IL-2/Neuroantigen Fusion Proteins as Antigen-Specific Tolerogens in Experimental Autoimmune Encephalomyelitis (EAE): Correlation of T Cell-Mediated Antigen Presentation and Tolerance Induction[1]

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The purpose of this study was to assess whether the Ag-targeting activity of cytokine/neuroantigen (NAg) fusion proteins may be associated with mechanisms of tolerance induction. To assess this question, we expressed fusion proteins comprised of a N-terminal cytokine domain and a C-terminal NAg domain. The cytokine domain comprised either rat IL-2 or IL-4, and the NAg domain comprised the dominant encephalitogenic determinant of the guinea pig myelin basic protein. Subcutaneous administration of IL2NAg (IL-2/NAg fusion protein) into Lewis rats either before or after an encephalitogenic challenge resulted in an attenuated course of experimental autoimmune encephalomyelitis. In contrast, parallel treatment of rats with IL4NAg (IL-4/NAg fusion protein) or NAg lacked tolerogenic activity. In the presence of IL-2R$^+$ MHC class II$^+$ T cells, IL2NAg fusion proteins were at least 1,000 times more potent as an Ag than NAg alone. The tolerogenic activity of IL2NAg in vivo and the enhanced potency in vitro were both dependent upon covalent linkage of IL-2 and NAg. IL4NAg also exhibited enhanced antigenic potency. IL4NAg was ~100-fold more active than NAg alone in the presence of splenic APC. The enhanced potency of IL4NAg also required covalent linkage of cytokine and NAg and was blocked by soluble IL-4 or by a mAb specific for IL-4. Other control cytokine/NAg fusion proteins did not exhibit a similar enhancement of Ag potency compared with NAg alone. Thus, the IL2NAg and IL4NAg fusion proteins targeted NAg for enhanced presentation by particular subsets of APC. The activities of IL2NAg revealed a potential relationship between NAg targeting to activated T cells, T cell-mediated Ag presentation, and tolerance induction. ***The Journal of Immunology,*** **2007, 178: 2835–2843.**

The function of T cell-mediated Ag presentation is currently unknown. Several studies have implicated T cell APC (T-APC)[3] as mediators of immunological tolerance in vitro and in vivo. MHC class II (MHCII)-dependent Ag presentation by T cells often results in anergy or desensitization of Ag-specific T cell responders in vitro (1–14). Likewise, adoptive transfer of MHCII$^+$ T cells presenting neuroantigenic peptides of myelin basic protein (MBP) conferred protection to recipients subsequently challenged with neuroantigen (NAg) in CFA (15–18). These findings suggested that MHCII-dependent Ag presentation by T cells may represent a mechanism involved in the resolution of cell-mediated immune responses (19).

To assess a possible relationship between T cell-mediated Ag presentation and immunological tolerance, cytokine/NAg fusion proteins were devised that had the potential of targeting Ag to activated T cells in vivo. This study focused on two fusion proteins, IL2NAg and IL4NAg. The hypothesis was that the cytokine domain would interact with the respective cell surface cytokine receptors on T cells and on other types of APC to facilitate targeting of the covalently attached Ag to those APC. The cytokine domain may not only target Ag to particular types of APC but may also facilitate APC activities engendering anti-inflammatory or tolerogenic activity by those APC. For example, interactions of the IL-2 domain of IL2NAg with T-APC may promote the presentation of NAg and simultaneously enhance cytotoxicity and the killing of NAg-specific responder T cells.

Both fusion proteins were comprised of an N-terminal cytokine domain and a C-terminal NAg representing the encephalitogenic GP73–87 sequence of GPMBP. Both fusion proteins targeted the covalently linked NAg to particular APC subsets for enhanced presentation. For example, in a T cell cytotoxic assay, IL2NAg exceeded the antigenic potency of NAg by >1,000-fold. Administration of the fusion proteins in vivo showed that IL2NAg had tolerogenic activity that reduced the subsequent induction of experimental autoimmune encephalomyelitis (EAE). Overall, this study revealed a correlation between the targeting of NAg to activated T cells, Ag presentation by MHCII$^+$ T cells, and tolerance induction in the Lewis rat model of EAE.

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[3] Abbreviations used in this paper: T-APC, T cell APC; DHFR, dihydrofolate reductase; EAE, experimental autoimmune encephalomyelitis; GPMBP, guinea pig myelin basic protein; IL2EKdel, IL2NAg fusion protein with deletion of the enterokinase cleavage site; IL2NAg, IL-2/neuroantigen fusion protein; IL4NAg, IL-4/neuroantigen fusion protein; MHCII, MHC class II glycoprotein; MBP, myelin basic protein; NAg, neuroantigen; EK, enterokinase cleavage site.

Table I. *Cytokine/NAg fusion proteins*

Descriptor	Name	N to C Terminus Order of Domains
IL2NAg[a]	IL2.7	IL2-EK-NAg-6his
IL2NAg[a]	IL2Ekdel	IL2-NAg-6his
IL2 alone[a]	IL2	IL2-6his
IL4NAg[a]	IL4.4	IL4-EK-NAg-6his
IL4NAg[a]	IL4Ekdel	IL4-NAg-6his
IL4 alone[a]	IL4	IL4-6his
IL10NAg[a]	IL10.6	IL10-EK-NAg-6his
IL13NAg[a]	IL13.6	IL13-EK-NAg-6his
NAgIL16[b]	NAgIL16-S	HBM ss-7His-NAg-C-terminal IL16

[a] The rat IL2.7, IL4.4, IL10.6, and IL13.6 proteins consisted of the native signal sequence, the full length mature cytokine, a GDDDDKG enterokinase (EK) domain, the major encephalitogenic peptide of GPMBP (PQKSQRSQDENPVVH), and a 6-His terminal tag. IL2Ekdel and IL4Ekdel had a deletion of the EK domain but were otherwise identical to IL2.7 and IL4.4 respectively. IL-2 and IL-4 lacked the EK-NAg domain.

[b] NAgIL16-S consisted of the honey bee mellitin (HBM) signal sequence (MK FLVNVALVFMVVYISYIYA), a 7-His tag the encephalitogenic peptide (YGSLPQ KSQRSQDENPVVH), and the C-terminal 118-aa sequence of rat IL16 (excepting the mouse C-terminal serine residue).

Materials and Methods

Animals and reagents

A colony of Lewis rats was maintained in the Department of Comparative Medicine at East Carolina University School of Medicine (Greenville, NC). GPMBP was purified from guinea pig spinal cords (Rockland). OX6 anti-I-A (RT1B) IgG1, OX17 anti-I-E (RT1D) IgG1 (20), and OX81 anti-IL-4 IgG1 (21) mAbs were concentrated by the ultrafiltration of B cell hybridoma supernatants through Amicon spiral wound membranes (100-kDa exclusion). The respective hybridomas were obtained from the European Collection of Cell Cultures. Aluminum hydroxide gel and PMA were purchased from Sigma-Aldrich. The domain structures of cytokine/NAg fusion proteins used in this study are shown in Table I. Derivations of baculovirus expression systems, the protein purification procedures, and the biological activities for each of these proteins were described elsewhere (22). The synthetic peptide GP69–88 (YGSLPQKSQRSQDENPVVHF) was obtained from Quality Controlled Biologicals.

