-off (\(\sim 60\) kDa), and increased serum half-life through reduced renal clearance and FcRn recycling\(^{(31)}\).

To reduce binding of our IL-2-Fc construct to CD25\(^+\) cells (and Tregs in particular), we introduced mutations directed at disrupting the IL-2/CD25 interaction (Fig. 1a, Supplementary Fig. 1). Inspection of the quaternary IL-2/IL-2R complex structure\(^{(32)}\) revealed that many of the residues in the IL-2/CD25 interface are charged and participate in electrostatic interactions with the receptor. This motivated us to introduce charge-reversal mutations at contact positions in order to considerably reduce binding affinity and activation of CD25\(^+\) cells (see Supplementary Discussion). Our strategy relied on the introduction of charge-reversal substitutions directly into bivalent IL-2-Fc constructs, thus differing from previous approaches that have targeted both charged and aromatic residues in monovalent unfused IL-2 (refs 15,33).

Although binding was considerably reduced, residual affinity to CD25 was observed for all designed single mutations, as well as for a previously reported F42A mutant\(^{(34)}\), particularly when expressed bivalently in an Fc-fusion format (Supplementary Fig. 1). To further reduce CD25 interactions, we next combined single mutations in a step-wise manner, first into double (Supplementary Figs 1–3), and then into triple mutants (Supplementary Fig. 4). Scanning mutagenesis using up to 14 different amino acid substitutions at targeted residues revealed strong positional effects and the requirement for at least three mutations in the interface to abolish activation of CD25\(^+\) cells both in vitro and in vivo (Supplementary Figs 4 and 5).

We then benchmarked the activity of a novel IL-2-Fc trimer mutant (R38D, K43E, E61R; Fig. 1a) against IL-2-WT-Fc and IL-2/mAb immune complexes consisting of human IL-2 and the mouse anti-human antibody MAB602 (see Methods). Single-dose IL-2-Fc induced robust expansion of MP CD8 and NK cell subsets in the spleens of C57BL/6 mice, substantially higher than what was observed not only for IL-2-WT-Fc, but also for treatment with IL-2/mAb immune complexes (Fig. 1b). The superior activity of mutant IL-2 fusion protein in a single-dose setting was consistent with a prolonged serum half-life relative to IL-2/mAb complexes and IL-2-WT-Fc (Supplementary Figs 3E and 8). Notably, IL-2-Fc administration induced minimal expansion of Tregs confirming its high level of selectivity for CD8 and NK subsets (Fig. 1b).

IL-2-Fc also drove potent expansion of cytotoxic subsets in multiple low-dose treatments, similar to what was observed for IL-2/mAb, but substantially higher than the parental IL-2-WT-Fc protein (Fig. 1c). However, while IL-2/mAb complexes caused considerable Treg expansion (\(\sim 5\)-fold), treatment with multiple low doses of IL-2-Fc failed to induce Treg expansion (Fig. 1c).
Taken together, these experiments demonstrated that our design objectives had been achieved; with IL-2\(^{3XFc}\) treatment causing prominent expansion of CD25\(^{+}\) MP CD8 and NK cells but no expansion of CD25\(^{+}\) Tregs.

**Low toxicity and potent antitumour activity of IL-2\(^{WTFc}\).** Having successfully designed a highly active and selective IL-2\(^{3XFc}\) triple mutant we proceeded to evaluate its therapeutic potential. First, we examined mice for signs of treatment-associated toxicity. Notably, multiple injections of IL-2\(^{3XFc}\) resulted in weight loss, suggesting that this variant induces systemic toxicity at the administered dose (Fig. 2a). Next, we assessed mice for pulmonary oedema and compromised hepatic function as a measure of experimentally induced vascular leak syndrome, a hallmark side effect of IL-2 therapy\(^{13,35}\). Treatment with IL-2/mAb or IL-2\(^{3XFc}\) induced pulmonary oedema, as evidenced by increases in lung water content (Fig. 2b; Supplementary Fig. 6A). By contrast, lung water weight in mice treated with IL-2\(^{WTFc}\) remained largely unchanged relative to PBS controls, either as absolute weight or as percentage of total body weight (Fig. 2b; Supplementary Fig. 6A). Assessment of liver weight and aspartate aminotransferase activity in serum revealed no differences to PBS controls across all treatment groups (Fig. 2c; Supplementary Fig. 6B). However, a significant elevation in alanine aminotransferase activity in serum was observed in the IL-2/mAb group only (Fig. 2d; one-way analysis of variance, \(P = 0.0065\)). These low levels of hepatic toxicity may reflect the lower maximal serum concentrations of administered cytokine relative to experimental high-dose IL-2 (refs 14,35). Taken together, these results suggest a broad correlation between the magnitude of immune cell expansion and the development of treatment-associated toxicities, with low-dose IL-2/mAb or IL-2\(^{3XFc}\) treatments displaying higher levels of toxicity, particularly pulmonary oedema, compared to IL-2\(^{WTFc}\).

We next evaluated the therapeutic efficacy of IL-2/mAb, IL-2\(^{WTFc}\) and IL-2\(^{3XFc}\) in the B16F10 melanoma model. For this purpose, we utilized a dosing regime consisting of five consecutive daily intraperitoneal (i.p.) injections of 1 \(\mu\)g antibody-complexed IL-2 or IL-2-Fc molar equivalent starting 1 day after subcutaneous injection of tumour cells. Strikingly, treatment with IL-2\(^{WTFc}\) resulted in a substantial reduction of tumour growth compared to either IL-2/mAb immune complex or the IL-2\(^{3XFc}\) triple mutant (Fig. 2f). This apparent superior therapeutic efficacy was observed despite the lower potential of IL-2\(^{WTFc}\) to induce expansion of cytotoxic immune cell subsets (Fig. 1c).

