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CUTTING EDGE

Cutting Edge: Mechanism of Enhancement of In Vivo Cytokine Effects by Anti-Cytokine Monoclonal Antibodies[1]

James D. Phelan, Tatyana Orekov,†‡ and Fred D. Finkelman[2†‡§]*

***Inhibitory anti-cytokine mAbs are used to treat cytokine-mediated disorders. Recently, however, S4B6, an anti-IL-2 mAb that blocks IL-2 binding to IL-2Rα, a receptor component that enhances affinity but is not required for signaling, was shown to enhance IL-2 agonist effects in vivo. We evaluated how S4B6 enhances IL-2 effects and whether a similar mechanism allows mAbs to IL-4 to enhance IL-4 effects. Induction of T cell proliferation by IL-2/S4B6 complexes did not require complex dissociation and was IL-2Rα independent. S4B6 increased IL-2 agonist effects by increasing in vivo half-life, not by focusing IL-2 onto cells through Fc receptors. In contrast to IL-2/S4B6 complexes, anti-IL-4 mAb enhancement of in vivo IL-4 effects required IL-4/anti-IL-4 mAb complex dissociation. Thus, agonist effects observed with high doses of anti-IL-2 mAb are most likely only applicable for mAbs that maintain cytokine half-life without blocking binding to receptor signaling components. The Journal of Immunology,* 2008, 180: 44–48.**

Antagonistic anti-cytokine mAbs are useful for treating cytokine-mediated diseases such as rheumatoid arthritis (1–4) and other inflammatory arthritides (5), inflammatory bowel disease (6), and periodic fevers (5) and are being evaluated for many other inflammatory disorders (7). Recently, however, S4B6, an anti-mouse IL-2 mAb that was long thought to enhance in vivo immune responses by blocking regulatory effects of IL-2 (8, 9), was shown to greatly increase direct stimulatory effects of IL-2 on T cells (10). This was particularly marked when mice were simultaneously treated with IL-2 and S4B6 and was even true when mice were injected with a large excess of S4B6. This last observation indicated that the stimulatory effect of the IL-2/S4B6 complex did not require dissociation of the complex. The stimulatory effect of S4B6 was associated with selective inhibition of IL-2 binding to the α-chain of the IL-2R heterotrimer, which is expressed on regulatory $CD4^+$ T cells (11), and, to a lesser extent, on effector $CD4^+$ T cells (9, 12, 13). The IL-2Rα-chain increases IL-2R affinity for IL-2 by ~100-fold (14) but does not participate in signaling (15). Because a $F(ab')_2$ of S4B6 had greatly reduced agonist activity compared with the intact molecule, it was suggested that S4B6 enhances IL-2 activity by focusing IL-2 onto cells that express FcγRs. In addition, because a complex of IL-4 with an anti-IL-4 mAb enhanced IL-4 effects in vivo, as had been shown previously (16), it was suggested that FcγR-dependent focusing of cytokine/anti-cytokine mAb complexes might be a general mechanism that enhances in vivo cytokine effects (10). This concept suggested the troubling possibility that many anti-cytokine mAbs might function as agonists, rather than antagonists, in vivo.

In this study, we confirm that the induction of T cell proliferation by IL-2/S4B6 complexes does not require IL-2/S4B6 complex dissociation and is IL-2Rα independent. However, we show that S4B6 promotes the agonist effects of IL-2 by increasing its in vivo half-life rather than by focusing IL-2 onto cells through FcγRs. Furthermore, we show that complexes prepared from IL-4 and a neutralizing anti-IL-4 mAb, unlike IL-2/S4B6 complexes, must dissociate to signal through IL-4Rs. Thus, the agonist effects observed with high doses of S4B6 are FcγR independent and are most likely only applicable for mAbs that can protect a cytokine from breakdown or excretion without blocking cytokine binding to a signaling component of its receptor.

