Selective Stimulation of T Cell Subsets with Antibody-Cytokine Immune Complexes

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B cell neoplasms in infected people (*1*). Therefore, more in-depth studies are required to unravel the various factors that influence the relation between xCT regulation and KSHV entry into cells of the B lymphocyte lineage.

The receptor function of xCT suggests new perspectives on the role of other cell surface molecules previously implicated in KSHV entry, particularly heparan sulfate (*15*, *16*) and integrin α3β1 (*17*). Sulfated proteoglycans and integrins play critical roles in enhancing attachment and internalization of diverse viruses (*18*–*20*); furthermore, accumulating evidence points to complex signaling pathways triggered by KSHV interaction with integrin α3β1 (*17*, *21*). Coupled with the recent observation (*22*) that 4F2hc forms multimeric complexes containing HAT light chains (including xCT) plus β1 integrins (including α3β1), our findings suggest a possible interplay between integrin signaling events and xCT-mediated KSHV entry.

By mediating cystine uptake, a rate-limiting step for glutathione (GSH) biosynthesis, the x_c^- system plays a central role in maintaining intracellular GSH levels during oxidative stress. xCT is up-regulated in response to GSH depletion, e.g., on exposure to reactive oxygen species (ROS) (*23*, *24*). Redox conditions also have substantial influences during KSHV infection, because hypoxia can induce lytic replication in chronically infected cell lines by activating hypoxia response elements in key lytic-phase genes (*25*). Moreover, it has been reported that KSHV induces ROS production in cultured endothelial cells and that ROS exposure results in enhanced virus entry (*26*). In light of these complex virus-host interactions, the identification of xCT as a KSHV receptor suggests novel pathogenic mechanisms whereby the virus might induce or exploit physiologic responses (i.e., ROS production and xCT up-regulation) that favor its own reactivation and dissemination. It is also noteworthy that intracellular GSH levels are progressively depleted during the course of HIV disease (*27*), at least in part because of HIV Tat-mediated down-regulation of GSH biosynthesis (*28*) and regeneration (*29*), plus enhancement of extracellular GSH hydrolysis (*30*). These concerted activities presumably contribute to the recently described Tat stimulation of xCT expression (*30*), as well as KSHV entry and infectivity (*31*). Thus, beyond the obvious consequences of immunosuppression, HIV coinfection might foster clinically aggressive HIV/AIDS-associated Kaposi's sarcoma by the additional mechanism of KSHV receptor up-regulation.

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Supporting Online Material

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Selective Stimulation of T Cell Subsets with Antibody-Cytokine Immune Complexes

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Interleukin-2 (IL-2), which is a growth factor for T lymphocytes, can also sometimes be inhibitory. Thus, the proliferation of CD8$^+$ T cells in vivo is increased after the injection of a monoclonal antibody that is specific for IL-2 (IL-2 mAb), perhaps reflecting the removal of IL-2–dependent CD4$^+$ T regulatory cells (T regs). Instead, we show here that IL-2 mAb augments the proliferation of CD8$^+$ cells in mice simply by increasing the biological activity of preexisting IL-2 through the formation of immune complexes. When coupled with recombinant IL-2, some IL-2/IL-2 mAb complexes cause massive (>100-fold) expansion of CD8$^+$ cells in vivo, whereas others selectively stimulate CD4$^+$ T regs. Thus, different cytokine-antibody complexes can be used to selectively boost or inhibit the immune response.

Contact with certain cytokines, notably interleukin-2 (IL-2) and IL-15, maintains the survival of T cells, especially CD8$^+$ T cells (*1*–*5*). The responsiveness to these two cytokines is controlled largely by a shared dimeric receptor composed of a β chain (CD122) and a common γ chain (*2*, *6*, *7*). CD122 expression is especially high on many "memory" CD8$^+$ cells primed against defined antigens and also on a naturally occurring population of CD8$^+$ cells with a similar phenotype. These latter CD122$^{high(hi)}$ memory-phenotype (MP) CD8$^+$ cells proliferate in response to IL-2 or IL-15 in vitro (*1*, *8*), and IL-15 controls their survival and intermittent proliferation (turnover) in vivo (*4*).