**IL-2\(^{WTFc}\) targets Tregs for FcγR-mediated depletion.** The observation that both IL-2/mAb immune complexes and the IL-2\(^{3XFc}\) triple mutant displayed greater toxicity and less efficient
protection against tumour growth than wild-type IL-2-Fc-fusion protein was unexpected and led to further investigation. We focused our attention on the role of the Fc part of the molecule, and in particular its interaction with FcγRs. For this purpose, we investigated mutations that abolish antibody-dependent cell-mediated cytotoxicity/phagocytosis (ADCC/ADCP). More specifically, we mutated conserved residues in the Fc region of IL-2-Fc mediating binding to FcγRs (L234A, L235E, and G237A)\(^{36,37}\). Accordingly, we assessed the activity of IL-2WTFcnil for binding to the C1q complement component, we also focused our attention on the role of the Fc part of the molecule, specifically, we mutated conserved residues in the Fc region of IL-2-WTFc (normal Fc) and IL-2WTFcC1q (no effector functions) and IL-2WTFc (normal Fc) for binding to the C1q complement component, we also generated a mutant (IL-2WTFcC1q\(^{+}\)) in which C1q binding was restored through mutation (see Methods). Disruption of the FcγR and/or C1q binding sites was validated through macrophage and C1q binding assays, respectively (Supplementary Fig. 7A). Importantly, mutation of the Fc region did not affect interaction with the CD25\(^{high}\) MP CD8 and NK cells, but no increase in the numbers or percentages of Tregs (Fig. 3c,d). In view of the similar levels of MP CD8 and NK cell expansion across all constructs and the lack of Treg expansion induced by IL-2WTFcnil. Hence the Fc-mediated depletion seemed to be largely restricted to IL-2-activated Tregs. We should emphasize, however, that this depletive effect was quite limited relative to PBS controls (Fig. 3e,f) and only became prominent when compared with the marked Treg expansion induced by IL-2WTFcnil. Hence the Fc-mediated depletion seemed to be largely restricted to IL-2-activated Tregs. Furthermore, Treg depletion was predominantly FcγR-mediated rather than C1q-mediated since the ability to bind C1q (IL-2WTFcC1q\(^{+}\)) did not result in increased elimination of Tregs (Fig. 3c,d). Notably, a large proportion of Tregs in mice treated with IL-2WTFc or IL-2WTFcC1q\(^{+}\) displayed high levels of the cell proliferation factor Ki-67 (Supplementary Fig. 7E). This finding

In vivo assessments in C57BL/6 mice receiving multiple low-dose IL-2-Fc treatment revealed similar increases in spleen lymphoid cellularity for all designed variants (Fig. 3b). Notably, the effector-less IL-2WTFcnil fusion protein readily expanded not only CD122\(^{high}\) MP CD8 and NK cells but also CD4\(^+\) CD25\(^+\) Tregs (Fig. 3c,d). By contrast, treatment with IL-2WTFc and IL-2WTFcC1q\(^{+}\) led to comparable increases in MP CD8 and NK cells, but no increase in the numbers or percentages of Tregs (Fig. 3c,d). In view of the similar levels of MP CD8 and NK cell expansion across all constructs and the lack of Treg expansion in the presence of Fc-mediated effector functions, we concluded that IL-2WTFc and IL-2WTFcC1q\(^{+}\) were able to selectively deplete Tregs. We should emphasize, however, that this depletive effect was quite limited relative to PBS controls (Fig. 3e,f) and only became prominent when compared with the marked Treg expansion induced by IL-2WTFcnil. Hence the Fc-mediated depletion seemed to be largely restricted to IL-2-activated Tregs.
IL-2

CD8 (CD8^marker (top row, shown as proportion of CD4^plots displaying the frequency of regulatory T-cells in treated mice as defined by the co-expression of CD4, FoxP3 and the IL-2-inducible CD25 surface status. Mutated residues within the Fc region are illustrated in Supplementary Fig. 7A. (b) Analysis of splenocytes (day 5) collected from C57BL/6 mice treated with five consecutive 5.6 g i.p. injections of IL-2^WTFc, IL-2^WTFc^C1q+ or PBS control on days 0–4 (n = 4). (c) Total live splenocytes counts showing increased lymphoid cellularity after IL-2-Fc treatment. (c-f) Flow cytometric analysis of collected splenocytes. (c) Total cell numbers of MP CD8 (CD8^+ CD44^high CD122^high), NK cell (CD3^- NK1.1^+ CD122^high^) and Treg (CD4^+ FoxP3^+ CD25^+ ) cell subsets. (d) Frequencies of MP CD8 (shown as proportion of CD8^-), NK cells (proportion of CD3^-) and Tregs (proportion of CD4^+). (e) Frequencies of CD4^+ FoxP3^- cells within the lymphocyte compartment showing depletion of this subset after treatment with FcR-binding IL-2-Fc constructs. (f) Representative flow cytometry dot plots displaying the frequency of regulatory T-cells in treated mice as defined by the co-expression of CD4, FoxP3 and the IL-2-inducible CD25 surface marker (top row, shown as proportion of CD4^+) or by expression of CD4 and FoxP3 (bottom row, shown as proportion of total lymphocytes). Data are displayed as mean ± s.e.m. Asterisks indicate significant differences relative to PBS controls (c,d) or between specified groups (b,e) as determined by one-way analysis of variance with Bonferroni post hoc test for multiple comparisons (**P < 0.01, ***P < 0.001, ****P < 0.0001).

