Published in final edited form as:

Immunol Cell Biol. 2012 August ; 90(7): 743–747. doi:10.1038/icb.2011.83.

***In vivo* accumulation of T cells in response to IL-2/anti-IL-2 mAb complexes is dependent in part on the TNF family ligand 4-1BBL**

Gloria HY Lin¹, John C Stone², Charles D Surh^{2,3}, and Tania H Watts¹

¹Department of Immunology, University of Toronto, Toronto, Ontario, Canada

²Department of Immunology and Microbial Science, The Scripps Research Institute, La Jolla, CA, USA

³Division of IBB, POSTECH (WCU), Pohang, Korea

Abstract

Immune complexes combining IL-2 with particular anti-IL-2 antibodies can be used to selectively expand regulatory T cells or memory T cells. Combining IL-2 with anti-IL-2 (Clone S4B6) greatly enhances the biological potency of IL-2 *in vivo* leading to selective expansion of CD8 memory T cells and NK cells compared with regulatory T cells. Here we show that *in vivo* administration of IL-2/anti-IL-2 mAb (IL-2/mAb) complexes induces 4-1BB expression on both adoptively transferred antigen-specific memory CD8 T cells as well as on endogenous memory phenotype cells. Remarkably, the accumulation of adoptively transferred memory CD8 T cells following *in vivo* IL-2/mAb-complex treatment was found to be dependent in part on the presence of 4-1BBL in the host. These effects were independent of IL-2-induced cell division, suggesting that 4-1BBL-induced survival signals contribute to IL-2/mAb-complex-induced T-cell accumulation *in vivo*.

Keywords

IL-2-antibody complex; therapy; 4-1BBL; CD137; T cell

IL-2 can signal by binding to a high-affinity trimeric receptor consisting of the α -chain (CD25), β -chain (CD122) and the common γ -chain (γ_C) or by binding to an intermediate-affinity receptor, consisting of CD122 and γ_C only.^{1–3} Whereas FoxP3-positive regulatory T cells express the trimeric high-affinity IL-2 receptor, memory CD8 T cells express the intermediate-affinity CD122/ γ_C receptor.^{4,5} Studies using anti-IL-2 *in vivo* have demonstrated that immune complexes consisting of IL-2 and anti-IL-2 antibodies can be used to enhance the effects of the cytokine with differential effects on cells that express high-affinity or intermediate-affinity IL-2 receptors.⁶ Some antibodies to IL-2 selectively enhance the expansion of regulatory T cells in response to the cytokine antibody complex, whereas the anti-IL-2 antibody S4B6 when combined with IL-2 potently expands memory CD8 T cells.⁶

4-1BB (CD137) is a member of the TNFR family with costimulatory activity for CD8 and to a lesser extent CD4 T cells (reviewed in Watts⁷). It is not expressed on naive T cells but is

CONFLICT OF INTEREST

CDS is a shareholder of Nascent Biologics Inc. The remaining authors declare no conflict of interest.

induced upon their antigen-dependent activation.⁸ 4-1BB can also be induced by IL-15 on CD44^{hi} memory T cells, independently of antigen⁹ and contributes to the survival of both effector and memory T cells.¹⁰ Here we show that IL-2/mAb-complex administration *in vivo* also increases 4-1BB expression on both adoptively transferred antigen-specific memory T cells as well as on endogenous memory phenotype CD8 T cells. Moreover, we find that the increased recovery of adoptively transferred memory T cells upon *in vivo* treatment with IL-2/mAb complexes is dependent in part on the presence of 4-1BBL in the host. The results show that 4-1BBL is dispensable for the IL-2-dependent proliferation of the T cells *in vivo*, but rather affects their accumulation, suggesting that 4-1BB/4-1BBL-dependent survival signals contribute to IL-2/ mAb-complex-mediated memory T-cell expansion.