Methods

Mice

Female BALB/c, C57B/6, and β_2-microglobulin-deficient B6.129P2-*B2m*^{tm1Jae} N12 mice were purchased from Taconic Farms and used at ages of 8–12 wk. FcRγ-deficient C.129P2(B6)-*Fcer1g*^{tm1Rav} N12 mice were a gift of R. Strait (Cincinnati Children's Hospital, Cincinnati, OH). All mice were housed at the Cincinnati Children's Research Foundation in an specific pathogen-free facility. All experiments used four mice per group.

Reagents

S4B6 (rat IgG2a anti-IL-2), PC-61 (rat IgG1 anti-IL-2Rα), GL113 (rat IgG1 anti-*Escherichia coli* β-galactosidase), GL117 (rat IgG2a anti-*E. coli* β-galactosidase), BVD4-1D11 (rat neutralizing IgG2b anti-IL-4), BVD6-24G2.3 (rat non-neutralizing IgG1 anti-IL-4), 2.4G2 (rat IgG2b anti-FcγRII/RIII), MAR

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18.5 (mouse anti-rat κ), and J1.2 (rat IgG2b anti-NP ((4-hydroxy-3-nitrophenyl)acetyl) were grown as ascites and purified by ammonium sulfate fractionation and DE-52 ion exchange chromatography. Fluorochrome-labeled mAbs to CD4, CD8, CD25, Foxp3, CD19, B220, CD49b, Ia^d, and BrdU were obtained from BD Pharmingen and eBioscience. BrdU was purchased from Sigma-Aldrich. IL-2 and IL-4 were purchased from Peprotech.

Flow cytometry

Spleen cells were stained according to the manufacturers' protocols. Stained cells were analyzed with a FACSCalibur flow cytometer and CellQuest software.

ELISA

IL-2 levels in serum and urine were determined by ELISA using microtiter plate wells coated with anti-mouse IL-2 (clone Jes-1A12) and biotinylated anti-mouse IL-2 (clone Jes-5H4) to detect bound IL-2. S4B6 did not interfere with this assay (data not shown). Biotin-BVD6-24G2.3 levels in mouse serum were determined using wells coated with mouse anti-rat κ (clone MAR 18.5) and HRP-streptavidin to detect bound biotin-rat IgG. Biotin-BVD6-24G2.3/IL-4 complexes were captured onto wells coated with BVD4-1D11 and detected with HPO-streptavidin. Total biotin-BVD6-24G2.3 in serum was quantitated by a similar assay, except that recombinant mouse IL-4 was added to the serum before performing the assay.

Inhibition of PAF

Because 2.4G2 can induce shock by causing macrophages to produce platelet-activating factor (PAF), 2.4G2-injected mice were pretreated with the platelet-activating factor antagonist CV-6209 (Biomol).

Statistical analysis

One-tailed Student *t* tests were performed to test the hypothesis that an anti-cytokine mAb has a stimulatory effect. Prism 4 software was used to perform *t* tests and one-way ANOVAs. Figures show means ± SEMs. $p < 0.05$ was considered significant.

Results

S4B6 selectively increases $CD8^+$ T cell proliferation in vivo

An initial experiment determined whether S4B6 enhances $CD8^+$ T cell proliferation in a dose-dependent manner. BALB/c mice were injected with 50, 500, or 5,000 μg of S4B6 or an isotype control mAb and splenic T cell proliferation from day 2 through day 3 was analyzed by BrdU incorporation. Even 50 μg of S4B6 increased $CD8^+$ T cell proliferation (determined as a percentage of $BrdU^+$ cells), but proliferation increased further with increasing doses of S4B6 (Fig. 1*A*). In contrast, S4B6 slightly inhibited BrdU incorporation by $CD4^+$ T cells and had no significant effect on the $Foxp3^+$ subpopulation of $CD4^+$ T cells (regulatory T cells (Tregs)[3]; Fig. 1*B*).