This supports the notion that these FcR-binding constructs did stimulate Tregs to divide but also eliminated a large proportion of these cells via Fc-mediated killing, resulting in little or no change in Treg numbers.

We further investigated the observed preferential depletion of Tregs by assessing the interaction of IL-2-Fc with different immune cell subsets. Evaluation in an ex vivo binding assay revealed that IL-2^WTFc preferentially bound CD4^+ CD25^+ Tregs over the CD25^- MP CD8 or NK cell subsets (Fig. 4a). To explore differential targeting of lymphocyte subsets in vivo, we compared the cellular biodistribution profiles of fluorescently labelled IL-2^WTFc and IL-2^WTFc^nil fusion proteins (Fig. 4b). We found that IL-2-Fc proteins targeted a low proportion of total CD8^+ T-cells, which is consistent with only a small fraction of this compartment expressing high levels of the dimeric IL-2Rb^y_c (that is, MP CD8 cells). Similarly, ~12% of total CD4^+ T-cells bound to IL-2-Fc proteins, in agreement with typical proportions of Tregs expressing the trimeric IL-2Rg^b^b^y_c. Accordingly, IL-2-Fc fusions were found to efficiently target CD25^+ FoxP3^- Tregs (>85%, Fig. 4b), despite reductions in IL-2-Fc fluorescence intensity after sample fixation for FoxP3 immunostaining (Supplementary Fig. 5C). We observed that labelled IL-2-Fc proteins, regardless of their ability to bind FcR, targeted ~95% of NK cells, thus suggesting that these constructs bind to this subset predominantly through the IL-2R (IL-2Rb^y_c) rather than via FcR. By contrast, disruption of FcR binding substantially reduced the proportion of macrophages and neutrophils associated with IL-2^WTFc^nil (Fig. 4c), suggesting that...
these myeloid subsets may function as effector cells in FcγR-dependent Treg depletion. Finally, similar frequencies of NKT cells (~50%), dendritic cells (~3%) and B-cells (~1%) were found to be targeted by both IL-2WTFC and IL-2WTFcnil proteins (Fig. 4b).

Having examined the frequencies of IL-2-Fc+ cells, we next compared the intensity of IL-2-Fc fluorescent signals, specifically on the NK cell and Treg subsets. To exclude any potential for FcγR-mediated binding on NK cells, fluorescently labelled IL-2WTFcnil was used for this comparison. Interestingly, the mean fluorescence intensity levels observed on IL-2-Fc+ CD4+ T-cells, of which the vast majority are Tregs (see Supplementary Fig. 5C), were nearly fivefold higher compared to IL-2-Fc+ NK cells (Fig. 4d,e). Likewise, fluorescently labelled IL-2WTFC and IL-2WTFcnil proteins were both observed at higher amounts on Tregs than on CD8+ T-cells and NKT cells in addition to NK cells (Fig. 4f,g).

Collectively, our ex vivo (Fig. 4a) and in vivo (Fig. 4c–g) analyses demonstrate substantially stronger binding of IL-2-WTFC proteins to high-affinity IL-2Rβγc on Tregs than to CD25− subsets expressing intermediate-affinity IL-2Rβγc, thus explaining the selective opsonization of Tregs by IL-2WTFc constructs.

**IL-2-Fc antitumour effects require CD25 and FcγR interaction.** To further investigate the influence of Fc-mediated effector functions on antitumour effects, we compared the efficacy of IL-2WTFC and IL-23X proteins expressed as fusions to Fc (able to bind FcγRI) or Fcnil (abolished effector functions). As previously observed (Fig. 2f), treatment with IL-2WTFC resulted in superior antitumour activity against B16F10 melanoma in comparison to IL-23XFc (Fig. 5a), despite the latter variant mediating considerably higher peripheral expansion of cytotoxic subsets (Fig. 1b,c). Remarkably, the efficacy of IL-2WTFc was critically
dependent on FcγR binding, since treatment with IL-2^{WT}Fcnil resulted in no detectable differences in tumour growth or survival compared to PBS controls (Fig. 5a). Furthermore, as observed in non-tumour bearing mice (Fig. 2a), treatment with the IL-2^{3X}Fc variant caused notable reductions in body weight (Fig. 5a, bottom right). This effect was accentuated in mice receiving IL-2^{3X}Fc treatment, possibly due to the elevated levels of fusion protein detected in serum compared to IL-2^{3X}Fc (Supplementary Fig. 8).

Flow cytometric analyses of spleen, draining lymph node (dLN) and B16F10 tumour tissue were performed in order to gain insights into mechanisms underpinning the efficacy of IL-2^{WT}Fc treatment. For this purpose, we compared the levels of lymphocyte expansion in mice treated with either IL-2^{WT}Fc or IL-2^{3X}Fcnil relative to PBS control treatment 48 h after the last IL-2-Fc dose (Fig. 5b–g). In agreement with our previous results (Fig. 3), we found that IL-2^{3X}Fcnil treatment readily expanded splenic Tregs, while IL-2^{WT}Fc failed to expand this subset (Fig. 5b, left). While these results reinforce the previously observed Treg-depleting activity of IL-2^{WT}Fc in the spleen (Fig. 3), this effect was less pronounced in dLN (Fig. 5b, middle) and not evident in tumour lesions (Fig. 5b, right) at this time point. Notably, however, flow cytometric analysis performed 24 h after the last administered dose of fusion protein revealed that IL-2^{WT}Fc is indeed able to mediate Treg depletion in the spleen, dLN and, crucially, at the tumour site itself (Supplementary Fig. 9A,B).