Tolerance induction

To determine whether cytokine/NAg proteins prevent the active induction of EAE in Lewis rats, rats were given a total of three s.c. injections of a given cytokine/NAg protein. A dose of 0.5–1 nmol of cytokine/NAg was delivered during each injection at 1-to 2-wk intervals as designated. The cytokine/NAg proteins were either solubilized in saline or emulsified in alum. At least 7 days after the last injection, rats were challenged with NAg in CFA (day 0) to induce EAE. Alternatively, rats were challenged with NAg in CFA (day 0) and injected s.c. with fusion proteins or controls (1 nmol in saline) beginning on day 5 and then every other day through day 9, 11, or 13 as designated.

Induction and clinical assessment of EAE

EAE was induced in Lewis rats by the injection of an emulsion containing 25 or 50 μg of GPMBP in CFA (200 μg of *Mycobacterium tuberculosis*). In designated experiments, rats were challenged with an emulsion containing 50 μg of the dihydrofolate reductase (DHFR)-NAg fusion protein in CFA. DHFR-NAg was comprised of the mouse DHFR as the N-terminal domain and the encephalitogenic GP69–87 peptide of GPMBP as the C-terminal domain. The emulsion (total volume of 0.1 ml per rat) was injected in two 0.05-ml volumes on either side of the base of the tail. The following scale was used to assign intensity of EAE: 0.25, paralysis in the distal tail; 0.5, limp tail; 1.0, ataxia; 2.0, hind leg paresis (retained some voluntary movement in the hind limbs but could not ambulate upright); and 3.0, full hind leg paralysis. The cumulative score for each rat consisted of the sum of daily scores for each animal. The median cumulative score and median maximal scores were the respective medians for all scores in each group.

Lewis rat T cells

The RsL.11 T cell and the *R1-trans* clone were described previously (14). T cells were assayed in complete RPMI 1640 medium and were maintained in same medium supplemented with recombinant rat IL-2. The complete RPMI 1640 medium consisted of 10% heat-inactivated FBS, 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin (BioWhittaker), and 50 μM 2-ME (Sigma-Aldrich). Rat IL-2 was obtained from a recombinant baculovirus expression system (23).

In vitro proliferation

Responder T cells (2.5×10^4/well) were cultured with irradiated splenocytes (5×10^5/well) and designated concentrations of Ag. After 2 days of culture, T cells were pulsed with 1 μCi of [^{3}H]thymidine (6.7 Ci/mmol; PerkinElmer). After another 1 day of culture, T cells were harvested onto filters to measure [^{3}H]thymidine incorporation by scintillation counting. Error bars portray SD values.

Statistical analysis

The median cumulative score was used as a measure of overall disease for a group of rats. This measure was based on the assumption that the ordinal scale used for the assessment of EAE (0, 0.25, 0.5, 1.0, 2.0, and 3.0) was an approximate quantitative representation of disease severity. Based on this assumption, the daily scores for each rat were summed to calculate the cumulative score for each rat. The median of all scores within a group represented an overall measure of EAE for a treatment group. Significant differences between median values for each treatment group were assessed by nonparametric ANOVA based on ranks. The median maximal intensity was used as a measure of peak disease severity for a group of rats. Each rat was scored based on the most severe clinical sign exhibited by the rat during the evaluation period (0, 0.25, 0.5, 1.0, 2.0, or 3.0). Median values for the treatment groups were compared for statistical significance by nonparametric ANOVA based on ranks.

The onset of disease was reported as the mean ($\pm$SD) for each treatment group, and differences between groups were assessed by parametric ANOVA. Comparisons restricted to two groups were assessed with a Mann-Whitney *U* Test or an unpaired *t* test. Incidence of severe EAE was based on whether rats exhibited an impaired gait or worse (i.e., 1.0, 2.0, or 3.0; ataxia to full hind limb paralysis). Incidence of severe EAE was assessed by Fisher's exact test or the χ^2 test for independence. The "mean number of days with severe EAE" was the average duration (no. of days $\pm$ SD) that rats in a group had severe EAE. Differences between groups were assessed by parametric ANOVA.

Two-way ANOVA was used when data from two separate experiments were compiled (Tables II, III, and V; comparison of experiment no.1 vs experiment no.2 and comparison of compiled treatment groups). Two-way ANOVA was performed with the Bonferroni post hoc test. Otherwise, one-way ANOVA was used to compare medians or means from different treatment groups (Table IV and VI). Kruskal-Wallis nonparametric ANOVA based on ranks with a Dunn's multiple comparison test was used to assess ranked data (median cumulative score and median maximal intensity). Parametric ANOVA with a Tukey-Kramer multiple comparison test was used to assess data based on a ratio scale (mean day of onset; mean number of days with severe EAE).

Results

Cytokine/NAg "vaccines" protect against the subsequent active induction of EAE

Subcutaneous injection of the IL2NAg or IL4NAg fusion proteins in saline or in alum did not cause EAE and did not cause adverse reactions at the injection site (data not shown). Pretreatment of rats with IL2NAg (IL2.7) in saline or alum significantly attenuated the subsequent induction of EAE (Table II). A total of three s.c. injections of IL2.7 (0.5 or 1 nmol per injection) were administered to each rat over the course of 1–2 mo and then the rats were then challenged with GPMBP in CFA to elicit EAE. Pretreatment of IL2.7 in saline or alum significantly reduced the median cumulative score and the median maximal intensity of EAE. When administered in saline, IL2.7 also resulted in a delayed onset of disease. Pretreatment with either IL2NAg in saline or IL2NAg in alum significantly reduced incidence of severe EAE and reduced the mean number of days that rats exhibited severe EAE. The mechanism by which IL2.7 inhibited EAE most likely involved tolerance induction because at least 1 wk elapsed between the last fusion protein injection and the encephalitogenic challenge, a delay that allowed ample time for clearance of the fusion proteins before challenge.

Table II. *Vaccination with the IL2NAg fusion protein protected against EAE*

Exp. No.	Treatment[a]	Incidence of EAE	Median Cumulative score[b]	Median Maximal Intensity[b]	Mean Day of Onset[b]	Incidence of Severe EAE[c]	Mean No. of Days with Severe EAE[d]
1	Saline alone	6 of 6	9.5	3.0	11.5 ± 1.5	6 of 6	4.7 ± 1.5
	IL2.7-saline	3 of 4	1.0	0.3	15.3 ± 1.5	0 of 4	0.0
2	Saline alone	6 of 6	11.1	3.0	8.7 ± 0.8	5 of 6	3.3 ± 1.8
	IL2.7-saline	5 of 6	1.1	0.4	12.8 ± 1.3	2 of 6	1.0 ± 1.6
	IL2.7-alum	1 of 6	0.0	0.0	8.0 ± 0.0	0 of 6	0.0
1&2	Saline alone	12 of 12	10.4	3.0	10.1 ± 1.9	11 of 12	4.0 ± 1.7
	IL2NAg-saline	8 of 10	1.0	0.3	13.8 ± 1.8	2 of 10	0.6 ± 1.4
	IL2NAg-alum	1 of 6	0.0	0.0	8.0	0 of 6	0.0

[a] Data were pooled from two independent experiments. In experiment (Exp.) no. 1, rats were pretreated with saline or 0.5 nmol of IL2.7 (IL2NAg) on days −60, −42, and −20 and were challenged with 50 μg of GPMBP in CFA on day 0. In experiment no. 2, rats were pretreated with saline or 1 nmol of IL2.7 (in saline or alum) on days −35, −21, and −7 and were challenged with 25 μg of GPMBP in CFA on day 0.