IL-2/S4B6 complexes are functionally active and their activity is IL-2Rα independent

S4B6 enhances $CD8^+$ T cell proliferation by forming complexes with IL-2 that are more stimulatory in vivo than free IL-2 (10). IL-2/S4B6 complexes might directly activate T cells or S4B6 might act as a carrier protein that increases in vivo IL-2 half-life but must release free IL-2 to stimulate these cells. To distinguish between these possibilities, BALB/c mice were injected i.p. with IL-2 plus S4B6 or control mAb at a relatively low mAb to IL-2 ratio (20:1, an ~4-fold molar excess of mAb, which should allow some dissociation of IL-2/S4B6 complexes) or a much higher ratio (5,000:1, an ~1,000-fold molar ratio, which should keep much more of the IL-2 in the complexed form). Mice were injected with BrdU 2 days later and BrdU incorporation was determined 1 day after that. Both low- and high-dose S4B6 greatly enhanced T cell proliferation, but a significantly greater effect was observed with the higher dose (Fig. 2*A*). Consequently, S4B6/IL-2 complexes do not need to dissociate to stimulate T cells.

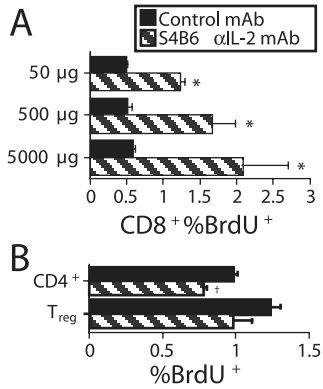

FIGURE 1. S4B6 αIL-2 mAb selectively increases $CD8^+$ T cell proliferation in vivo. BALB/c mice were injected i.p. with the doses of anti-IL-2 (αIL-2) mAb S4B6 or isotype control mAb (GL117) shown (*A*) or with 5000 μg of these mAbs (*B*). Two and 2.5 days later, mice were injected i.p. with 600 μg of BrdU. Splenocytes were harvested 1 day after that, stained for CD4, CD8, and BrdU (*A*) or CD4, CD25, Foxp3, and BrdU (*B*) and analyzed by flow cytometry. An asterisk (*) signifies a significant increase in the percentage of $BrdU^+$ cells vs mice treated with the same dose of control mAb; a cross (†) indicates a significant decrease in the percentage of $BrdU^+$ cells vs mice treated with the same dose of control mAb.

Because S4B6 blocks IL-2 binding to the IL-2Rα-chain, which increases IL-2R affinity and is expressed in the greatest amount by Tregs, S4B6 might enhance T cell responses to IL-2 by blocking Treg stimulation or blocking IL-2 binding to soluble IL-2Rα in blood. To eliminate these possibilities, all mice in the preceding experiment, except the group that received only control mAb, were treated 2 days before IL-2 stimulation with anti-IL-2Rα mAb, which blocks IL-2Rα enhancement of IL-2R affinity and kills $CD25^+$ Tregs. An additional experiment that specifically evaluated the effect of anti-IL-2Rα mAb on IL-2-induced T cell proliferation confirmed that S4B6 enhancement of IL-2-induced $CD8^+$ T cell proliferation is IL-2Rα independent (Fig. 2*B*). Thus, S4B6 enhancement of the in vivo stimulatory effects of IL-2 is not due to killing or inhibiting Tregs.

The stimulatory activity of IL-2/S4B6 complexes is not limited to T cells; treatment of BALB/c mice with these complexes also induces large increases in splenic NK cell numbers (Fig. 2*C*) and proliferation (Fig. 2*D*).