Steady-state levels of IL-2 in vivo are normally too low to stimulate MP CD8$^+$ cells but are vital for the survival of CD4$^+$ T regulatory cells (T regs) (*9*, *10*). These latter cells are characterized by strong constitutive expression of IL-2Rα (CD25), which enables the cells to express a high-affinity trimeric αβγ receptor (IL-2Rαβγ) and thereby use low levels of IL-2. Reflecting their dependency on IL-2, CD4$^+$ T regs disappear after the injection of IL-2 mAb (*11*, *12*). However, such treatment surprisingly accentuates the turnover of MP CD8$^+$ cells (*3*, *13*). This effect of IL-2 mAb may indicate that CD4$^+$ T regs have a direct inhibitory influence on MP CD8$^+$ cell turnover (*11*) or that

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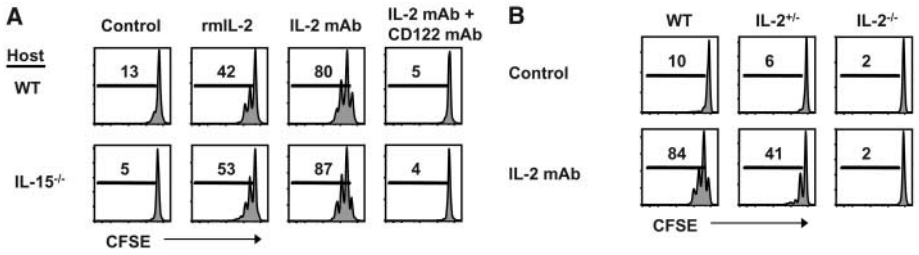

Fig. 1. Stimulation of MP CD8$^+$ cells in vivo by IL-2 or IL-2 mAb. CFSE-labeled purified Thy1.1 MP (CD44^{hi} and CD122^{hi}) CD8$^+$ T cells were transferred intravenously (iv) to (**A**) wild-type (WT) or IL-15$^{-/-}$ mice, which then received daily intraperitoneal (ip) injections of phosphate-buffered saline (PBS) (control), rmIL-2, S4B6 IL-2 mAb, or IL-2 mAb plus CD122 mAb, or to (**B**) WT, IL-2$^{+/-}$, or IL-2$^{-/-}$ mice, followed by daily injections of S4B6 IL-2 mAb or control mAb. Donor cells were analyzed on day 7 by flow cytometry. Numbers represent percentages of divided (CFSE^{lo}) donor Thy1.1$^+$ CD8$^+$ cells. All data in this and the following figures represent at least two separate experiments.