[b] Combined experiments. Median cumulative scores and median maximal intensity scores of rats pretreated with IL2NAg/saline ($p < 0.001$) or IL2NAg/alum ($p < 0.001$) were significantly less than the respective scores for rats treated with saline (two-way nonparametric ANOVA on ranks; Bonferroni post hoc test). The mean day of onset of rats treated with IL2NAg in saline was significantly delayed compared to that for rats pretreated with saline (unpaired t test, $p = 0.0004$).

[c] Combined experiments. Rats that exhibited ataxia, hind limb paresis, or full hind limb paralysis were scored as positive for severe EAE. Incidence of severe EAE in rats pretreated with IL2NAg/alum ($p = 0.0004$) or with IL2NAg/saline ($p = 0.0015$) was significantly less than the respective incidence in rats pretreated with saline (pairwise comparisons by Fisher's exact test).

[d] Each rat was scored for the total number of days that the rat exhibited severe EAE. The mean number of days that IL2NAg/alum-pretreated rats ($p < 0.001$) and IL2NAg/saline-treated rats ($p < 0.001$) exhibited severe EAE was significantly less than that for saline pre-treated rats (two-way parametric ANOVA; Bonferroni post hoc test).

The tolerogenic activity of the IL-2NAg fusion protein was contingent upon the covalent linkage of IL-2 and NAg (Table III). Rats pretreated with IL2EKdel (IL2NAg fusion protein with deletion of the enterokinase cleavage site) had a lower median cumulative score and a reduced median maximal intensity compared with rats pretreated with saline, the encephalitogenic GP69–88 synthetic peptide, purified rat IL-2, or the combination of IL-2 and GP69–88. Rats pretreated with IL2EKdel also had a delayed onset of EAE and had shorter duration of severe EAE compared with rats pretreated with the relevant controls. These findings indicated that the tolerogenic activity of IL2NAg was Ag-specific rather than a cytokine-dependent, Ag-nonspecific activity. The tolerogenic activity of IL2NAg also could not be attributed to the Ag alone. Rather, the tolerogenic activity reflected a synergy attributed to the

Table III. *Covalent tethering of IL-2 and NAg was necessary for tolerogenic activity*

Exp. No.	Treatment[a]	Incidence of EAE	Median Cumulative Score[b]	Median Maximal Intensity[b]	Mean Day of Onset[c]	Incidence of Severe EAE[d]	Mean No. of Days with Severe EAE[d]
1	Saline alone	5 of 5	9.5	2.0	8.2 ± 0.8	5 of 5	4.0 ± 1.0
	GP69–88	5 of 5	9.8	3.0	8.8 ± 2.2	5 of 5	3.8 ± 0.8
	IL2	5 of 5	6.3	2.0	8.8 ± 1.3	5 of 5	4.4 ± 1.1
	IL2 & GP69–88	4 of 4	10.8	2.5	8.8 ± 1.0	4 of 4	4.3 ± 1.0
	IL2Ekdel	4 of 4	3.6	1.3	10.0 ± 0.8	2 of 4	1.5 ± 1.7
2	Saline alone	7 of 7	9.3	3.0	11.0 ± 1.2	7 of 7	3.3 ± 1.0
	GP69–88	7 of 7	8.5	3.0	10.7 ± 1.0	7 of 7	3.3 ± 0.5
	IL2	8 of 8	11.3	3.0	9.8 ± 0.9	8 of 8	3.5 ± 0.5
	IL2 & GP69–88	6 of 6	12.0	3.0	11.3 ± 0.8	6 of 6	3.3 ± 0.8
	IL2Ekdel	8 of 8	4.6	2.0	12.5 ± 0.5	7 of 8	2.0 ± 1.2
1&2	Saline alone	12 of 12	9.4	3.0	9.8 ± 1.7	12 of 12	3.6 ± 1.0
	NAg	12 of 12	9.0	3.0	9.9 ± 1.8	12 of 12	3.5 ± 0.7
	IL2	13 of 13	9.3	3.0	9.4 ± 1.1	13 of 13	3.8 ± 0.9
	IL2 & NAg	10 of 10	12.0	3.0	10.3 ± 1.6	10 of 10	3.7 ± 0.9
	IL2NAg	12 of 12	4.3	2.0	11.7 ± 1.4	9 of 12	1.8 ± 1.3

[a] Rats were pretreated with saline, 1 nmol of IL2Ekdel (IL2NAg), 1 nmol of GP69–88 (NAg), 1 nmol of IL-2 (without NAg), or the combination of GP69–88 and IL-2. Rats (IL-2 and GP69–88) were treated with separate injections of 1 nmol of IL-2 and 1 nmol of GP69–88 at a distance of <0.5 cm apart near the base of the tail. In experiment no. 1, rats were pretreated on days −27, −20, and −13 and were challenged with 50 μg of GPMBP in CFA on day 0. In experiment no. 2, rats were pretreated on days −35, −21, and −7 and were challenged with 50 μg of DHFR-NAg in CFA on day 0.

[b] Combined experiments. The median cumulative score ($p < 0.002$, all comparisons) and the median maximal score ($p < 0.02$, all comparisons) of rats pretreated with IL2NAg was significantly less than the respective scores of rats treated with saline, NAg, IL-2 alone, or the combination of IL-2 and NAg (two-way nonparametric ANOVA on ranks; Bonferroni post hoc test).

[c] Combined experiments. The mean day of onset of rats pretreated with IL2NAg was significantly delayed compared to the respective means of rats treated with either saline ($p = 0.049$) or IL-2 alone ($p = 0.005$) (two-way parametric ANOVA; Bonferroni post hoc test).

[d] Combined experiments. Rats that exhibited ataxia, hind limb paresis, or full hind limb paralysis were scored as positive for severe EAE. Shown is the incidence of severe EAE together with the mean number of days each group exhibited severe EAE. The mean number of days that IL2NAg-treated rats were severely afflicted with EAE was significantly less than the respective means for groups pretreated with saline, NAg, IL-2, or the combination of IL-2 and NAg ($p \leq 0.001$ for all comparisons) (two-way parametric ANOVA; Bonferroni post hoc test).