RESULTS AND DISCUSSION

IL-15 induces 4-1BB on memory CD8 T cells independently of the presence of antigen.⁹ As IL-2 and IL-15 can both use CD122 and CD132 for signaling to T cells, we asked whether IL-2/mAb-complex administration-induced 4-1BB on T cells *in vivo*. We generated OT-I memory T cells *in vitro*⁹ and transferred them i.v. into naive mice followed by i.p. treatment with IL-2/mAb complexes. 4-1BB expression on the transferred T cells as well as on endogenous memory phenotype T cells was analyzed a day later. In the absence of IL-2/ mAb-complex treatment, the transferred memory T cells express low levels of 4-1BB, attributed to their previous *in vitro* culture with IL-15 (Figure 1a left and data not shown). However, *in vivo* administration of the IL-2/mAb complexes led to further upregulation of 4-1BB on the adoptively transferred memory CD8 T cells in LN, spleen and most dramatically in the bone marrow (Figure 1a, left). We also observed induction of 4-1BB on endogenous CD8⁺ T cells upon IL-2/ mAb-complex treatment (Figure 1 right). Most of the 4-1BB was induced on the CD44^{hi} population, consistent with a preferential effect on memory T cells. These increases were reflected in both the percent staining (Figure 1a), as well as in the median fluorescence intensity (Figure 1b). Of note, we consistently observed a lower proportion of CD8⁺ CD44^{Hi} cells early after IL-2/mAb treatment, likely due to activation-induced cell death of a proportion of CD8⁺ CD44^{Hi} cells in response to IL-2 (Figure 1a). However, at later time points (days 4 and 8), consistent with the study performed by Boyman *et al.*,⁶ we observed a dramatic expansion of CD8⁺ CD44^{Hi} cells and they outnumbered the CD8⁺ CD44^{Hi} cells in the mice that had not received IL-2 treatment (data not shown).

We next asked whether 4-1BB induction has any functional role in the expansion of CD8 memory T cells. To this end, we adoptively transferred *in vitro* generated memory cells into wildtype (WT) or 4-1BBL-deficient hosts and then administered IL-2/mAb complexes on day 0, 2 and 5 post transfer and analyzed T-cell recovery on day 4 and day 8. The absence of 4-1BBL in the host resulted in a decrease in the number of cytokine expanded cells in the spleen, LN and BM at both these time points (Figure 2a). By day 4 and 8, the 4-1BB was no longer detectable on the transferred T cells, consistent with its upregulation being transient and perhaps explaining why there is no difference in 4-1BBL-dependence between day 4 and 8. The results show that 4-1BBL contributes substantially to the cytokine-dependent accumulation of the T cells, although there are also 4-1BBL-independent effects of IL-2 (as seen by comparing T-cell numbers from IL-2 treated and untreated 4-1BBL-deficient mice). In addition, there was also an effect of 4-1BBL on T-cell numbers even in mice that had not been treated with IL-2, likely due to the persistence of 4-1BB on the *in vitro* generated OT-I memory T cell as a result of their *in vitro* preparation in IL-15, which itself induces 4-1BB expression (Figure 1a).⁹ However, in some experiments, we clearly observed effects of 4-1BBL that were completely dependent on the *in vivo* IL-2 treatment (data not shown).

Thus, the induction of 4-1BB by IL-2/mAb-complex therapy allows its binding to 4-1BBL thereby contributing to T-cell expansion or persistence *in vivo*.

To determine whether 4-1BBL was required for the IL-2-dependent proliferation, we analyzed carboxyfluorescein succinimidyl ester (CFSE) dilution on the adoptively transferred cells on day 4 post-treatment. The results showed that IL-2/mAb-complex-dependent proliferation was independent of 4-1BBL in the host (Figure 2b). The similar decrease in T-cell numbers in bone marrow, spleen and lymph node suggests that 4-1BBL is not affecting the trafficking of the cells between lymphoid compartments (Figure 2a). Thus, it appears that 4-1BBL contributes to the survival of the IL-2/mAb-complex-expanded cells. These findings are consistent with previous studies showing a role for 4-1BB in the survival of activated and memory T cells.^{9,11-13} The present results indicate that 4-1BBL/4-1BB contributes to cell survival during antigen-independent IL-2/mAb-complex-dependent T-cell expansion.