We observed an increased proportion of CTLA-4^{+} Ki-67^{high} Tregs in the tumours of mice treated with IL-2^{3X}Fcnil but not with IL-2^{WT}Fc (Fig. 5c). Interestingly, this difference was tumour-specific and was not observed in the spleen or dLN (Supplementary Fig. 9C). Thus, the reduced frequency of highly activated CTLA-4^{+} Ki-67^{high} Tregs in the tumours of IL-2^{WT}Fc-treated mice may provide a basis for improved antitumour responses. In line with this observation, B16F10 tumours collected from mice receiving IL-2^{WT}Fc displayed a pronounced increase in the numbers of infiltrating leukocytes (Fig. 5d) with a clear enrichment of cytotoxic CD8{\(^+\)} T-cells, both in terms of numbers (Fig. 5e) and frequency (Fig. 5f). This enrichment translated into an increased intratumoural ratio of CD8{\(^+\)} to regulatory T-cells, as calculated from their relative proportions in the CD45.2{\(^+\)} compartment. dLNs, draining lymph nodes; one mouse in the IL-2^{WT}Fc group did not develop a tumour. Data are displayed as mean ± s.e.m. Asterisks indicate significant differences between specified groups (* \(P < 0.05\), ** \(P < 0.01\), *** \(P < 0.001\), **** \(P < 0.0001\)) as determined by one-way analysis of variance (ANOVA) (b–g) or two-way ANOVA (a) with Bonferroni post hoc test for multiple comparisons. Survival (a, top right) is displayed using Kaplan–Meier plots and compared by the Gehan–Breslow–Wilcoxon test.

We next investigated the potential of the IL-2-Fc-fusion proteins for therapy in the B16F10 model in combination with tumour-targeting antibodies.\(^30\) For this purpose IL-2/mAb, IL-2^{WT}Fc or IL-2^{3X}Fc treatment was administered in combination with a monoclonal antibody targeting the B16F10 tumour antigen tyrosinase-related protein 1 (TRP-1) (Fig. 6a). In this setting, IL-2^{WT}Fc again provided the largest reduction in tumour growth and was the only treatment to significantly improve survival compared to anti-TRP-1 monotherapy (Gehan–Breslow–Wilcoxon test, \(P = 0.0446\)).
In addition to B16F10 melanoma, the efficacy of IL-2-Fc-fusion proteins was also investigated in the CT26 colorectal carcinoma syngeneic tumour model (Fig. 6b). Results in this model closely resembled what had been observed for B16F10, with IL-2WTFc providing substantial reductions in tumour growth, while all other treatments displayed no detectable effects. Remarkably, some of the mice treated with IL-2WTFc displayed complete rejection of subcutaneous tumours (Fig. 6b, right), further highlighting the potential of IL-2WTFc therapy.

**Discussion**

Considered the first effective cancer immunotherapy, high-dose IL-2 is able to mediate durable responses in a small subset of metastatic melanoma and metastatic renal cancer patients. However, IL-2 suffers from sub-optimal therapeutic properties arising from a short serum half-life and pleiotropic biological actions, which lead to toxicity and the unwanted expansion of immunosuppressive Tregs.

Here we have generated highly selective IL-2-Fc-fusion proteins through introduction of CD25-disrupting mutations into the cytokine component. Using a combination of structural design, in vitro biosensor measurements and in vivo screening, we developed a novel IL-2-Fc triple mutant (IL-23XFc). Disruption of CD25 binding resulted in prolongation of serum half-life, large increases in biological activity and preferential expansion of CD25<sup>+</sup> CD122<sup>high</sup> MP CD8 and NK cells. Notably, IL-23XFc displayed enhanced activity and selectivity compared to not only wild-type IL-2-Fc, but also previously reported IL-2/mAb complexes.

Further analyses revealed that the antitumour effects and toxicity profile of the IL-23XFc triple mutant were comparable to that of IL-2/mAb complexes, indicating that our initial design objectives had been achieved. However, intriguingly, these experiments also revealed that both IL-23XFc and IL-2/mAb were in fact less effective than the parental IL-2WTFc protein in the B16F10 melanoma model. Furthermore, unlike IL-23XFc and IL-2/mAb treatment, IL-2WTFc did not induce pulmonary oedema or reductions in body weight. This indicated that toxicities arising from these low-dose treatments might be largely immune-related, likely due to excessive activation of CD8<sup>+</sup> T-cells and NK cells (although residual effects on the lung endothelium could not be excluded).13.