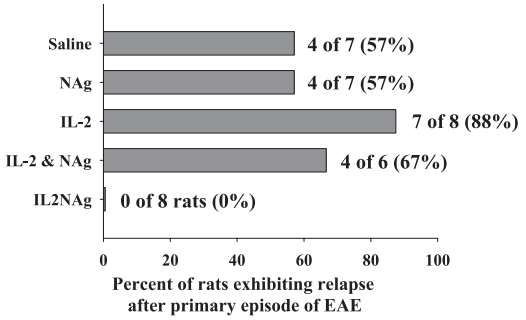

FIGURE 1. A prechallenge treatment regimen of IL2NAg prevented a subsequent relapse of EAE. Shown are data from experiment no.2 of Table III. The frequency of relapse was significantly less in IL2NAg-treated rats compared with saline-treated or NAg (GP69–88)-treated rats ($p = 0.0256$), IL2-treated rats ($p = 0.0014$), or IL2 and NAg-treated rats ($p = 0.0150$). Pairwise comparisons were performed by Fisher's exact test.

covalent linkage of IL-2 and NAg. Lastly, these data provide evidence that two independently derived IL2NAg fusion proteins, (IL2.7 (Table II) and IL2EKdel (Table III)) had tolerogenic activity.

Rats challenged with DHFR-NAg in CFA often had a single relapse marked by a milder second course of EAE. Pretreatment with IL2EKdel completely prevented relapse in rats challenged with DHFR-NAg, whereas >50% of rats in the other four groups had spontaneous relapses (Fig. 1). The primary bout of EAE ranged from day 7 through day 16 with peak disease from day 12 to day 15, whereas the secondary bout of EAE ranged from day 20 through day 30 with peak disease from day 23 to day 26. In this experiment, IL2NAg was administered on days −35, −21, and −7, whereas the initiation of the second bout of EAE began on day 20. The finding of reduced disease severity in primary EAE (Table III, experiment no.2) and abrogation of EAE in the subsequent relapse (Fig. 1) indicated that the tolerogenic consequences of IL2NAg treatment endured for >1 mo.

When delivered in either saline or alum, the IL4.4 fusion protein lacked tolerogenic activity (Table IV). Rats were given s.c. injections of IL4.4, IL2.7, or GPMBP at a dose of 1 nmol on days −42, −28, and −14 and then were challenged with 25 μg of GPMBP in CFA on day 0. Again, pretreatment of rats with IL2.7 significantly decreased the median cumulative score and the median maximal intensity of EAE and delayed the onset of EAE compared with control groups. Furthermore, IL2NAg pretreatment abrogated the incidence of severe EAE, whereas >50% of rats in the three other pooled treatment groups exhibited severe EAE. These data indicate that IL-2 is more efficient than IL-4 as a fusion partner for the induction of tolerance to NAg.

When cytokine/NAg fusion proteins were injected in alum rather than saline (Tables II and IV), the encephalitogenic challenge resulted in an accelerated onset of EAE. The ability of alum to accelerate the course of disease was independent of the ability of IL2.7 to suppress disease. Thus, pretreatment with IL2.7 in alum decreased the incidence and severity of EAE but nonetheless accelerated disease onset. The alum adjuvant did not appear to consistently augment the tolerogenic activity compared with injection of the tolerogen in saline (compare Tables II and IV). Because alum promoted accelerated disease, the alum adjuvant was contraindicated for use in tolerogenic vaccines.

Data shown in Tables II–IV indicated that IL2NAg treatment before encephalitogenic challenge significantly suppressed disease severity. IL2NAg was also an effective inhibitor of EAE when delivered after an encephalitogenic challenge (Tables V and VI). Rats treated with IL2NAg on days 5, 7, and 9 (experiment no.1) or on days 5, 7, 9, and 11 (experiment no.2) had significantly reduced median cumulative scores and median maximal intensities compared with rats treated with either NAg (GP69–88) or IL4NAg. Rats treated with IL2NAg also had significant reductions in the number of days in which these rats were afflicted with severe EAE compared with either control group. The covalent linkage of IL2 and NAg was required for the immunosuppressive activity of the postchallenge treatment regimen (Table VI). IL2NAg substantially

Table IV. *IL2NAg was a more effective tolerogen than the IL4NAg fusion protein*

Treatment[a]	Incidence of EAE	Mean Day of Onset[b]	Pooled Treatment Groups[c]	Median Cumulative Score[c]	Median Maximal Intensity[c]	Incidence of Severe EAE[d]	Mean No. of Days with Severe EAE[e]
Saline alone	15 of 15	10.1 ± 1.5	Saline	2.5	1.0	8 of 15 (53%)	1.6 ± 1.5
GPMBP/saline	3 of 4	12.3 ± 2.5	GPMBP	2.5	1.0	5 of 9 (56%)	1.2 ± 1.3
GPMBP/alum	4 of 5	7.0 ± 1.4					
IL4.4/saline	9 of 10	11.0 ± 3.3	IL4NAg	3.1	1.0	11 of 16 (69%)	1.6 ± 1.2
IL4.4/alum	6 of 6	7.0 ± 0.6					
IL2.7/saline	2 of 5	16.5 ± 2.1	IL2NAg	0.3	0.1	0 of 10 (0%)	0.0 ± 0.0
IL2.7/alum	3 of 5	7.3 ± 1.5					

[a] Rats were given subcutaneous injections of IL4.4 (IL4NAg), IL2.7 (IL2NAg), or GPMBP at a dose of 1 nmol on days −42, −28, and −14 and then were challenged with 25 μg of GPMBP in CFA on day 0.

[b] The mean day of onset of rats treated with IL2NAg in saline was significantly delayed compared to that for rats treated with IL4NAg in saline ($p = 0.0364$) (Mann-Whitney *U* Test). To assess the effects of the alum adjuvant on the mean day of onset, pooled data for the GPMBP/saline, IL4NAg/saline, and IL2NAg/saline groups ($n = 14$) revealed a significant delay compared to that for pooled data of the GPMBP/alum, IL4NAg/alum, and IL2NAg/alum groups ($n = 13$) ($p = 0.0002$). Unpaired *t* tests were used to confirm these differences: GPMBP/saline vs GPMBP/alum; $p = 0.0153$; IL4NAg/saline vs IL4NAg/alum, $p = 0.0128$; and IL2NAg/saline vs IL2NAg alum, $p = 0.0105$.

[c] Because the median cumulative score and the median maximal intensity were not affected by the saline or alum adjuvant despite differences in the mean day of onset, the data for each respective protein injected in saline or alum were pooled for statistical analysis of disease intensity. The median cumulative score and the median maximal intensity score for rats injected with IL2NAg (pooled saline and alum groups) was significantly less than the respective medians of the control group (saline only, $p < 0.01$), the pooled GPMBP group ($p < 0.05$), and the pooled IL4NAg group ($p < 0.001$) (Kruskal-Wallis nonparametric ANOVA on ranks; Dunn's multiple comparison test).

[d] Rats that exhibited ataxia, hind limb paresis, or full hind limb paralysis were scored as positive for severe EAE. The incidence of severe EAE in rats treated with IL2NAg was significantly less than the respective incidences of rats treated with saline ($p = 0.0077$), GPMBP ($p = 0.0108$), or IL4NAg ($p = 0.0007$) by Fisher's exact test. These differences were confirmed for groups possessing a sufficient n (saline, IL4NAg, and IL2NAg) by the χ^2 test for independence.