S4B6 promotes IL-2 agonist effects by increasing IL-2 half-life

Because cytokine/mAb complexes can have a longer in vivo half-life than free cytokine molecules (16), we evaluated whether S4B6 increases the serum half-life of IL-2. BALB/c mice were injected i.v. with IL-2 and/or S4B6 and sera were obtained 1 day later and assayed for IL-2 concentration by using an ELISA that detects free IL-2 and S4B6-complexed IL-2 with equal sensitivity. S4B6 administration increased serum IL-2 concentration ~30,000-fold, indicating that it greatly prolongs the in vivo half-life of IL-2 (Fig. 3*A*). This did not seem to be accomplished by decreasing urinary excretion of IL-2, because S4B6 administration actually increased the IL-2 content of urine excreted during the 24 h subsequent to IL-2 injection. We

[3] Abbreviations used in this paper: Treg, regulatory T cell; FcRn, neonatal Fc receptor.

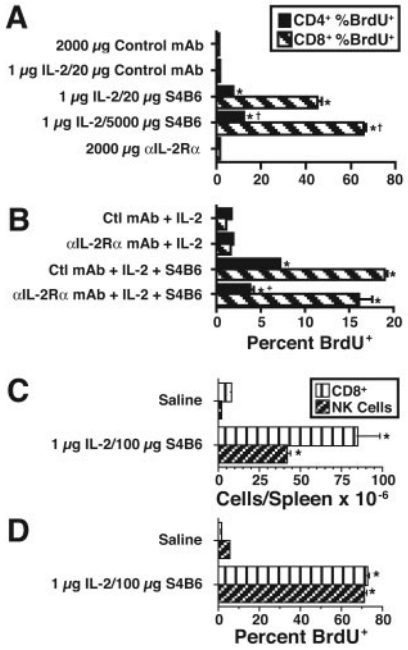

FIGURE 2. IL-2/S4B6 complexes are functionally active and act independently of IL-2Rα. *A*, BALB/c mice were injected with 2,000 μg of anti-IL-2Rα (αIL-2Rα) or saline 2 days before and at the time of injection of 1 μg of IL-2 plus 20 or 5,000 μg of S4B6 or 20 μg of isotype control mAb. Mice were injected with BrdU 2 days later. Their spleen cells were stained for CD4, CD8, and BrdU 1 day after that and analyzed by flow cytometry. *B*, BALB/c mice were injected with 2,000 μg of anti-IL-2Rα or isotype control mAb 1 day before and at the time of injection of 1 μg of IL-2 with or without 5 μg of S4B6, and proliferative responses by their spleen cells were evaluated as in *A*. *C* and *D*, In a separate experiment, BALB/c mice were injected with saline or 100 μg of S4B6 plus 1 μg of IL-2 on days 0 and 2 and with BrdU on day 2, and sacrificed on day 3. Spleen cells were counted, stained for CD3, CD8, CD49b, and BrdU, and analyzed by flow cytometry for the numbers of $CD8^+$ T cells ($CD3^+CD8^+$) and NK cells ($CD3^-CD49b^+$) per spleen and for the percentage of these cells that were $BrdU^+$. An asterisk (*) indicates a significant increase in cell number or the percentage of $BrdU^+$ cells vs mice treated with the same dose of control mAb; a cross (†) indicates a significant increase in the percentage of $BrdU^+$ cells vs mice treated with IL-2 plus 20 μg of S4B6; a plus sign (+) indicates a significant decrease in the percentage of $BrdU^+$ cells vs mice treated with control mAb instead of anti-IL-2Rα mAb.

