SUPPRESSOR T CELL GROWTH AND DIFFERENTIATION

Identification of a Cofactor Required for Suppressor T Cell Function and Distinct from Interleukin 2*

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Proliferative and differentiative signals required for expression of helper and cytolytic T cell functions have been increasingly discerned through analysis of a family of multiple and functionally distinct monocyte- and lymphocyte-derived interleukins (1–8). Antigen-nonspecific cofactors also affect B cells and a complex series of differentiative and growth factors (9) have been identified as requisite signals in the antigen-initiated sequence that culminates in clonal expansion and antibody secretion. In contrast, the identity and role of such soluble factors in the expression of suppressor T cell (Ts)1 effector function are poorly understood.

Interleukin 2 (IL-2) appears to be an obligate stimulus to the proliferation of helper/inducer (10, 11) and cytolytic (5–8) T cells, as well as natural killer cells (11, 12); in addition, culture supernates that contain IL-2 also support Ts growth (11, 13–16). However, studies involving the fungal metabolite cyclosporin A (CsA) suggest that Ts may have distinct or additional interleukin requirements in comparison with those of helper or cytolytic T cells. Interference with IL-2 production (17–20), possibly in concert with effects on IL-2 receptor display (20–22) and IL-1 generation (17, 19), appears to underlie CsA-mediated interference with helper and cytolytic T cell activation (17, 23–25). In contrast, Ts function is unaffected (20, 23, 26, 27), implying that Ts may use additional interleukins produced through CsA-resistant mechanisms.

Thus, to define specific and possibly unique features of differentiative and proliferative signals required by Ts, we have developed a Ts costimulator assay. Initial studies using this assay have identified an antigen-nonspecific, genetically unrestricted soluble factor that is required, in addition to specific alloantigens on fixed stimulator cells, for the activation of alloantigen-primed Ts, designated MLR-Ts and characterized in previous studies (28). Ts costimulator activity has been identified in primary mixed leukocyte reaction (MLR) supernates and is

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1 Abbreviations used in this paper: CsA, cyclosporin A; FCS, fetal calf serum; IFN-γ, gamma interferon; IL-1, IL-2, interleukin 1 and 2; LISM, low ionic strength medium; MLR, mixed leukocyte reaction; MLR-Ts, alloantigen-activated suppressor T cell suppressive of MLR; MLR-TsF, MLR suppressor T cell factor; PMA, phorbol myristate acetate; Ts, suppressor T cell; TsDF, suppressor T cell differentiative factor; TsF, suppressor T cell factor; TsI, T suppressor inducer cell; Ts1, first-order suppressor T cell.
found in addition in supernates of the murine thymoma EL4. In both preparations, however, Ts costimulator is distinct from IL-2. Ts activation to suppressor factor (MLR-TsF) production is promoted by MLR-derived Ts costimulator in the absence of Ts proliferation, implying a differentiative rather than proliferative function, which suggests the provisional designation, Ts differentiative factor (TsDF). This initial characterization thus identifies one of a family of interleukins that are used, perhaps selectively, in the expression of Ts cell function.

Materials and Methods

**Mice.** BALB/cCrj (BALB) mice were obtained from the Department of Cell Biology, Baylor College of Medicine. All other mice were purchased from The Jackson Laboratory, Bar Harbor, ME. All mice used were 6–10-wk old.

**Antibodies.** Monoclonal anti-Lyt-1.2 and anti-Lyt-2.2 hybridoma antibodies were purchased from New England Nuclear, Boston, MA. Monoclonal anti-Thy-1.2 antibody was prepared by ammonium sulfate fractionation of spent culture medium from the HO13.4.9 hybridoma obtained originally from the Salk Institute, La Jolla, CA and provided by Dr. Ellen Vitetta, University of Texas Health Science Center, Dallas, TX. Monoclonal anti-I-J	extsuperscript{8}, an ascites preparation of hybridoma WF18.2815, was the generous gift of Dr. Carl Waltenbaugh, Northwestern University Medical School, Chicago, IL.

**Cyclosporin A.** Cyclosporin A (CsA), obtained as a gift from Sandoz Ltd., Basel, Switzerland, was diluted to 5 mg/ml in 95% ethanol, stored at −70°C, and further diluted in supplemented Eagle's minimum essential medium (MEM) to 1.0 µg/ml in culture, an optimal concentration established by titration for nontoxic inhibition of MLR proliferation and IL-2 production.