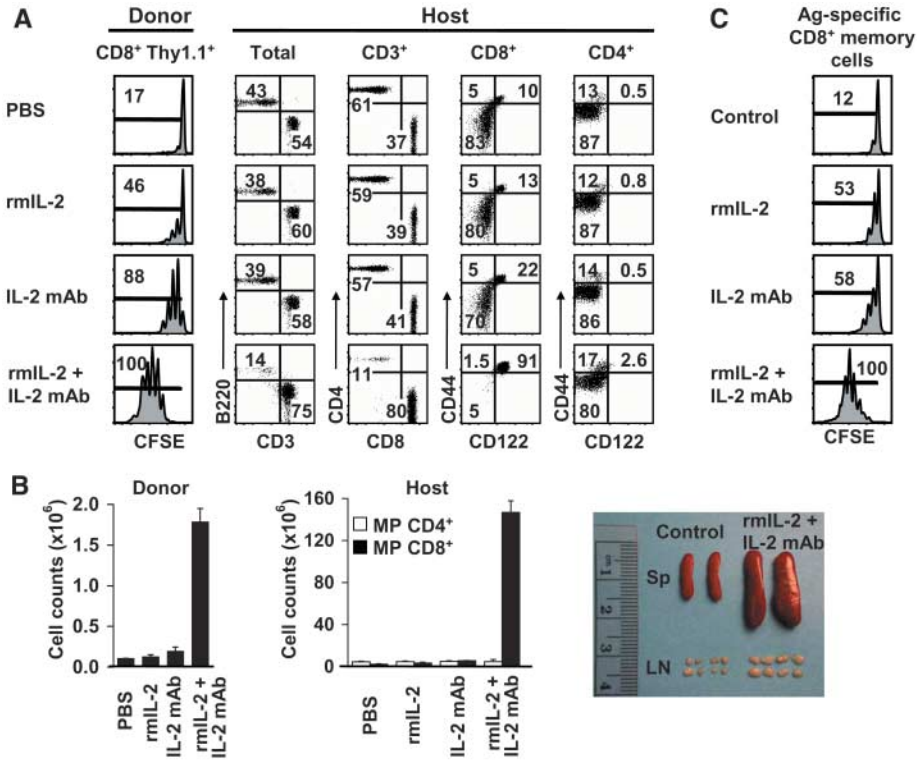

Fig. 2. Marked selective expansion of MP and Ag-specific memory CD8$^+$ T cells in vivo by a combination of IL-2 and IL-2 mAb. (**A**) CFSE-labeled MP CD8$^+$ T cells were transferred to B6 mice, followed by daily ip injections of PBS, rmIL-2, S4B6 IL-2 mAb, or rmIL-2 plus IL-2 mAb. Donor and host cells from LNs were examined for the markers shown on day 7. Comparable results were obtained for spleen cells. (**B**) Total spleen and LN cell numbers of donor and host CD44^{hi} T cells from mice in (A) (+SD, two mice per group). A photograph of two representative spleens and LNs from the injected mice is shown at the right. (**C**) CFSE-labeled Ag lymphocytic choriomeningitis virus–specific memory CD8$^+$ T cells were transferred to B6 mice, followed by daily injections as described above. Donor cells were analyzed on day 7 by flow cytometry. Numbers indicate percentages of divided (CFSE^{lo}) cells in the left column of (A) and in (C).

IL-2 suppresses the synthesis of a putative novel cytokine that stimulates via CD122 (*13*).

The paradox, therefore, is that the turnover of MP CD8$^+$ cells in vivo can be increased by injecting either IL-2 or IL-2 mAb (Fig. 1) (*14*). For IL-2, the proliferation of CD8$^+$ cells in vivo—which is measured by dilution of the dye carboxy-fluorescein diacetate succinimidyl ester (CFSE) (Fig. 1, A and B) or by the incorporation of bromodeoxyuridine (BrdU) (fig. S1)—was prominent after the injection of recombinant mouse IL-2 (rmIL-2) and was largely restricted to MP CD8$^+$ cells, both for host and adoptively transferred, purified CD8$^+$ cells. In contrast, stimulation of naïve T cells, as defined by low expression of CD122 and CD44, was minimal (fig. S1). Confirming previous findings (*3*, *13*), even greater proliferation occurred after the injection of IL-2 mAb, specifically by the anti-mouse IL-2 mAb S4B6 (Fig. 1 and fig. S1). This effect was also seen in IL-15$^{-/-}$ hosts and was blocked by CD122 mAb (Fig. 1A), confirming that the effector cytokine for proliferation is not IL-15 but nevertheless stimulates via CD122 (*3*, *13*).

The unexpected finding was that the stimulation of MP CD8$^+$ cells by IL-2 mAb on adoptive transfer was abolished in IL-2$^{-/-}$ hosts and considerably reduced in IL-2$^{+/-}$ hosts (Fig. 1B). This finding implies that despite its reported neutralizing function in vitro (*15*), S4B6 mAb functions in vivo by increasing the biological activity of preexisting IL-2, perhaps through the formation of immune complexes. To assess this possibility, we used a regime of daily injections of IL-2 and IL-2 mAb in mice. The resulting proliferation of adoptively transferred and host MP CD8$^+$ cells was dramatically enhanced over that seen with single IL-2 or IL-2 mAb administration (Fig. 2A) and led to a very large (>100-fold) increase in the total numbers of MP CD8$^+$ cells in the spleen and lymph nodes (LN) on day 7, with marked enlargement of these organs (Fig. 2B). The combined regime of IL-2 and IL-2 mAb also caused a marked (20- to 30-fold) increase in total numbers of another CD122^{hi} population, namely natural killer (NK) (CD3$^-$, NK1.1$^+$, and DX5$^+$) cells (*16*), but had minimal effects on other cells, including MP CD44^{hi} CD4$^+$ cells and B220$^+$ B cells (Fig. 2, A and B). The proliferation of transferred naïve CD8$^+$ cells was relatively low, suggesting that the IL-2/IL-2 mAb combination was acting largely on preexisting CD122^{hi} cells rather than on naïve CD122^{lo} precursors (fig. S2A). Proliferation was independent of IL-15 because comparable data occurred with transfer of MP CD8$^+$ cells to IL-15$^{-/-}$ hosts (fig. S2B). There was also a strong stimulation of primed virus-specific CD8$^+$ cells (Fig. 2C), indicating that the proliferation of CD122^{hi} CD8$^+$ cells applied to defined antigen (Ag)-specific memory cells as well as to MP cells. For the latter, proliferation did not lead to CD25 up-regulation and was unimpaired with CD25$^{-/-}$ MP CD8$^+$ cells, indicating that stimulation occurred only via IL-2Rβγ (CD122) and not by IL-2Rαβγ (fig. S3).