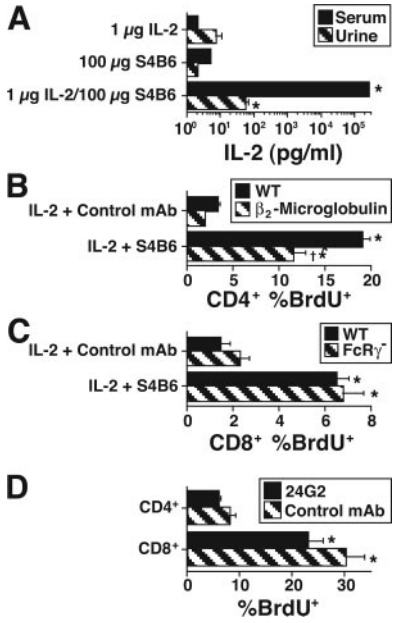

FIGURE 3. S4B6 increases IL-2 effects by increasing serum IL-2 half-life. *A*, BALB/c mice were injected i.v. with IL-2 (1 μg) S4B6 (100 μg) or IL-2/S4B6; serum and urine were collected 1 day after injection and IL-2 levels were determined by ELISA. IL-2 was undetectable (<10 pg/ml) in the urine of untreated mice. *B*, β_2-microglobulin-deficient B6.129P2-*B2m*^{tmJae} N12 mice and WT mice were injected i.p. with IL-2/S4B6 or IL-2/GL117 (1 μg/20 μg). Mice were injected with BrdU 2 days later. Spleen cells obtained 1 day after that were stained for CD4, CD8, and BrdU and analyzed by flow cytometry. *C*, FcγR-deficient C.129P2(B6)-*Fcer1g*^{tm1Rav} N12 and wild-type (WT) mice were injected i.v. with 66 μg of CV6209 and 2 min later with 500 μg of 2.4G2. One hour later, mice were injected i.p. with IL-2/S4B6 or IL-2/GL117 (1 μg/5 μg). Two days later, mice were injected with BrdU. Spleen cells obtained 1 day later were stained for CD4, CD8, and BrdU and analyzed by flow cytometry. The effects on $CD4^+$ T cells were smaller than but consistent with the effects on $CD8^+$ T cells (data not shown). *D*, BALB/c mice were treated with CV6209 and 500 μg of 2.4G2 or control mAb. Mice were injected with IL-2/S4B6 (1 μg/5 μg) 1 h later and BrdU 2 days later. Spleen cells obtained 1 day later were stained for CD4, CD8, and BrdU and analyzed by flow cytometry. An asterisk (*) indicates a significant increase vs mice that did not receive both IL-2 and S4B6; a cross (†) indicates a significant decrease vs wild-type mice.

cannot eliminate the possibility, however, that free IL-2 is broken into undetectable fragments as it is excreted.

Because the long in vivo half-life of IgG is partially dependent on the neonatal Fc receptor (FcRn) (17), an MHC-I-related heterodimer that contains β2-microglobulin (18), we evaluated whether S4B6 enhancement of IL-2 effects was decreased in β2-microglobulin-deficient B6.129P2-*B2m*^{tmJae} N12 mice. S4B6 still enhanced IL-2 stimulation of $CD4^+$ T cells in these mice (which lack $CD8^+$ T cells), although less so than in wild-type mice (Fig. 3*B*). Thus, FcRn enhancement of IgG half-life may account for some but not all of the enhancing effect of S4B6.

Because it has been proposed that anti-cytokine mAbs enhance cytokine effects in vivo by focusing them onto target cell FcγRs (10), we also evaluated whether S4B6 enhancement of IL-2 effects is FcγR dependent. This was accomplished by evaluating $CD8^+$ T cell proliferation in wild-type or FcRγ-deficient C.129P2(B6)-*Fcer1g*^{tm1Rav} N12 mice (which lack all stimulatory FcRs) that were treated with anti-FcγRII/RIII mAb (clone 2.4G2) and then stimulated with IL-2/control mAb or IL-2/S4B6. S4B6 still enhanced IL-2 effects in 2.4G2-treated FcRγ-deficient mice (Fig. 3*C*), and 2.4G2 did not inhibit the enhancing effect of S4B6 in wild-type mice (Fig. 3*D*). Thus, S4B6 enhancement of IL-2 effects is FcγR independent.