[e] Each rat was scored for the total number of days that the rat exhibited severe EAE. Shown are the mean and SD for each pooled group (saline and alum). The mean number of days that the IL2NAg-treated rats were severely afflicted with EAE was significantly less than the respective means of the groups pretreated with saline ($p < 0.05$), GPMBP ($p < 0.05$), or IL4NAg ($p < 0.01$) (ANOVA; Tukey-Kramer multiple comparisons test).

Table V. *Administration of IL2NAg after encephalitogenic sensitization also attenuated EAE*

Exp. No.	Treatment[a]	Incidence of EAE	Median Cumulative Score[b]	Median Maximal Intensity[b]	Mean Day of Onset[c]	Incidence of Severe EAE[d]	Mean No. of Days with Severe EAE[d]
1	GP69–88	8 of 8	11.3	3.0	10.9 ± 1.4	8 of 8	3.1 ± 0.6
	IL4Ekdel	6 of 6	7.6	3.0	12.8 ± 1.2	6 of 6	2.8 ± 0.4
	IL2Ekdel	6 of 6	3.8	2.0	13.5 ± 0.8	5 of 6	1.8 ± 1.0
2	GP69–88	8 of 8	7.5	3.0	9.9 ± 1.1	8 of 8	2.6 ± 0.9
	IL4Ekdel	9 of 9	6.3	2.0	12.4 ± 1.3	7 of 9	3.0 ± 1.3
	IL2Ekdel	8 of 9	1.0	1.0	12.5 ± 2.6	3 of 9	1.1 ± 1.2
1&2	NAg	16 of 16	9.4	3.0	10.4 ± 1.3	16 of 16	2.9 ± 0.8
	IL4NAg	15 of 15	7.3	3.0	12.6 ± 1.2	14 of 15	2.9 ± 1.0
	IL2NAg	14 of 15	3.3	2.0	12.9 ± 2.0	10 of 15	1.4 ± 1.1

[a] Rats were sensitized with 50 μg of DHFR-NAg in CFA on day 0. Rats were then subcutaneously injected with 1 nmol of GP69–88 (NAg), IL4NAg (IL4Ekdel), or IL2NAg (IL2Ekdel) in saline on days 5, 7, and 9 (experiment no. 1) or on days 5, 7, 9, and 11 (experiment no. 2).

[b] Combined experiments. The median cumulative score and the median maximal intensity of rats treated with IL2NAg was significantly less than the respective medians for rats treated with either IL4NAg or NAg ($p < 0.001$ for all comparisons) (two-way nonparametric ANOVA on ranks; Bonferroni post hoc test).

[c] Combined experiments. The mean day of onset of groups treated with IL2NAg or IL4NAg was significantly delayed compared to rats treated with NAg ($p \leq 0.001$ for both comparisons) (two-way parametric ANOVA; Bonferroni post hoc test).

[d] Combined experiments. Rats that exhibited ataxia, hind limb paresis, or full hind limb paralysis were scored as positive for severe EAE. The mean number of days that IL2NAg-treated rats were severely afflicted with EAE was significantly less than the respective means for groups treated with IL4NAg or NAg ($p < 0.001$ for each comparison) (two-way parametric ANOVA; Bonferroni post hoc test).

reduced the median cumulative score, the median maximal intensity, the incidence of severe EAE, and the mean number of days that rats were afflicted with severe EAE, whereas the delivery of IL-2 and NAg simultaneously as separate molecules had no tolerogenic activity. These data indicate that IL2NAg is a highly effective inhibitor when delivered after encephalitogenic challenge and that IL2NAg can attenuate an ongoing encephalitogenic immune response. Comparison of the postchallenge treatment regimens (days 5, 7, and 9; days 5, 7, 9, and 11; days 5, 7, 9, 11, and 13) for data displayed in Tables V and VI showed progressively more effective inhibition of EAE as treatments were extended into the phase of disease onset and maximal paralysis. These data provide suggestive evidence that IL2NAg directly impairs the effector phase of an ongoing encephalitogenic immune response.

Cytokine/NAg fusion proteins target NAg to APC

This study was based on the hypothesis that the cytokine moiety of the cytokine/NAg fusion protein would target NAg to APC and thereby result in enhanced T cell Ag recognition. As shown in Fig. 2*A*, purified cytokine/NAg fusion proteins stimulated proliferation of an encephalitogenic $CD4^+$ clone specific for the 72–86 region of MBP in the presence of irradiated splenic APC. The IL4.4 fusion protein was >100-fold more potent than GPMBP based on a comparison of the Ag concentrations eliciting a half-maximal response. The proliferative response to IL2.7 was bimodal. Concentrations of IL2.7 in the 10 pM to 1 nM range stimulated ~20,000 cpm of [^{3}H]thymidine incorporation. IL2.7 concentrations of 1 nM to 1 μM stimulated the second mode of responsiveness.

IL4.4 and IL2.7 stimulated T cell proliferation by a mechanism restricted by MHCII I-A but not I-E (Fig. 2*B*). Responses elicited by IL4.4 and IL2.7 were completely abrogated by the OX6 mAb (anti-I-A MHCII) but not affected by the OX17 (anti-I-E MHCII) mAb. Indeed, the antigenic reactivity of IL4.4 was inhibited by over five orders of magnitude by OX6, whereas the response to GPMBP was inhibited by an ~100-fold margin. These findings were in accordance with the known Ag restriction of the RsL.11 clone. Thus, when cultured with these cytokine/NAg fusion proteins and irradiated splenic APC, RsL.11 T cells exhibited Ag-stimulated proliferation rather than a mitogenic response to the cytokine domain. Other control cytokine/NAg fusion proteins

Table VI. *Covalent tethering of IL-2 and NAg was necessary to inhibit EAE when IL2NAg was administered during onset of EAE*

Treatment[a]	Incidence of EAE	Median Cumulative Score[b]	Median Maximal Intensity[b]	Mean Day of Onset[c]	Incidence of Severe EAE[d]	Mean No. Days with Severe EAE[d]
Saline alone	10 of 10	8.6	3.0	9.8 ± 0.6	10 of 10	3.0 ± 0.7
IL2 and GP69–88	10 of 10	7.9	3.0	9.9 ± 0.9	9 of 10	3.0 ± 1.3
IL2NAg	8 of 10	0.6	0.3	11.5 ± 1.6	2 of 10	0.5 ± 1.1

[a] Rats were sensitized with 50 μg of DHFR-NAg in CFA on day 0. Rats were then injected with saline, 1 nmol of IL2NAg (IL2Ekdel) in saline, or with separate injections of 1 nmol of IL-2 and 1 nmol of GP69–88 in saline s.c. at a distance of <0.5 cm apart near the base of the tail on days 5, 7, 9, 11, and 13.

[b] The median cumulative score of rats treated with IL2NAg was significantly less than the respective scores of rats treated with saline or the combination of IL-2 and GP69–88 ($p < 0.01$ or $p < 0.05$, respectively). The median maximal score ($p < 0.01$ all comparisons) of rats pretreated with IL2NAg was significantly less than the respective scores of rats treated with saline or the combination of IL-2 and GP69–88 (Kruskal-Wallis nonparametric ANOVA on ranks; Dunn's multiple comparison test).