Near-optimal expansion of CD122^{hi} CD8$^+$ cells occurred with daily injections of a premixed 2:1 molar ratio of IL-2 to IL-2 mAb for 1 week (fig. S4A, arrow, and fig. S4B). At this ratio, even a single injection of IL-2/IL-2 mAb complex caused considerable expansion of CD122^{hi} CD8$^+$ cells (fig. S4C). Based on the results of injecting IL-2/IL-2 mAb complexes at various times before T cell transfer, the biological half-life of IL-2/IL-2 mAb complexes was determined to be relatively short, i.e., <4 hours (fig. S4D).

In addition to S4B6, we also observed equivalent proliferation with the injection of the following: anti-mouse IL-2 mAb JES6-5H4 (JES6-5) plus rmIL-2 (Fig. 3A), and an anti-human IL-2 mAb (MAB602) plus recombinant human IL-2 (rhIL-2) (Fig. 3B). When complexed with IL-2, each of these three mAbs (S4B6, JES6-5, and MAB602) caused marked

expansion of CD122^{hi} CD8^{+} cells on adoptive transfer (Fig. 3, A and B) and strong and selective expansion of host CD122^{hi} cells, including both MP CD8^{+} cells and NK cells (*16*).

The results with a third anti-mouse IL-2 mAb, JES6-1A12 (JES6-1), were quite different (Fig. 3A). IL-2/JES6-1 complexes caused lower proliferation of CD122^{hi} CD8^{+} cells than IL-2 alone, indicating that JES6-1 blocked the in vivo response to IL-2. However, JES6-1 plus IL-2 injection led to mild proliferation of a different IL-2–responsive population, namely CD25^{+} CD4^{+} cells (Fig. 3, C and D). These cells were predominantly Foxp3^{+} and thus resembled T regs. Expansion of these cells was also seen with injection of the other IL-2 mAbs, although this effect was dwarfed by the huge expansion of CD122^{hi} CD8^{+} cells (Fig. 3E).

The above results suggested that S4B6 and related mAbs may bind to a different site on IL-2 than JES6-1 does. IL-2/IL-2 mAb sandwich enzyme-linked immunosorbent assays (ELISAs) provided direct support for this possibility (fig. S5). Similar to its function in vivo, JES6-1 totally blocked the response of both normal and CD25$^{-/-}$ MP CD8^{+} cells to IL-2 in vitro via CD122 (IL-2Rβγ) (Fig. 4, A and B). However, as for CD4^{+} CD25^{+} T regs in vivo, JES6-1/IL-2 complexes were able to induce weak but significant in vitro stimulation of cells expressing high-affinity IL-2Rαβγ, namely CD25^{+} CD3-activated naïve CD8^{+} cells (fig. S6); these cells were very sensitive to IL-2 and were easily inhibited by CD25 mAb. Thus, JES6-1 mAb apparently binds to an IL-2 site that is crucial for interaction with CD122 but less crucial for binding to CD25 (IL-2Rαβγ). In contrast, S4B6 failed to inhibit (or enhance) the response of MP CD8^{+} cells to IL-2 in vitro (Fig. 4, A and B) but strongly inhibited the IL-2 response of CD3-activated CD8^{+} cells (fig. S6). Hence, S4B6 binds to an IL-2 site that partly occludes binding to CD25 but does not impede binding to CD122. When not complexed with exogenous IL-2, a mixture of JES6-1 and S4B6 mAbs caused near abolition of T cell proliferation in vivo, both for MP CD8^{+} cells and for T regs (fig. S7), further suggesting that S4B6 and JES6-1 recognize different sites on IL-2.