Subsequent analyses revealed that selective Treg depletion was critically dependent on the high affinity of IL-2WTFc towards...
CD25⁺ cells, as well as the interaction of the Fc region with FcγR. Crucially, the potent antitumour activity displayed by IL-2/Fc was also dependent on these two properties, with mutation of either the CD25- or FcγR-binding sites resulting in reduced treatment efficacy in the B16F10 melanoma and CT26 colorectal carcinoma models. Analysis of B16F10 tumours revealed reduced frequencies of CTLA-4⁺ and FcγR⁺ Tregs after IL-2/Fc treatment, as well as a large increase in total leukocyte infiltration dominated by cytotoxic CD8⁺ T-cells. Our results therefore suggest that IL-2/Fc administration contributes to the establishment of a highly immunogenic tumour microenvironment that favours the infiltration of immune cells in general, and of CD8⁺ T-cells in particular, leading to enhanced antitumour responses. This is consistent with previously reported increases in CD8⁺ T-cell tumour infiltration and subsequent tumour rejection following in vivo Treg depletion in transgenic mouse models. The data are also in agreement with the finding that, in the context of immune checkpoint blockade, effective antitumour immunity correlates with a high ratio of CD8⁺ T-cells to Tregs in tumour-infiltrating lymphocytes.

In addition to the depletion of Tregs, IL-2/Fc treatment also resulted in expansion of splenic MP CD8 and NK cells, although to a lesser extent than what was observed for IL-2/Fc or IL-2/mAb complexes. The provision of dual activities, which can be either depleting or proliferative depending on the cellular subset, differentiates IL-2/Fc from existing therapeutic modalities, such as the anti-CD25 monoclonal antibody daclizumab (Zenapax). This property may also provide advantages over IL-2-toxin fusions, which target Tregs for depletion but also mediate collateral elimination of CD8⁺ T-cells and NK cells. Further, pre-administration of IL-2/Fc could be used to improve the tumour-targeting properties of IL-2 immunocytokines, as recently reported by Wittrup and colleagues. Finally, based on our findings, ultra-low doses of the highly active and selective IL-2/Fc variant may prove effective in combination with Treg-depleting antibodies, a strategy that we are currently evaluating.

Taken together, our results on the engineering of IL-2/Fc-fusion proteins outline an effective strategy to enhance the therapeutic properties of this key immunomodulatory cytokine. Recent clinical successes of immune checkpoint inhibitors highlight the potential of novel immune modulating agents in cancer immunotherapy, either as stand-alone therapeutics or in combination with anti-CD154 and anti-CTLA-4 therapy. The IL-2/Fc fusion protein described here displays favourable properties, as characterized by a long serum half-life, low levels of toxicity, increased antitumour activity and the ability to selectively deplete Tregs. The role of Treg depletion in the IL-2 system had so far remained elusive: indeed, IL-2/mAb complexes have been reported to act independently of Fc-mediated effector functions and previous reports of IL-2/Fc-fusion proteins have failed to identify any evidence of Treg depletion. By contrast, such a mechanism has been apparent for other targets and therapeutic modalities. In particular, FcγR-mediated Treg depletion has been recently described as a critical component in the antitumour activity of antibodies targeting CTLA-4,OX40 and GITR.

In summary, here we describe the development and characterization of IL-2/Fc-fusion proteins that exceed the specificity (IL-2/Fc) and efficacy (IL-2/Fc) of IL-2/mAb complexes. Moreover, we identify the depletion of Tregs, rather than the expansion of cytotoxic CD8⁺ T-cells and NK cells, as a major determinant for antitumour activity, providing important guidance for the future development of IL-2 reagents for cancer immunotherapy.

**Methods**

**Mutagenesis and production of recombinant proteins.** For the generation of IL-2-Fc-fusion proteins, regions encoding human IL-2 (residues Ala1–Thr133) were genetically fused to murine Fc (IgG2c Glu216-Lys447, Eu antibody numbering) containing a mutated C1q-binding site (E318A, K320A and K322A) by means of a short glycine-serine linker (GSQ). The construct was generated by gene synthesis (GeneArt) and cloned into the mamalian expression vector pCPE4 (Thermo Fisher). Disruption of the CD25 and FcγR binding sites and restoration of the C1q binding site were performed using the Q5 site-directed mutagenesis kit (NEB) using custom-designed primers, followed by validation of the mutation by Sanger sequencing. IL-2-Fc constructs were used to transfect suspension-adapted HEK293 cells using the Exp293 expression system (Thermo Fisher). Protein purification was performed with protein G agarose beads (ACRObiosystems) using disposable columns (Thermo Fisher). All protein preparations were quality controlled for endotoxin levels, as measured by chromogenic LAL assay (Lonza). Genetic constructs coding for the ectodomains of hCD25 and hCD122 (in pCEP4, C-terminal His-tagged) were purchased from the Animal Resources Centre (Canning Vale, WA, Australia) and used at 8–10 weeks of age. IL-7 transgenic mice (IL-7 Tg; B6 background, Thy-1.1-congenic) and transgenic mice expressing DTR-GFP under the FoxP3 promoter were bred at the Australian Bioresources facilities (Moss Vale, NSW, Australia). Mice were housed under conventional barrier protection and handled in accordance with protocols approved by the Garvan Institute of Medical Research Animal Ethics and Use Committee, which comply with the Australian code of practice for the care and use of animals for scientific purposes.