[c] The mean day of onset of rats pretreated with IL2NAg was significantly delayed compared to the respective means of rats treated with either saline ($p < 0.01$) or the combination of IL-2 and GP69–88 ($p < 0.05$) (one-way parametric ANOVA; Tukey-Kramer multiple comparisons test).

[d] Rats that exhibited ataxia, hind limb paresis, or full hind limb paralysis were scored as positive for severe EAE. The incidence of severe EAE in rats treated with IL2NAg was significantly less than the incidence for rats treated with the combination of IL2 and NAg ($p = 0.0055$, Fisher's Exact Test). The mean number of days that IL2NAg-treated rats were severely afflicted with EAE was significantly less than the respective means for groups pretreated with saline or the combination of IL2 and GP69–88 ($p \leq 0.001$ for all comparisons) (one-way parametric ANOVA; Tukey-Kramer multiple comparisons test).

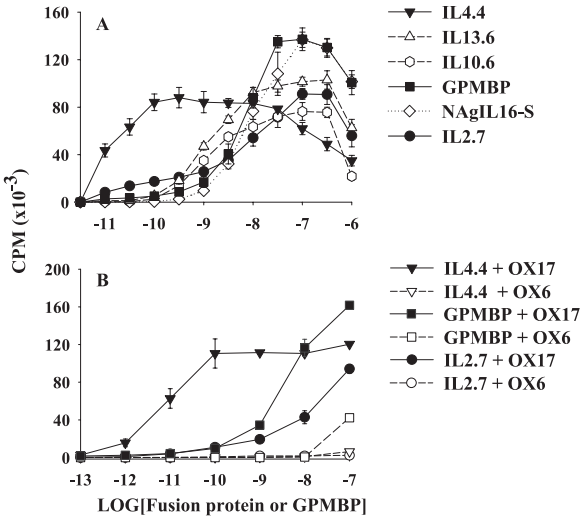

FIGURE 2. Purified cytokine/NAg fusion proteins stimulated the antigenic proliferation of an MBP-specific T cell clone. *A*, RsL.11 T cells (25,000/well) and irradiated splenocytes (500,000/well) were cultured with designated concentrations of the respective fusion protein. *B*, RsL.11 T cells and irradiated splenic APC were cultured with designated concentrations of GPMBP, IL4.4, or IL2.7 in the presence or absence of the anti-rat I-A MHCII OX6 mAb or anti-rat I-E MHCII OX17 mAb. Cultures were pulsed with [^{3}H]thymidine during the last 24 h of a 72-h culture. These data are representative of three experiments.

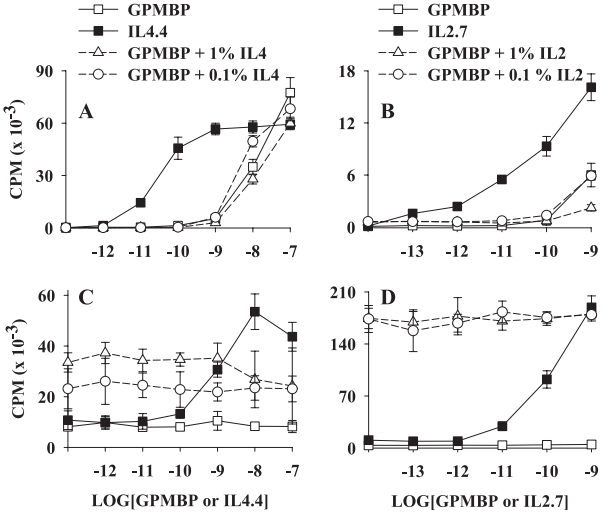

FIGURE 3. Enhanced potency of IL4.4 and IL2.7 was dependent upon the covalent linkage of cytokine and NAg. *A* and *B*, RsL.11 T cells (25,000/well) and irradiated splenocytes (500,000/well) were used to assay antigenic activity of the NAg. *C*, Thymocytes (1×10^6/well) cultured with 1 μM PMA and rat IL-2 (0.4% baculovirus supernatant) were used to assay IL-4 activity. *D*, CTLL cells (1×10^4/well) were used to assay IL-2 activity. All cells in the figure were cultured with or without designated concentrations of GPMBP, IL4.4, or IL2.7 in the presence or absence of IL-4 or IL-2 (1% or 0.1% baculovirus supernatants). Cultures were pulsed with [^{3}H]thymidine during the last 24 h of a 72-h culture. Different scales were used for the y-axes. These data are representative of three experiments.

(IL10.6, IL13.6, and NAgIL16-S) had antigenic reactivity similar to that of GPMBP (Fig. 2*A*). These responses were also fully blocked by an anti-MHCII I-A mAb (OX6) and thereby represented antigenic responses to the encephalitogenic peptide (not shown). These data indicated that the encephalitogenic GP73–87 sequence in each fusion protein was processed and presented on MHCII glycoproteins.

Covalent linkage of the respective cytokine with the NAg was required for the enhanced potency of Ag recognition. IL4.4 was ~100-fold more potent than GPMBP even when GPMBP was added in culture with saturating concentrations of IL-4 as a separate molecule (Fig. 3*A*). IL-4 activity of the IL4.4 protein and the 1% and 0.1% IL4.4 baculovirus supernatants was confirmed in a mitogenesis assay of thymocytes (Fig. 3*C*). Because the thymocytes were costimulated with PMA and saturating concentrations of IL-2 in all wells, the assay specifically detected IL-4 activity but not IL-2 activity. The enhanced antigenic potency of IL4.4 could not be explained by an independent action of IL-4 on either APC or T cell responders. For example, the ability of IL-4 to induce MHCII on B cells could not explain the enhanced potency of the IL4.4 Ag, because IL4-induced MHCII induction would increase antigenic potency without requirement for cytokine-Ag linkage. The antigenic activity of IL4.4 was also substantially more active than the IL-4 activity of IL4.4 (compare Fig. 3, *A* and *C*). The interpretation most consistent with these data is that the IL-4 domain of IL4.4 directly interacted with IL-4 receptors on APC to catalyze the uptake of the tethered NAg into the MHCII Ag processing pathway, thereby resulting in enhanced presentation of the relevant NAg.

Covalent linkage of IL-2 and NAg was also critical for the enhanced potency of IL2.7 (Fig. 3*B*). That is, IL2.7 was ~100-fold more potent than GPMBP even when GPMBP was added to culture with saturating concentrations of IL-2 as a separate molecule. The IL-2 activity of IL2.7 and the 1% and 0.1% IL2.7 baculovirus supernatants was confirmed in a mitogenesis assay of CTLL cells (Fig. 3*D*). IL-2 did not directly stimulate RsL.11 T cells, because these T cells were rested and had low concentrations of IL-2 receptors. Thus, the enhanced potency of IL2.7 compared with that of GPMBP could not be explained by the mitogenic activity of IL-2. Rather, the covalent tethering of IL-2 and NAg enabled synergistic antigenic activity that could not be duplicated by adding IL-2 and NAg to a culture as separate molecules. Again, the most consistent interpretation is that the IL-2 moiety interacted with IL-2R on APC to target the covalently linked NAg into the MHCII Ag processing pathway.