Why IL-2/IL-2 mAb complexes are so potent in vivo is unclear. It was reported previously that binding to antibody can increase the half-life of IL-2 (and also IL-4 and IL-6) in vivo; but, other than inducing a mild increase in NK cell–mediated tumor rejection, the effects of IL-2/IL-2 mAb complexes on T cells were not mentioned (*17*–*20*). For the marked expansion of CD122^{hi} MP CD8^{+} cells reported here, F(ab′)$_2$ mAb fragments were much less stimulatory than intact mAb (fig. S8), suggesting that the complexes became bound to cells via the mAb Fc region. Such presentation may be unusually efficient and explain why IL-2/IL-2 mAb complexes are far more stimulatory in vivo than in vitro.

The stimulatory effects of IL-2/IL-2 mAb complexes also applied to complexes of IL-4 and IL-4 mAb (Fig. 4C) and IL-7 and IL-7 mAb (*16*). Thus, the proliferation of CD8^{+} cells was much higher after injection of these cytokine/mAb complexes than with cytokine or mAb alone.

For S4B6 and related antibodies, injecting IL-2/IL-2 mAb complexes might be clinically useful for tumor immunotherapy and for expanding T cell numbers after bone marrow (BM) transplantation. In support of this latter idea, irradiated mice given unseparated BM cells and then a course of IL-2/S4B6 injections showed a rapid restoration of mature T cell numbers, especially CD8^{+} cells, as early as 1 week post-transfer (Fig. 4D). Conversely, the expansion of CD4^{+} T regs by IL-2 and JES6-1 or related mAbs could be useful for treating autoimmune disease.