**Production of MLR Supernates with Ts Costimulator Activity.** Bulk primary MLR cultures were established with 10 × 10⁶/ml responder spleen cells and 5 × 10⁶/ml irradiated (1,500 rad) allogeneic or syngeneic stimulator spleen cells in supplemented MEM containing 5% heat-inactivated fetal calf serum (FCS). MLR culture supernates were harvested after a 48-h incubation at 37°C in 10% CO₂, 83% N₂, and 7% O₂ by centrifugation at 1,000 rpm followed by a second centrifugation of the supernate at 2,500 rpm. Supernates were stored at −70°C until use.

**Production of IL-2-containing Supernates.** EL4 thymoma cells of a cloned subline developed by Dr. J. Farrar, NIH and obtained from Dr. E. Vitetta were resuspended to 1 × 10⁹/ml in supplemented RPMI 1640 containing 1% FCS and 12.5 µg/ml phorbol myristate acetate (PMA) (Sigma Chemical Co., St. Louis, MO). Supernatants were harvested after a 24-h incubation, and the IL-2-containing fraction was isolated by 50–85% saturated ammonium sulfate precipitation. The precipitate was dissolved and dialyzed against phosphate-buffered saline (PBS) before final dialysis into Heps-buffered Hanks' balanced salt solution (HBSS) and storage at −20°C. This EL4 supernate preparation was used as described below for the assessment of Ts costimulator activity.

Additionally, lectin-free concanavalin A (Con A) supernate was used as a standard in IL-2 quantitation by HT2 proliferation. Mouse splenocytes were incubated in supplemented RPMI 1640 containing 2% FCS and 10 µg/ml Con A (Sigma Chemical Co.) for 2 h at 37°C in an atmosphere of 5% CO₂. The cells were then centrifuged, washed, and resuspended in supplemented RPMI 1640 containing 10% FCS and incubated for an additional 20 h. Supernates were harvested, filtered, and stored at −20°C until use.

**Ts Costimulator Assay.** Primed spleen cell populations containing MLR-Ts were prepared from mice immunized in the footpads 4 d previously with 40 × 10⁶ allogeneic splenocytes. Spleen cells from these animals or normal control animals were enriched for viable cells with low ionic strength medium (LISM) (29), depleted of erythrocytes with Tris-NH₄Cl buffer, and resuspended to 40 × 10⁶ cells/ml in supplemented MEM with 2% FCS. Stimulator spleen cells of the MLR-Ts priming strain were irradiated (1,500 rad) or glutaraldehyde fixed by incubation of 15 × 10⁶ cells/ml in freshly prepared 0.05% glutaraldehyde in HBSS for 15 min at 22°C. Fixed cells were washed twice in HBSS, and
both irradiated and fixed stimulator cells were resuspended to $40 \times 10^6$/ml in supplemented MEM-2% FCS. Additional treatment of fixed cells with irradiation before or after fixation provided results identical to those achieved with fixation alone. Equal numbers of primed MLR-Ts-containing spleen cells and irradiated or fixed stimulator cells were combined, usually $20 \times 10^6$ of each, in a total volume of 1.0 ml, and an equal volume of control medium or Ts costimulator-containing preparation was added in varying concentrations. MLR supernates were typically used at 1:2 and EL4 supernates at 1:100 final concentration. These cultures were incubated for 48 h at $37°C$ in 10% CO$_2$, 85% N$_2$, and 7% O$_2$ and supernates were harvested by centrifugation at 2,500 rpm and stored at $-70°C$ until assay for MLR-TsF activity, a period usually <2 wk. Negative control cultures contained unprimed cells with syngeneic irradiated stimulators and control medium. Additional controls included control or Ts costimulator-containing supernates incubated 48 h as above in the absence of suppressor and stimulator cells. Primed MLR-Ts are designated in the text as the MLR-Ts strain followed by a superscript indicating the allogeneic priming strain (e.g., BALB/c).

Proliferation in Ts costimulator assays was assessed by parallel culture of triplicate or quadruplicate 0.2-ml aliquots of the MLR-Ts stimulator/MLR supernate mixtures that were cultured for MLR-TsF production. Cultures were incubated for 72 h under conditions used for MLR-TsF production, with 1 μCi $[^3H]$thymidine ($[^3H]$TdR) added during the final 18 h. Cultures were harvested and processed for liquid scintillation counting as described below for MLR assays.

Antibody and Complement Depletion of MLR-Ts Cells. Viable, primed MLR-Ts-containing spleen cells prepared by LISM and Tris-NH$_4$Cl buffer treatment were incubated at $20 \times 10^6$/ml with monoclonal anti-Thy-1.2 (1:30), anti-Lyt-1.2 or -2.2 (1:500), or anti-I-J$^d$ (1:100) antibodies at $4°C$ for 30 min and washed once. They were resuspended at $20 \times 10^6$/ml in prescreened young rabbit serum (1:10) as a complement source, obtained through the generosity of Dr. R. Baughn, Veterans Administration Medical Center, Houston, TX, and were incubated for 45 min at $37°C$. After all cells were washed once, cells exposed to anti-Thy-1.2 and anti-Lyt antibodies were treated a second time with antibodies and complement as before, and all treated groups were washed two to three additional cycles, depleted of dead cells by LISM treatment, and remaining viable cells resuspended as above for culture with irradiated or fixed stimulator cells for MLR-TsF production.