The IL2.7 fusion protein was also substantially more potent than GPMBP in a T cell-mediated cytotoxic assay (Fig. 4). In these experiments, blastogenic MHCII$^+$ T cells from the *R1-trans* T cell

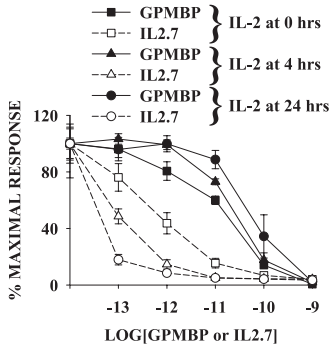

FIGURE 4. T cell-mediated presentation of IL2.7 was inhibited by soluble IL-2. The MHCII$^+$ blastogenic *R1-trans* T cell clone was starved of IL-2 for 24 h before the assay. At the initiation of the assay, *R1-trans* T cells were cultured with irradiated MBP-specific RsL.11 responders and designated concentrations of IL2.7 or GPMBP (*x*-axis). Rat IL-2 (0.4% of a baculovirus supernatant) was added at 0, 4, or 24 h after initiation of culture. Cultures were pulsed with [^{3}H]thymidine during the last 24 h of culture of a 72-h assay. These data are representative of three experiments.

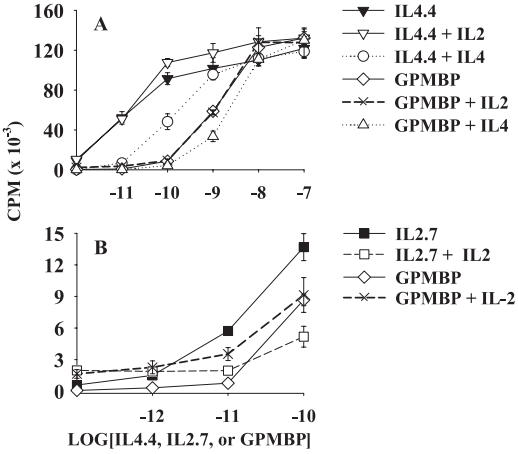

FIGURE 5. In the presence of irradiated splenic APC, the presentation of IL2.7 and IL4.4 were inhibited by IL-2 (*B*) and IL-4 (*A*), respectively. RsL.11 T cells and irradiated splenic APC were cultured for 3 days with designated concentrations of GPMBP, IL4.4, or IL2.7 in the presence or absence of 1% (v/v) baculovirus supernatant containing IL-2 or IL-4. IL-2 or IL-4 was added to culture 3 h before Ag. Cultures were pulsed with [^{3}H]thymidine during the last 24 h of a 72 h culture. Different scales were used for the *y*-axes. These data are representative of three experiments.

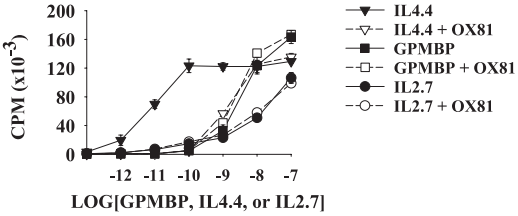

FIGURE 6. IL4.4 and MBP are equally accessible to Ag processing. RsL.11 T cells and irradiated splenic APC were cultured for 3 days with designated concentrations of GPMBP, IL4.4, or IL2.7 in the presence or absence of the OX81 mAb against rat IL-4. The mAb were added to culture 3 h before Ag. Cultures were pulsed with [^{3}H]thymidine during the last 24 h of a 72-h culture. These data are representative of three experiments.

clone were the APC. Previous studies showed that the presentation of Ag by *R1-trans* T cells to irradiated Ag-specific responders resulted in the Ag-specific killing of *R1-trans* T cells by a MHCII-restricted mechanism. In this assay, *R1-trans* T cells grew rapidly in the presence of IL-2 unless these T-APC were killed by irradiated RsL.11 responders (14, 24). The assays were devised such that the IL2.7 fusion protein competed with IL-2 for the IL-2R on T-APC. Cultures were supplemented with rat IL-2 at the initiation of culture (IL-2 at 0 h), 4 h after the initiation of culture (IL-2 at 4 h), or 24 h after initiation of culture (IL-2 at 24 h). *R1-trans* T-APC mediated rapid IL-2 dependent growth (*y*-axis) unless the irradiated RsL.11 T cells killed *R1-trans* T cells upon recognition of the Ag on *R1-trans* T cells. Irradiation of RsL.11 T cells prevented their Ag-specific proliferation but not cytotoxicity. The data indicated that when the addition of IL-2 was delayed until 24 h after the initiation of culture, IL2.7 was >1000 times more potent than GPMBP. However, when IL-2 was added at the initiation of culture with IL2.7 or GPMBP, IL2.7 was only ~32-fold more potent than GPMBP. These data indicate that the IL2.7 fusion protein competed with IL-2 for cell surface IL-2 receptors and that this competition determined the quantity of the IL2.7-associated NAg loaded into the MHCII-Ag processing pathway. Overall, these data support the hypothesis that IL-2 receptors on T cells can be used to target Ag to the MHCII-Ag processing pathway of activated T cells to enhance Ag recognition.

The potentiated responses of RsL.11 T cells to IL4.4 and IL2.7 that were stimulated in the presence of irradiated splenic APC were also inhibited in part by IL-4 and IL-2, respectively. For example, IL-4 inhibited the response to IL4.4 by ~10-fold but only slightly inhibited the response to GPMBP (Fig. 5*A*) or IL2.7 (not shown). Likewise, IL-2 inhibited the IL2.7 proliferative activity by ~10-fold but slightly enhanced the proliferative responses to GPMBP (Fig. 5*B*) and IL4.4 (not shown). These data provide additional evidence that the enhanced antigenic potency of IL4.4 and IL2.7 was due to the targeting of fusion proteins to the respective cytokine receptors.

The availability of a mAb specific for IL-4 (OX81) enabled another approach to assess the ability of the cytokine domain of the fusion protein to target the NAg to APC. This mAb enabled neutralization of the IL-4 domain in the IL4NAg fusion protein. The antigenic activity of IL4.4 was inhibited by the OX81 mAb by ~100-fold to the extent that IL4.4 was rendered equipotent to GPMBP (Fig. 6). The inhibitory action of OX81 was specific in that OX81 did not affect antigenic responses stimulated by either GPMBP or IL2.7. These data indicate that in the presence of OX81, the NAg in IL4.4 had antigenic reactivity that was essentially equal to GPMBP and that the encephalitogenic 73–89 sequences in IL4.4 and GPMBP were processed and presented equally as measured by the responses of cloned RsL.11 T cells.