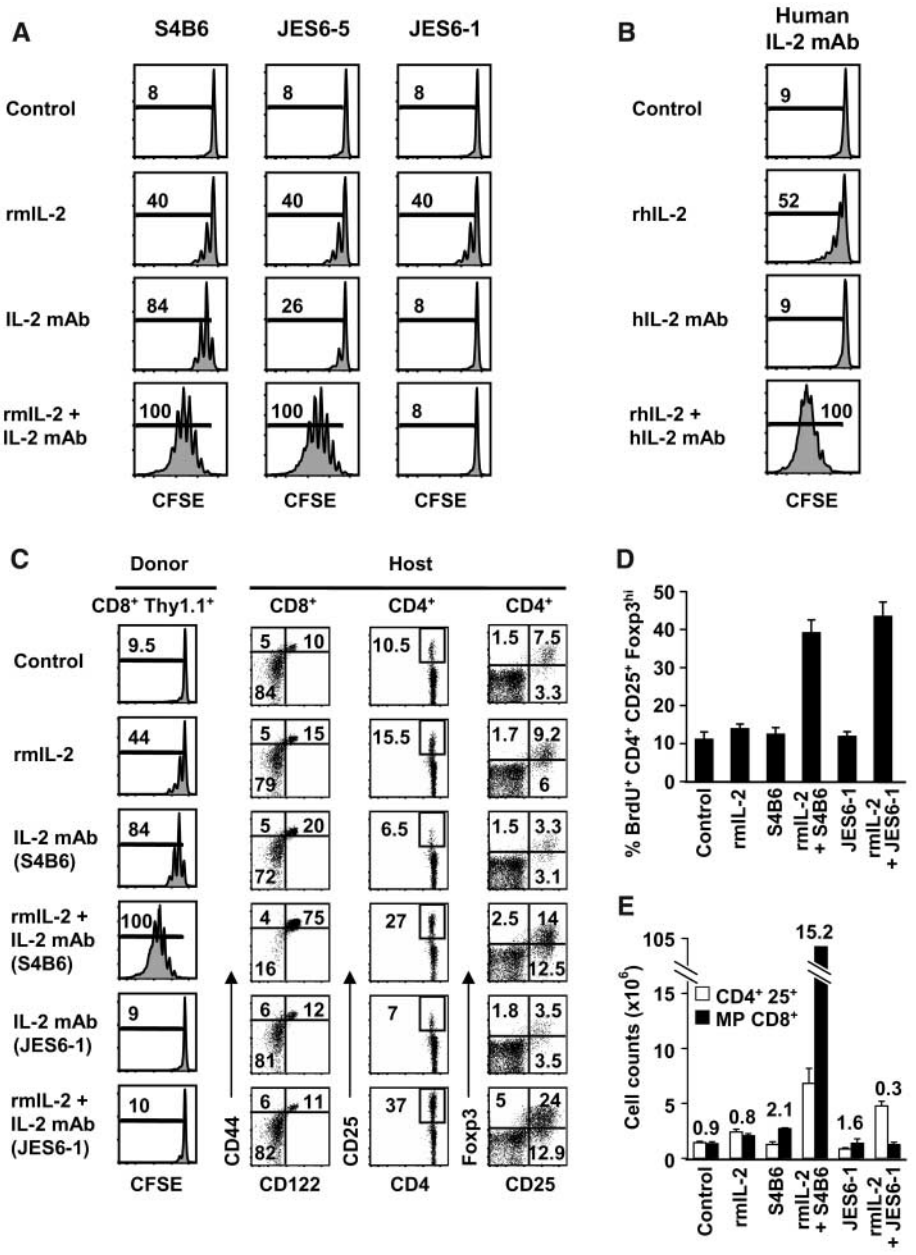

Fig. 3. Selective stimulation of T cell subsets by different IL-2/IL-2 mAb complexes. CFSE-labeled MP CD8^{+} T cells were transferred to B6 mice, followed by (**A** and **B**) daily ip injections of control mAb, IL-2 ([mIL-2 in (A), rhIL-2 in (B)], IL-2 mAb, or IL-2 plus IL-2 mAb as in Fig. 2A. The IL-2 mAbs used were (A) anti-mouse S4B6, JES6-5, or JES6-1, or (B) anti-human MAB602. (**C**) MP CD8^{+} T cells were transferred to B6 mice, followed by daily injections of control mAb, rmIL-2, IL-2 mAb, or rmIL-2 plus IL-2 mAb as above. Donor and host cells from the spleen were examined for the markers shown on day 7. (**D**) Mice treated as in (C) were given BrdU in the drinking water for the last 3 days. Shown are the percentages of CD3^{+} CD4^{+} CD25^{+} Foxp3^{hi} cells that were BrdU^{+} (+SD, 2 mice/group). (**E**) Total cell counts of CD4^{+} CD25^{+} and MP CD8^{+} cells in spleen from mice in (C). The numbers on top of the bars indicate the ratios of MP CD8^{+} to CD4^{+} CD25^{+} cells. Mice were analyzed on day 7 in all panels. Numbers indicate percentages of divided (CFSE^{lo}) cells in (A) to (C), left column.