The ability of IL-2/mAb complexes to enhance T-cell expansion is of interest as a potential therapy for chronic infections and cancer.¹⁴⁻¹⁶ Although high-dose IL-2 is toxic owing to its induction of vascular leakage syndrome (VLS), recent data suggest that IL-2/mAb complexes by acting selectively on cells that express the intermediate-affinity IL-2R avoid VLS, which has recently been attributed to CD25 expression on vascular endothelial cells.¹⁵ Thus, by increasing the potency of IL-2 without inducing VLS, IL-2/mAb complexes could be useful for human therapy. Our study suggests that the induction of 4-1BB by IL-2/mAb complexes and its binding to host 4-1BBL could contribute to T-cell survival during IL-2/mAb-complex therapy. 4-1BB-induced survival signals critically depend on the signaling adaptor TRAF1.^{13,17} As TRAF1^{18,19} and the 4-1BB locus²⁰ both show polymorphisms in humans, the role of 4-1BB/4-1BBL in IL-2/mAb-complex-dependent T-cell accumulation should be taken account when evaluating heterogeneity in human responses to this treatment.

METHODS

Mice

C57BL/6 wild-type mice were obtained from Charles River Laboratories (St Constant, QC, Canada). 4-1BBL-deficient (4-1BBL^{-/-}) mice on the C57BL/6 background (n=9) were obtained under a materials transfer agreement from Immunex (Amgen, Thousand Oaks, CA, USA). OT-I and CD45.1 congenic mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and crossed to generate CD45.1^{+/+} or CD45.1^{+/-} OT-I mice. All the Mice were maintained under specific pathogen free conditions in sterile microisolators at the University of Toronto. All mouse experiments were approved by the University of Toronto animal care committee in accordance with the regulations of the Canadian Council on animal care (University of Toronto approved protocol no. 20007828).

Generation of memory T cells *in vitro*

CD8 T cells with a central memory phenotype were generated from OT-I splenocytes by stimulation with 0.1 $\mu\text{g ml}^{-1}$ SIINFEKL peptide and 1 $\mu\text{g ml}^{-1}$ of LPS for 1 day, then the non-adherent cells were rested for 2 days followed by live cell enrichment using lympholyte (Cedarlane, Hornby, Ontario, Canada). Live cells were cultured in media containing 20 ng ml^{-1} rhIL-15 (R&D, Minneapolis, MN, USA) for another 6 days. For CFSE (Molecular Probes, Eugene, OR, USA) labeling, $5 \times 10^7 \text{ ml}^{-1}$ T cells were incubated in 1 μM CFSE for 10 min at 37 °C and then washed extensively.

IL-2/mAb-complex treatment

IL-2/mAb complexes were delivered by i.p. injection of 1.5 µg of recombinant mouse IL-2 (eBioscience, San Diego, CA, USA) premixed with 7.5 µg of anti-IL2mAb (clone S4B6).⁶ Mice were treated on day 0, 2 and 5 post adoptive transfer of OT-I memory CD8 T cells.

Flow cytometry

Congenically marked OT-I TCR transgenic cells were tracked with anti-mouse CD45.1. labeled with PE or APC (ebioscience) or Pacific blue (BioLegend, San Diego, CA, USA) used in various combinations with PerCP-anti-CD8 (BD Biosciences, San Diego, CA, USA), PE-anti-mouse CD3 and PE-Cy-7 anti-CD44 (eBioscience). 4-1BB-deficient mice (kindly provided by Dr Byoung S Kwon, National Cancer Center, Korea), was used as a negative staining for analysis of 4-1BB expression on CD8 T cells, detected using biotinylated anti-4-1BB (Clone 3H3) and SA-PE or SA-APC. Samples were analyzed using a FACScalibur (BD Biosciences) or a FACS CANTO (BD Biosciences) and FlowJo (TreeStar Inc., Ashland, OR, USA) software.