**Flow cytometry and antibodies.** Flow cytometric analysis of mouse spleens and lymph nodes was performed according to standard protocols. The following antibodies were used for staining (all purchased from eBioscience unless stated otherwise): PE- or eFluor450-conjugated anti-CD8 (clone 53-6.7, used at 1.25 µg ml⁻¹); FITC- or eFluor450-conjugated anti-CD122 (clone TM-b1, 1 µg ml⁻¹); APC-conjugated anti-CD44 (clone IM7, 0.4 µg ml⁻¹); FITC- or eFluor450-conjugated anti-CD45.2 (clone 104, 1 µg ml⁻¹); PE- or eFluor450-conjugated anti-CD3e (clone 145-2C11, 1 µg ml⁻¹); PE- or APC-conjugated anti-NK1.1 (clone PK136, 1 µg ml⁻¹); BV421- or BV605-conjugated anti-CD4 (clone GK1.5, 1 µg ml⁻¹; BioLegend); PerCP/Cy5.5-conjugated anti-Thy-1.1 (clone HIS11, 0.5 µg ml⁻¹); PerCP/Cy5.5- or eFluor450-conjugated anti-CD25 (clone PC615, 1 µg ml⁻¹); PerCP-conjugated anti-B220 (clone RA3-6B2, 1 µg ml⁻¹; BioLegend); PE/Cy7-conjugated anti-Ly6G (clone 1A8, 1 µg ml⁻¹; BD); PE-conjugated anti-CD11b (clone M1/70, 1 µg ml⁻¹); PerCP-conjugated anti-CD11c (clone N418, 1 µg ml⁻¹; BioLegend); FITC-labelled anti-F4/80 (clone BM8, 5 µg ml⁻¹; BioLegend); PE-, APC- or eFluor450-conjugated anti-FoxP3 (clone FJK-16s, 5 µg ml⁻¹); FITC- or PE-conjugated anti-Ki-67 (clone SolA15, 0.25 µg ml⁻¹) and APC-conjugated anti-CTLA-4 (clone UC10-4F10-11, 1 µg ml⁻¹; BD); Intracellular staining with anti-FoxP3, anti-CTLA-4 and anti-CTLA-4 were performed after fixation/permeabilization with the FoxP3 buffer set (eBioscience).

**Cytokine treatments.** Mice received recombinant hIL-2, IL-2/mAb or IL-2/Fc treatments at specific quantities and dosing schedules, as described in the figure legends. Recombinant hIL-2 was obtained from Peprotech. IL-2/mAb complexes were prepared by mixing hIL-2 and the anti-hIL-2 MA602 (mouse IgG2a, clone 5355, R&D systems) at 2:1 molar ratio (for example, 1 µg IL-2 + 5 µg mAb), followed by incubation at 37 °C for 25 min prior to injection. Purified IL-2/Fc variants were stored at –80 °C, thawed, filtered (0.22 µm) and re-assessed for protein concentration prior to injection.

**Assessment of treatment-associated toxicity.** Mice were injected ip. once per day with PBS, 1 µg IL-2 + 2.5 µg mAb, 5.6 µg IL-2/Fc or 5.6 µg IL-2/mAb on days 0–4. On day 6, mice were killed for collection of blood (cardiac puncture), lungs, livers and spleens. Blood samples were allowed to clot for 2 h at room temperature (RT), followed by separation of serum by centrifugation. Aspartate aminotransferase and alanine aminotransferase activity assays (Teco Diagnostics) were performed on non-hemolyzed serum samples as per manufacturer’s instructions. Tissue wet weights were recorded using an analytical balance. To determine lung water content, lungs were dehydrated overnight at 42 °C using a Speedvac instrument (Savant) and the difference between lung wet weight and lung dry weight was calculated.

**Assessment of IL-2/Fc binding to mouse splenocytes ex vivo.** Red blood cell-depleted splenocytes from a FoxP3-DTR/GFP transgenic mouse were incubated with Fc-Block (anti-mouse CD16/CD32, BD), followed by washing in flow cytometry buffer and staining with fluorochrome-conjugated anti-CD3e, anti-CD4, anti-CD44 and anti-NK1.1 antibodies. Stained cells were washed, seeded in a 96-well plate (1 x 10⁶ cells per well) and re-suspended in serially diluted IL-2/Fc-Fc-biotin. After incubation for 30 min on ice, cells were
stained with APC-conjugated streptavidin (eBioscience). The levels of IL-2γFc bound to the surface of MP CD8 (CD3 + CD8 + CD44high), NK cells (CD3 + NK1.1 + ) and Tregs (CD3 + CD4 + Foxp3 + ) were determined by flow cytometry.

**IL-2-Fc fluorescent labelling and cellular biodistribution.** Purified IL-2-Fc variants in PBS were filtered through a syringe-driven 0.22 μm filter prior to labelling with Alexa Fluor 647-NHS (Thermo Fisher). IL-2-Fc concentration was adjusted to 300 μg ml⁻¹ in 500 μl, followed by addition of 50 μl 1 mg ml⁻¹ sodium bicarbonate to increase pH. Alexa Fluor 647-NHS was added at a 1:50 molar ratio and labelling was allowed to take place for 2 h at room temperature in the dark. After the labelling reaction, IL-2-Fc variants were buffer-exchanged into PBS using a Nanodrop instrument (Thermo Fisher). Fluorescently labelled IL-2-Fc variants were administered to mice via i.p. as a single 16.8 mg kg⁻¹ dose and spleens were collected 12 h post injection. Flow cytometric analysis of red blood cell-depleted splenocytes was performed after staining with antibodies conjugated to fluorophores with different emission spectra to that of Alexa Fluor 647. The frequencies and mean fluorescence intensity levels of IL-2γFcs were determined for the following populations: CD8^- T-cells (CD3^- CD8^-), CD4^- T-cells (CD3^- CD4^-), NK cells (CD3^- NK1.1^-), NKT cells (CD3^- NK1.1^-), B-cells (CD3^- B220^), dendritic cells (CD3^- CD11c^high), macrophages (CD11^-CD11b^mid high CD11c^-CD11b^- F4/80^- SSC^low-) and neutrophils (CD3^- CD11b^- CD11c^- Ly6G^-).