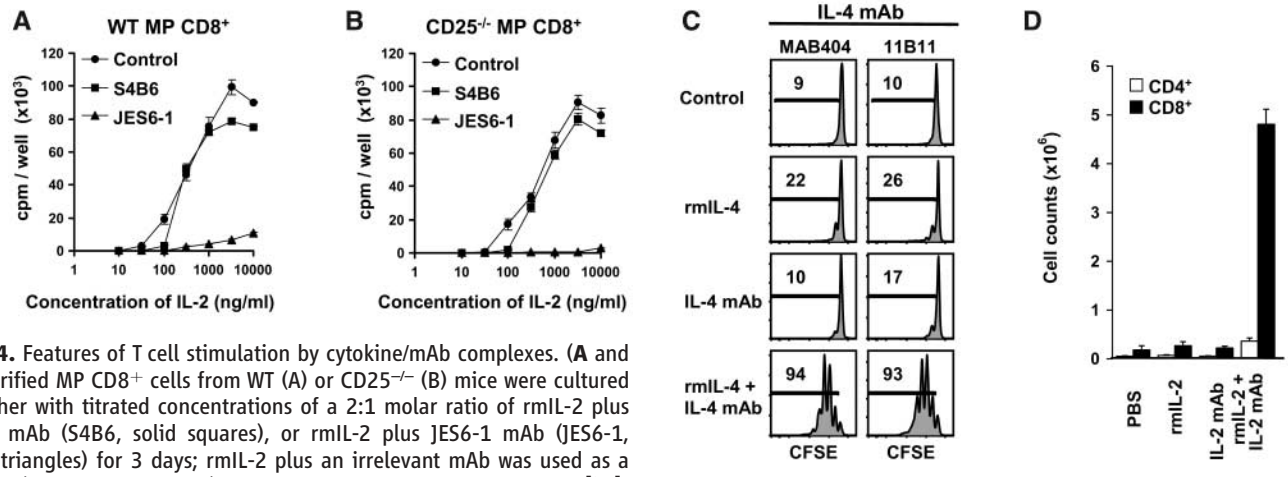

Fig. 4. Features of T cell stimulation by cytokine/mAb complexes. (**A** and **B**) Purified MP CD8$^+$ cells from WT (A) or CD25$^{-/-}$ (B) mice were cultured together with titrated concentrations of a 2:1 molar ratio of rmIL-2 plus S4B6 mAb (S4B6, solid squares), or rmIL-2 plus JES6-1 mAb (JES6-1, solid triangles) for 3 days; rmIL-2 plus an irrelevant mAb was used as a control (control, solid circles). Proliferation was measured by adding [^{3}H]-thymidine for the last 16 hours. (**C**) Purified CFSE-labeled MP CD8$^+$ cells were transferred to B6 mice, which were then given every other day ip injections of control mAb, rmIL-4, IL-4 mAb (MAB404 or 11B11), or rmIL-4 plus IL-4 mAb. Mice were analyzed on day 7. Numbers indicate percentages of divided (CFSE^{lo}) cells. (**D**) B6 mice were irradiated with 1000 centigray (cGy) and injected iv with unseparated versus T cell–depleted B6 BM cells, followed by daily ip injections of PBS, rmIL-2, S4B6 IL-2 mAb, or rmIL-2 plus S4B6 IL-2 mAb. Eight days after adoptive transfer, spleen cells were analyzed by flow cytometry. Shown are mean cell numbers of CD3$^+$ CD4$^+$ and CD3$^+$ CD8$^+$ cells from recipients of unseparated BM (+SD, two mice per group). With injection of T cell–depleted BM, no restoration of T cell numbers occurred (*16*).

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Figs. S1 to S8
References

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A Critical Role for the Innate Immune Signaling Molecule IRAK-4 in T Cell Activation

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IRAK-4 is a protein kinase that is pivotal in mediating signals for innate immune responses. Here, we report that IRAK-4 signaling is also essential for eliciting adaptive immune responses. Thus, in the absence of IRAK-4, in vivo T cell responses were significantly impaired. Upon T cell receptor stimulation, IRAK-4 is recruited to T cell lipid rafts, where it induces downstream signals, including protein kinase Cθ activation through the association with Zap70. This signaling pathway was found to be required for optimal activation of nuclear factor κB. Our findings suggest that T cells use this critical regulator of innate immunity for the development of acquired immunity, suggesting that IRAK-4 may be involved in direct signal cross talk between the two systems.

The innate immune system provides a critical front line of protection against infection (*1*, *2*). Among the mediators of innate immunity, the Toll-like receptors (TLRs) are prominent in pathogen recognition, resulting in the activation of transcription factors, mainly nuclear factor κB (NF-κB), through adaptor molecules and downstream kinases (*1*, *2*). Of these kinases, IRAK-4 plays a dominant role, as demonstrated by evidence that IRAK-4–deficient ($^{-/-}$) mice lack the ability to elicit innate immune responses (*3*). These mice fail to eliminate infected bacteria, are resistant to lipopolysaccharide-induced septic shock, and show no response to interleukin-1 (IL-1), IL-18, or most of the TLR ligands. IRAK-4 induces NF-κB activation through a signaling cascade involving MyD88, IRAK-4, and tumor necrosis factor receptor–associated factor 6 (TRAF6).

Vertebrates also develop adaptive immune responses that are initiated by T cell activation after T cell receptor (TCR) engagement by antigen (Ag) peptide–major histocompatibility complexes on Ag-presenting cells (APCs) (*4*). At the molecular level, TCR engagement results in phosphorylation of CD3 chains by the Src-family kinase Lck and recruitment of the Syk-family kinase Zap70 to the TCR complex, followed by the recruitment and phosphorylation of adaptor proteins, including linker of