Anti-IL-4 mAb complexes must dissociate to stimulate lymphocytes

IL-4/anti-IL-4 mAb complexes, like IL-2/S4B6 complexes, have a much longer in vivo half-life than free IL-2 or IL-4 and can stimulate T cell proliferation and B cell MHC class II (Ia) expression in vivo (10, 16). To determine whether IL-4/Ab complexes function similarly to IL-2/S4B6 complexes, BALB/c mice were injected i.p. with low (5:1) or high (5,000:1) weight ratios of blocking anti-IL-4 mAb (BVD4.1D11) to IL-4 or a high ratio of control mAb to IL-4. Mice were injected with BrdU 2 days later and sacrificed 1 day afterward. The low ratio mixture of anti-IL-4 mAb:IL-4 greatly increased T cell proliferation (Fig. 4*A*) and B cell Ia expression (Fig. 4*B*), while IL-4 plus the control mAb and the high ratio mixture of anti-IL-4 mAb:IL-4 had no stimulatory effect. Thus, IL-4/BVD4-1D11 complexes, unlike IL-2/S4B6 complexes, must dissociate to have an agonist effect.

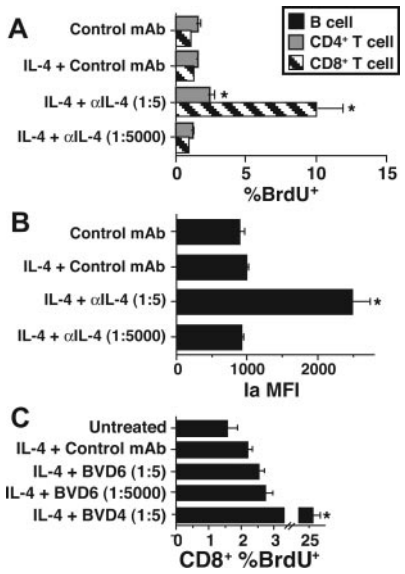

FIGURE 4. In vivo agonist effects of IL-4/blocking anti-IL-4 mAb complex require complex dissociation. *A* and *B*, BALB/c mice (four per group) were injected i.p. with IL-4/BVD4-1D11 anti-IL-4 (αIL-4) complexes or IL-4/control mAb complexes that contained 1 μg of IL-4 and 5 or 5000 μg of mAb. Mice were injected with BrdU 2 days later and splenocytes were harvested 1 day after that, stained for CD4, CD8, and BrdU (*A*) or CD19, B220, and Ia (*B*) and analyzed by flow cytometry. *C*, BALB/c mice (four per group) were injected i.p. with IL-4 and complexes of saline, control mAb (5000 μg), and BVD6-24G2 anti-IL-4 mAb at low (5 μg) or high (5000 μg) doses or with BVD4-1D11 anti-IL-4 mAb (5000 μg). Mice were injected with BrdU 2 days later and splenocytes were harvested 1 day after that, stained for CD8 and BrdU, and analyzed by flow cytometry. An asterisk (*) indicates a significant increase vs mice that received anything other than IL-4 plus BVD4-1D11 at a 1:5 weight ratio.

An additional experiment evaluated whether a nonblocking anti-IL-4 mAb (BVD6-24G2.3) mAb similarly enhanced IL-4 effects in vivo. Results demonstrate that the nonblocking anti-IL-4 mAb did not significantly up-regulate B cell Ia expression (data not shown) or enhance T cell proliferation (Fig. 4*C*) at low (5:1) or high (5,000:1) weight ratios of mAb to cytokine while complexes containing a 5:1 weight ratio of BVD4-1D11: IL-4 had a stimulatory effect similar to that observed in previous experiments (Fig. 4*C*). ELISAs performed 1 day after injecting mice with 1 μg of IL-4 plus 5 μg of biotin-BVD6-24G2.3 demonstrated serum biotin-BVD6-24G2.3 concentrations of 492 $\pm$ 59 ng/ml. The biotin-BVD6-24G2.3 in serum was no longer complexed with IL-4 ($<$ 10 pg/ml) but bound as much IL-4 in vitro as purified biotin-BVD6-24G2.3 that had a concentration of 419 $\pm$ 71 ng/ml. Thus, although BVD6-24G2.3 avidly binds IL-4 in vitro, BVD6-24G2.3/IL-4 complexes dissociate too rapidly in vivo to enhance in vivo effects of IL-4.