Discussion

The tolerogenic efficacy of IL2NAg fusion proteins may not be surprising, given that IL-2 is uniquely involved in active tolerogenic mechanisms that are requisite for the maintenance of self-tolerance (25–28). IL-2-deficient mice, for example, exhibit lymphoproliferation marked by lymphadenopathy and splenomegaly, infiltration of memory CD4$^+$ and CD8$^+$ T cells into peripheral organs, hemolytic anemia, systemic autoimmunity, and premature death. In IL-2$^{-/-}$ mice, CD4$^+$ T cells bearing autoreactive specificity mediate autoimmune pathogenesis, whereas the administration of IL-2 or the introduction of IL-2-expressing T cells prevents autoimmune disease (26, 29–31). In fact, IL-2$^{-/-}$ mice that were treated with IL-2 were able to generate regulatory T cells that conferred protection upon the adoptive transfer into IL-2$^{-/-}$ recipients (26). Similar to the IL-2$^{-/-}$ deficiency, genetic defects of IL-2 receptor signaling pathways, including deficiency of IL-2Rα (32), IL-2Rβ (33, 34), Stat5α, or Stat5β (35), also result in lymphoproliferative and autoimmune disorders. In humans, a mutation in the CD25 IL-2Rα-chain has also been associated with extensive lymphocytic infiltration and inflammation of peripheral tissues (36). Thus, an important branch of the IL-2 signal transduction pathway may be implicated in a common mechanism of self-tolerance. The IL-2Rα (CD25) chain has also emerged as a prototypic marker of regulatory T cells that actively mediate self-tolerance (37–39). CD4$^+$ T cells depleted of a small subset of CD25$^+$ T cells transfer systemic autoimmune disease to athymic nude mice, whereas the cotransfer of CD25$^+$ T cells prevents autoimmunity. Because IL-2 is required for the generation of regulatory T cells and the induction of immunological tolerance, IL2NAg fusion proteins may facilitate regulatory T cell responses.

Several lines of evidence indicated that the IL-2 moiety of IL2NAg targeted NAg to APC. First, the covalent link between IL-2 and the NAg was critical for tolerance induction (Table III). The IL2NAg fusion protein IL2EKdel was an effective tolerogen whereas rat IL-2 and synthetic peptide GP69–88, when delivered alone or injected together at the same site, lacked suppressive activity. Second, the physical linkage of IL-2 and NAg was also essential for the potentiation of Ag recognition by a MBP-specific

T cell clone (RsL.11) in vitro. These experiments showed that IL2NAg was more potent than GPMBP even when GPMBP was tested in the presence of saturating IL-2 (Fig. 3*B*). Third, the mechanism by which the IL-2 domain of IL2NAg potentiated the antigenic response to IL2NAg was dependent on cell surface IL-2 receptors. As shown in Fig. 4, IL-2 and IL2NAg competed for a limited pool of IL-2 receptors on *R1-trans* T cells, and IL-2 competitively antagonized antigenic recognition of IL2NAg. In this cytotoxicity assay, IL2.7 was either ~32-fold or ~1000-fold more potent than GPMBP, depending on whether IL-2 was added at either the initiation of culture or after 24 h, respectively. Likewise, IL-2 inhibited IL2NAg-stimulated proliferation of RsL.11 T cells in the presence of irradiated splenic APC, whereas IL-2 did not affect or enhanced antigenic responses to GPMBP or IL4NAg (Fig. 5). These data indicate that the IL-2 moiety of IL2NAg mediated the targeting of NAg to APC via high affinity, high capacity binding of cell surface IL-2 receptors.

The APC targeted by the IL2NAg fusion protein were activated T cells that expressed high surface concentrations of IL-2R and that synthesized MHCII. *R1-trans* T cells constitutively synthesized MHCII (14, 24, 40, 41), and RsL.11 T cells that were activated with GPMBP in the presence of irradiated splenic APC also synthesized MHCII (42). Thus, in cultures of *R1-trans* T cells and RsL.11 responders (Fig. 4) the only APC present in the culture were activated IL-2R$^+$MHCII$^+$ T cells. In cultures of irradiated splenic APC and RsL.11 T cells (Figs. 3 and 5), IL-2R$^+$ APC were primarily blastogenic MHCII$^+$ RsL.11 T cells. These data indicate that IL2NAg targeted NAg to activated T cell APC in vitro.

The IL4NAg also targeted NAg to APC. IL4NAg (IL4.4) was ~100-fold more potent than GPMBP in the presence of irradiated splenic APC (Fig. 2). Covalent linkage of IL-4 and NAg was required for antigenic potentiation because, when added as a separate molecule, IL-4 alone did not potentiate the antigenic response to GPMBP (Fig. 3*A*), nor did IL-4 elicit mitogenic responses of RsL.11 T cells. The mechanism by which the IL-4 moiety of IL4NAg potentiated the antigenic response to IL4NAg was also dependent on cell surface IL-4 receptors. That is, the response to IL4NAg was antagonized ~10-fold in the presence of IL-4, whereas the response to GPMBP was only marginally inhibited by IL-4 (Fig. 5*A*). The enhanced recognition of NAg in IL4NAg was reversed in the presence of a neutralizing anti-IL-4 OX81 mAb, whereas antigenic recognition of GPMBP or IL2.7 was unaffected by OX81 (Fig. 6). Indeed, the antigenic response to IL4NAg was equipotent with GPMBP when the IL-4 moiety of IL4NAg could not bind cell surface IL-4 receptors due to Ab-mediated neutralization. These data indicate that IL4NAg targeted NAg to IL-4 receptors on splenic APC to potentiate processing and Ag recognition of the covalently tethered NAg.

Although both IL2NAg and IL4NAg targeted NAg to APC to enhance NAg recognition, IL2NAg inhibited EAE (Tables II–VI), whereas IL4NAg was not effective as a tolerogen at the tested dosages (Tables IV and V). These data indicate that the potentiation of Ag recognition per se did not render tolerance. Rather, the specificity of APC targeting appeared to be a critical variable in the efficiency of tolerance induction. IL2NAg and IL4NAg appeared to target NAg to different APC types. IL2NAg efficiently and selectively targeted NAg to activated MHCII$^+$ T cells via interactions of the IL-2 domain to abundant IL-2 receptors on activated T cells. Conversely, IL4NAg exhibited the targeting of NAg to professional APC subsets that may include B cells or dendritic cells. Because many T cell subsets bear cell surface IL-4 receptors, IL4NAg, like IL2NAg, may also target NAg to activated T cells. Although the targeting of NAg to activated MHCII$^+$ T cells was correlated with the ability of IL2NAg to induce tolerance in vivo, other considerations may also be important. For example, IL2NAg may target unique T cell subsets such as IL2Rα^+ regulatory T cells and/or may condition activated T cell APC to express cytotoxic activity. Cytotoxic T cell APC that efficiently present NAg may efficiently kill NAg-specific responders to promote tolerance to NAg. Thus, selective targeting of Ag to particular T cell subsets coupled with the ability of IL-2 to functionally modify these APC may be critical aspects of IL2NAg-mediated tolerance.

IL-2 based fusion proteins have been used to generate immunity in murine models of cancer and infectious disease (43–48). However, the targeting of Ag to activated T cells in murine systems may not cause tolerance, because mouse T cells may lack the capacity to synthesize MHCII (49–51). In other species such as human and rat, activated T cells are known to synthesize MHCII, and in the rat, T cell-mediated Ag presentation has been implicated in tolerance induction (15–18). Although the strong proinflammatory activities of IL-2 may preclude the use of IL2NAg fusion proteins for the treatment of human autoimmune disease, this study nonetheless supports the principle that the targeting of autoantigen to activated T cells may be an efficacious route for Ag-specific tolerance induction.

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Disclosures

The authors have no financial conflict of interest.

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