Statistical analysis

Student's *t*-test was used for comparison of two groups (unpaired, two tailed and 95% confidence interval).

Acknowledgments

We thank Laura Snell and Michael Wortzman for help with tissue harvest, and Byoung S Kwon for provision of 4-1BB^{-/-} mice. This research was funded by grant number MOP-84419 from the Canadian Institutes of Health Research (CIHR) to THW, NIH grants to CDS and by WCU program, NRF, MEST, Korea (R31-10105). GHYL was funded by a CIHR doctoral award. THW holds the Sanofi Pasteur Chair in Human Immunology at the University of Toronto.

References

1. Waldmann TA. The multi-subunit interleukin-2 receptor. *Annu Rev Biochem.* 1989; 58:875–911. [PubMed: 2673025]
2. Takeshita T, Asao H, Ohtani K, Ishii N, Kumaki S, Tanaka N, et al. Cloning of the gamma chain of the human IL-2 receptor. *Science.* 1992; 257:379–382. [PubMed: 1631559]
3. Nakamura Y, Russell SM, Mess SA, Friedmann M, Erdos M, Francois C, et al. Heterodimerization of the IL-2 receptor beta- and gamma-chain cytoplasmic domains is required for signalling. *Nature.* 1994; 369:330–333. [PubMed: 8183373]
4. Malek TR. The biology of interleukin-2. *Ann Rev Immunol.* 2008; 26:453–479. [PubMed: 18062768]
5. Letourneau S, Krieg C, Pantaleo G, Boyman O. IL-2- and CD25-dependent immunoregulatory mechanisms in the homeostasis of T-cell subsets. *J Allergy Clin Immunol.* 2009; 123:758–762. [PubMed: 19348914]
6. Boyman O, Kovar M, Rubinstein MP, Surh CD, Sprent J. Selective stimulation of T cell subsets with antibody-cytokine immune complexes. *Science.* 2006; 311:1924–1927. [PubMed: 16484453]
7. Watts TH. TNF/TNFR family members in costimulation of T cell responses. *Ann Rev Immunol.* 2005; 23:23–68. [PubMed: 15771565]
8. Pollok KE, Kim Y-J, Zhou Z, Hurtado J, Kim KK, Pickard RT, et al. Inducible T cell antigen 4-1BB: Analysis of expression and function. *J Immunol.* 1993; 150:771–781. [PubMed: 7678621]
9. Pulle G, Vidric M, Watts TH. IL-15-dependent induction of 4-1BB promotes antigen-independent CD8 memory T cell survival. *J Immunol.* 2006; 176:2739–2748. [PubMed: 16493029]
10. Wang C, Lin GH, McPherson AJ, Watts TH. Immune regulation by 4-1BB and 4-1BBL: complexities and challenges. *Immunol Rev.* 2009; 229:192–215. [PubMed: 19426223]