Discussion

Our observations confirm the demonstration by Boyman et al. (10) that S4B6 enhances the in vivo agonist effect of endogenous or injected IL-2 by forming a complex with this cytokine that directly stimulates T cells, especially $CD8^+$ T cells, through the IL-2R $\beta\gamma$ heterodimer. However, we demonstrate that S4B6 enhances IL-2 in vivo agonist activity by increasing its half-life rather than by focusing it onto cells through IgG-FcγR interactions. This appears to contradict the observation that $F(ab')_2$ of S4B6, which bind IL-2 but cannot interact with FcRs, have considerably less IL-2 enhancing activity than intact S4B6. The substantially reduced in vivo half-life of IgG Fab and $F(ab')_2$, compared with intact IgG molecules (19), however, makes this observation consistent with our evidence that intact S4B6 promotes IL-2 agonist effects by increasing its in vivo half-life.

Although the main importance of Boyman's finding (10) was that it changed our understanding of how IL-2 affects in vivo immune responses, it also had implications for the clinical use of anti-cytokine mAbs to suppress inflammatory diseases. Cytokine/anti-cytokine mAb complexes often have much longer in vivo half-lives than free cytokines and accumulate in patients treated with some anti-cytokine mAbs (for example, anti-IL-6 or anti-TNF). Furthermore, other cytokine/anti-cytokine mAb complexes, including complexes of IL-4 with a neutralizing anti-IL-4 mAb, complexes of IL-3 with a neutralizing anti-IL-3 mAb, and complexes of IL-7 with a neutralizing anti-IL-7 mAb, have in vivo agonist effects (16). As a result, the observation that IL-2/S4B6 complexes directly activate T cells raises the concern that other anti-cytokine mAbs that inhibit cytokine-cytokine receptor interactions might also enhance rather than suppress the effect of their cytokine ligands even when present in great molar excess.

Our studies with IL-4 and a neutralizing anti-IL-4 mAb mitigate this concern by showing that the agonist effect of the neutralizing anti-IL-4 mAb was only seen when the mAb:IL-4 molar ratio was low and disappeared when it was raised to a level consistent with the therapeutic use of anti-cytokine mAbs in humans. This is not consistent with the possibility that the IL-4/neutralizing anti-IL-4 mAb complex is directly active and confirms our earlier suggestion (16) that neutralizing anti-cytokine mAbs can act as carrier proteins that protect a cytokine from degradation or excretion and slowly release bound cytokine to prolong its in vivo effect. The difference between the mechanisms by which S4B6 and BVD4-1D11 increase the in vivo effects of IL-2 and IL-4, respectively, is explained, at least in part, by differences in the structures of the IL-2 and IL-4 receptors. Although S4B6 blocks IL-2 binding to IL-2Rα, this chain is not required for IL-2 signaling. By causing endogenously produced IL-2 to accumulate in vivo to levels sufficient to signal through the lower affinity $\beta\gamma$ form of IL-2R, S4B6 eliminates the need for high receptor affinity and promotes a chronic IL-2 effect. Most neutralizing anti-cytokine mAbs, which inhibit the ability of a bound cytokine to signal through its receptor or totally prevent cytokine binding to its receptor, would not be able to form a cytokine/anti-cytokine mAb complex that is directly active. However, these anti-cytokine mAbs might have an agonist effect when, with time after injection, their concentrations decrease to the level where they can act as carrier proteins. This may account for some of the disease flares that are occasionally seen when arthritis patients are withdrawn from an anti-TNF mAb or soluble receptor (20).

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