**Assessment of binding and activity on CTLL-2 cells.** The murine CTLL-2 cell line (IL-2 dependent, CD25^high) was cultured in complete RPMI 1640 (10% FBS, 2 mM l-glutamine, 50 U ml⁻¹ penicillin, 50 μg ml⁻¹ streptomycin, 1 mM sodium pyruvate, 10 μM HEPES, 55 mM 2-mercaptoethanol supplemented with 1 ng ml⁻¹ hIL-2 (Peprotech). CTLL-2 proliferation was measured by incorporation of radiolabelled thymidine. Briefly, 5 × 10^5 cells were added to each well and allowed to take up antibody for 24 h, followed by addition of PHA and culturing for a further 24 h. Cells were collected onto a glass fibre filter and radioactivity was quantified using a liquid scintillation counter (Perkin Elmer). Prior to binding and signalling assays, CTLL-2 cells were starved of IL-2 for 6 h in order to remove surface-bound cytokine. Binding of IL-2-Fc variants to CTLL-2 cells (2 × 10^6 cells per well) was allowed for 20 min on ice. Unbound IL-2-Fc was removed by washing in flow cytometry buffer (2% FBS, 2 mM EDTA in PBS) and surface-bound IL-2-Fc was detected by flow cytometry using a FITC-conjugated anti-mIgG2a antibody (clone R19-15, BD). Staining in CTLL-2 cells was measured by flow cytometric detection of pSTAT5 after IL-2-Fc stimulation. Cells (2 × 10^6 per well) were stimulated with IL-2-Fc variants for 15 min and their proliferation measured by CFSE dilution.

**Fluorescence microscopy.** Spleen tissue from mice treated with Alexa Fluor 647-labelled IL-2-Fc was collected 12 h post injection and fixed in optimal cutting temperature (OCT) embedding medium (Sakura). Tissue sections (5 μm) were cut on a cryostat at -20°C and collected in OCT embedding medium and mounted with Fluoromount (Sigma) and analysed using a Leica DMIRE2 microscope. Sections were stained with anti-CD3ε, anti-CD4, anti-CD8, anti-CD11b, anti-F4/80, anti-CD44, anti-CD11c, anti-TCRγδ, and their proliferation measured by CFSE dilution.

**Adaptive adoptor of MP CD8 cells.** The MP CD8 reporter assay was performed as previously described. MP CD8 cells from IL-7 Tg mice were fluorescence-activated cell sorting-sorted and labelled with carboxyfluorescein succinimidyl ester (CFSE) for adoptive transfer into C57BL/6 recipients. After cytokine treatment, donor MP CD8 cells in the spleens of recipient mice were identified by flow cytometry as Thy-1.1^- and their proliferation measured by CFSE dilution.
IL-2-Fc binding, cells were washed in flow cytometry buffer and stained with 2 μg ml⁻¹ anti-human IL-2-biotin (clone 5344.111, BD). Finally, cells were stained with streptavidin-APC (eBioscience) prior to flow cytometric analysis.

**Complement deposition assay.** CTL2-2 cells were collected 2–3 days after passing and surface-bound Hil-2 was stripped by washing in RPMI 1640, 2% FBS, pH 3 for 20 s. Since complement deposition is calcium-dependent, all steps were performed in EDTA-free buffers. Accordingly, washing and incubation steps were performed in high-glucose DMEM supplemented with 0.5% bovine serum albumin and 0.08% sodium azide. IL-2-stripped CTL2-2 cells were aliquoted into 96-well plates (3.5 × 10⁵ cells per well) and re-suspended in 42 μg ml⁻¹ IL-2-Fc (500 nM). After IL-2-Fc binding, cells were washed in media and re-suspended in purified mouse complement (Cedarlane Laboratories) diluted 1:2. Complement deposition was allowed for 2 h on ice, followed by washing and staining with anti-mIgG2a-AF488 and anti-mC1q-biotin (clone RmC7H8, Cedarlane Laboratories). Cells were then washed and stained with streptavidin-APC (eBioscience). Finally, cells were re-suspended in HEPEs-buffered saline supplemented with 2% FBS and 1 mM CaCl₂ for flow cytometric analysis.

**Statistical analysis.** Data are displayed as mean ± s.e.m., (**P<0.05, ***P<0.01, ****P<0.001). Statistical analyses included one-way and two-way analysis of variance with Bonferroni post hoc test for multiple comparisons, and two-tailed unpaired Student’s t-tests in data sets comparing two groups. Survival was displayed using Kaplan–Meier plots and compared by the Gehan–Breslow–Wilcoxon test. Data were analysed using the Prism software (GraphPad).

**Data availability.** The data supporting the findings of this study are available within the article and its Supplementary Information files and from the corresponding authors on reasonable request.