11. Takahashi C, Mittler RS, Vella AT. Cutting edge: 4-1BB is a bona fide CD8 T cell survival signal. *J Immunol.* 1999; 162:5037–5040. [PubMed: 10227968]
12. Lee HW, Park SJ, Choi BK, Kim HH, Nam KO, Kwon BS. 4-1BB Promotes the Survival of CD8(+) T Lymphocytes by Increasing Expression of Bcl-x(L) and Bfl-1. *J Immunol.* 2002; 169:4882–4888. [PubMed: 12391199]
13. Sabbagh L, Pulle G, Liu Y, Tsitsikov EN, Watts TH. ERK-Dependent Bim Modulation Downstream of the 4-1BB-TRAF1 Signaling Axis Is a Critical Mediator of CD8 T Cell Survival *In Vivo.* *J Immunol.* 2008; 180:8093–8101. [PubMed: 18523273]
14. Tomala J, Chmelova H, Mrkvan T, Rihova B, Kovar M. *In vivo* expansion of activated naive CD8+ T cells and NK cells driven by complexes of IL-2 and anti-IL-2 monoclonal antibody as novel approach of cancer immunotherapy. *J Immunol.* 2009; 183:4904–4912. [PubMed: 19801515]
15. Krieg C, Letourneau S, Pantaleo G, Boyman O. Improved IL-2 immunotherapy by selective stimulation of IL-2 receptors on lymphocytes and endothelial cells. *Proc Natl Acad Sci USA.* 2010; 107:11906–11911. [PubMed: 20547866]
16. Molloy MJ, Zhang W, Usherwood EJ. Cutting edge: IL-2 immune complexes as a therapy for persistent virus infection. *J Immunol.* 2009; 182:4512–4515. [PubMed: 19342623]
17. Wang C, Wen T, Routy JP, Bernard NF, Sekaly RP, Watts TH. 4-1BBL Induces TNF Receptor-Associated Factor 1-Dependent Bim Modulation in Human T Cells and Is a Critical Component in the Costimulation-Dependent Rescue of Functionally Impaired HIV-Specific CD8 T Cells. *J Immunol.* 2007; 179:8252–8263. [PubMed: 18056369]
18. Kurreeman FA, Padyukov L, Marques RB, Schrodi SJ, Seddighzadeh M, Stoeken-Rijsbergen G, et al. A candidate gene approach identifies the TRAF1/C5 region as a risk factor for rheumatoid arthritis. *PLoS Med.* 2007; 4:e278. [PubMed: 17880261]
19. Cerhan JR, Ansell SM, Fredericksen ZS, Kay NE, Liebow M, Call TG, et al. Genetic variation in 1253 immune and inflammation genes and risk of non-Hodgkin lymphoma. *Blood.* 2007; 110:4455–4463. [PubMed: 17827388]
20. Maier LM, Smyth DJ, Vella A, Payne F, Cooper JD, Pask R, et al. Construction and analysis of tag single nucleotide polymorphism maps for six human-mouse orthologous candidate genes in type 1 diabetes. *BMC Genet.* 2005; 6:9. [PubMed: 15720714]

Figure 1. IL-2/mAb complexes upregulate 4-1BB on CD8 memory T cells *in vivo*
(a) 4-1BB expression on adoptively transferred OT-I memory T cells and endogenous CD8⁺ CD44^{Hi} cells from the LN, BM and spleen was analyzed 1 day following one dose of IL-2/mAb-complex treatment or after no treatment. The black lines on the histograms represent the staining of 4-1BB on adoptively transferred CD8 T cells, whereas the gray lines represent the staining of 4-1BB on 4-1BB-deficient CD8⁺ CD44^{Hi} cells. The gates were determined based on the staining of 4-1BB-deficient mice. Data are representative of two independent experiments, each with $n=3-4$ mice per group. **(b)** Summary of the MFI of 4-1BB on OT-I memory T cells or CD8⁺ CD44^{Hi} T cells from the spleen, LN and bone marrow, with each data point representing an individual mouse. Similar results were obtained in another independent experiment. The level of statistical significance is defined as ** $P<0.01$; *** $P<0.001$.

Figure 2. Accumulation of IL-2/mAb-complex-expanded CD8 memory T cells is dependent on 4-1BBL in the host

(a) *In vitro* generated OT-I memory T cells were adoptively transferred to WT and 4-1BBL^{-/-} hosts; some mice were treated with IL-2/mAb complexes. The total numbers of CD45.1⁺ CD8⁺ memory T cells recovered in the LN, spleen and BM on day 4 (received two doses) or day 8 (received three doses) post adoptive transfer were analyzed. Results from day 4 and day 8 were pooled from three and two experiments, respectively. The level of statistical significance is defined as * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$. Each data point represents an individual mouse. (b) CFSE staining of the OT-I memory T cells was analyzed on day 4 following adoptive transfer into WT or 4-1BBL^{-/-} hosts. A representative histogram is shown for each condition.