**References**

1. Boyman, O. & Sprent, J. The role of interleukin-2 during homeostasis and activation of the immune system. *Nat. Rev. Immunol.* 12, 180–190 (2012).
2. Morgan, D. A., Ruscetti, F. W. & Gallo, R. Selective complement deposition assay with streptavidin-APC (eBioscience) prior to flow cytometric analysis. *Nature Communication* 8:15373 | DOI: 10.1038/ncomms15373 | www.nature.com/naturecommunications
3. Lotze, M. T. et al. High-dose recombinant interleukin 2 in the treatment of patients with disseminated cancer: responses, treatment-related morbidity, and histologic findings. *JAMA* 256, 3117–3124 (1986).
4. Rosenberg, S. A. IL-2: the first effective immunotherapy for human cancer. *J. Immunol. 192*, 5431–5438 (2014).
5. Taniguchi, T. & Minami, Y. The IL-2/IL-2 receptor system: a current overview. *Cell 73*, 5–8 (1993).
6. Devis, R. et al. Molecular-cloning of human interleukin-2 carrier DNA and its expression in Escherichia coli. *Nucleic Acids Res. 11*, 4307–4323 (1983).
7. Taniguchi, T. et al. Structure and expression of a cloned cDNA for human interleukin-2. *Nature 302*, 305–310 (1983).
8. Devos, R. et al. IL-2 antagonists for the inhibition of regulatory T cells. *Biochemistry* 37, 1007–1008 (1998).
9. Weinblatt, M. E. et al. A trial of etanercept, a recombinant tumor necrosis factor receptor: Fc fusion protein, in patients with rheumatoid arthritis receiving MTX. *Arthritis Rheum.* 40, 252–259 (1999).
10. Krieg, C., Letourneau, S., Pantaleo, G. & Boyman, O. Improved IL-2 combination immunotherapy with anti-tumor antigen antibodies and extended survival half-life IL-2. *Cancer Cell 27*, 489–501 (2015).
11. Kontermann, R. E. Strategies for extended survival half-life of protein therapeutics. *Curr. Opin. Biotechnol.* 22, 866–876 (2011).
12. Wang, X., Kawan, B. H., Opel, C. F., Navaratna, T. & Wittrup, K. D. Antigen specificity can be irrelevant to immunocytokine efficacy and biodistribution. *Proc. Natl Acad. Sci. USA 112*, 3320–3325 (2015).
13. Liu, D. V., Maier, L. M., Hafler, D. A. & Wittrup, K. D. Engineered interleukin-15 to a superagonist by binding to neonatal FcR and protein A. *Proc. Natl Acad. Sci. USA 110*, 567–573 (2013).
14. Sondermann, P., Huber, R., Oosthuizen, V. & Jacob, U. The 3.2-Å crystal structure of the human IgG1 Fc fragment–FcRII complex. *Nature 406*, 267–273 (2000).
15. Wines, B. D., Powell, M. S., Parren, P. W., Barnes, N. & Hogarth, P. M. The IgG Fc contains distinct Fc receptor (FcR) binding sites: the leukocyte receptors FcγRI and FcγRIIa bind to a region in the Fc distinct from that recognized by neonatal FcR and protein A. *J. Immunol. 164*, 5313–5318 (2000).
16. Mercier, M. et al. Extravasation of intravascular fluid mediated by the systemic administration of recombinant interleukin-2. *J. Immunol. 137*, 1735–1742 (1986).
17. Sondermann, P., Haber, R., Oosthuizen, V. & Jacob, U. The 3.2-Å crystal structure of the human IgG1 Fc fragment–FcγRII complex. *Nature 406*, 267–273 (2000).
18. Li, X. et al. Synergistic innate and adaptive immune response to combination immunotherapy with anti-tumor antigen antibodies and extended survival half-life IL-2. *Cancer Cell 27*, 489–501 (2015).
19. Misra, S. et al. Interleukin-2 receptor α activity can be fine tuned with engineered receptor signaling clamps. *Immunity 42*, 826 (2015).
20. Rubinstein, M. P. & Pao, L. A strategy to convert IL-2 into a cytokine potent and selective for T cell development. *Nature 480*, 529–533 (2012).
46. Bulliard, Y. et al. OX40 engagement depletes intratumoral Tregs via activating FcγRs, leading to antitumor efficacy. *Immunol. Cell Biol.* **92**, 475–480 (2014).
47. Simpson, T. R. et al. Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. *J. Exp. Med.* **210**, 1695–1710 (2013).
48. Edelman, G. M. et al. The covalent structure of an entire γG immunoglobulin molecule. *Proc. Natl Acad. Sci. USA* **63**, 78–85 (1969).
49. Duncan, A. R. & Winter, G. The binding site for C1q on IgG. *Nature* **332**, 738–740 (1988).
50. Kim, J. M., Rasmussen, J. P. & Rudensky, A. Y. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat. Immunol.* **8**, 191–197 (2007).

**Acknowledgements**

This work was supported by the National Health and Medical Research Council. We thank Professor Alexander Rudensky (Howard Hughes Medical Institute and Memorial Sloan-Kettering Cancer Center, New York) for supplying the *Foxp3*ΔTET/GFP mice.

**Author contributions**

D.C. and J.S. conceived and supervised the study. R.V.-L. designed and performed most of the experiments. T.G.P., K.E.W., J.S. and D.C. contributed to experimental design. C.L., D.Z., J.J., P.S., E.K.D. and C.K. performed or contributed to specific experiments. R.V.-L., J.S. and D.C. wrote the manuscript.

**Additional information**

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

How to cite this article: Vázquez-Lombardi, R. et al. Potent antitumour activity of interleukin-2-Fc fusion proteins requires Fc-mediated depletion of regulatory T-cells. *Nat. Commun.* **8**, 15373 doi: 10.1038/ncomms15373 (2017).

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.