Assay of MLR-TsF in MLR cultures. MLR cultures in a final volume of 0.2 ml were established in 96-well flat-bottom microtiter plates with 10$^6$ each of responder and irradiated (1,500 rad) stimulator cells in supplemented MEM with 10% FCS and 0.1 ml of medium, control factor, or MLR-TsF in multiple concentrations. MLR responder cell strains were always H-2 syngeneic with the MLR-TsF-producing strain. MLR cultures were incubated at $37°C$ in 10% CO$_2$, 85% N$_2$, and 7% O$_2$ for 96 h, with 1 μCi $[^3H]$TdR (sp act, 2 Ci/mmol; New England Nuclear) added during the final 18 h. Cultures were harvested onto glass fiber filters using a MASH II apparatus (M. A. Bioproducts, Bethesda, MD) and processed for liquid scintillation counting. Data are expressed as Δcpm ± SEM, representing mean cpm of triplicate cultures with stimulator cells allogeneic to the responder cell strain minus mean cpm of cultures with syngeneic stimulator cells. SEM of Δcpm are calculated by the formula for the propagation of errors. Percent MLR suppression is calculated as follows: $[1 - (\text{Δcpm of MLR with MLR-TsF})/(\text{Δcpm of MLR with medium or control factor})] \times 100$.

IL-2 Assay. IL-2 activity was assayed by proliferation of the IL-2-dependent T cell line HT2, described by Dr. J. Watson (30) and obtained from Dr. J. Kappler and Dr. P. Marrack, National Jewish Hospital and Research Center, Denver, CO. HT2 cells were washed and resuspended in supplemented RPMI 1640 and $5 \times 10^5$ cells were cultured in the presence of serial twofold dilutions of IL-2-containing supernatants as previously described (31). $[^3H]$TdR incorporation was determined during the last 4 h of a 24 h culture period. Activity is presented as cpm vs. dilution of IL-2-containing supernatant.

Adsorption of IL-2. IL-2-containing supernates were depleted of IL-2 by incubation of MLR supernates (undiluted) or EL4 supernates (1:50) with $20 \times 10^6$/ml washed HT2
cells for 2.5 h at 4°C. Supernates were collected by centrifugation at 2,500 rpm and tested for residual IL-2 activity as above.

**Gel Filtration Analysis.** MLR supernates were concentrated by Minicon-B15 macr

solute concentration (Amicon Corp., Danvers, MA), dialyzed against 0.01 M PBS buffer,

and fractionated on a 1.6 x 100-cm calibrated Sephadex G100 column (Pharmacia Fine

Chemicals, Uppsala, Sweden) equilibrated in 0.01 M PBS. 3-ml fractions were collected,

dialyzed against culture medium, and assayed for IL-2 and Ts costimulator activity as

above.

**Results**

**Activation of MLR-TsF Production in a Ts Costimulator Assay.** A short-term Ts
cell costimulator assay was developed to identify stimuli required for Ts cell

activation to TsF production. This assay was based on previous observations that
glutaraldehyde-fixed stimulator cells fail to trigger the production of MLR-TsF

normally obtained from primed MLR-Ts cultured with irradiated or mitomycin

C-inactivated stimulator cells (32). It was possible that fixation prevented an

activity in the stimulator cell population that was required for primed MLR-Ts

proliferation or differentiation, and that was distinct from the display of alloan-
tigen, which is thought to be largely unaffected (33). Thus, various cell culture

supernates were tested for a soluble factor(s) that would reconstitute the missing

stimulus and promote MLR-TsF production when added to primed MLR-Ts

and fixed stimulator cells. In Fig. 1, C57BL/6 (B6)-primed BALB MLR-Ts

produced substantial MLR-TsF in response to irradiated but not glutaraldehyde-

fixed B6 (B6c) spleen cells. However, supernate from a 48-h primary BALB anti-



![Figure 1.](image-url)

Figure 1. Activation of MLR-TsF production in a Ts costimulator assay. B6-primed BALB

spleen cells containing MLR-Ts were cultured with irradiated (B6) stimulators (●) with

glutaraldehyde-fixed (B6c) stimulators alone (▲) or with the additions of a 48-h primary BALB

anti-B6 MLR supernate, 1:2 final concentration (▲), or with BALB anti-B6 MLR supernate in

the absence of stimulators (▲). BALB anti-B6 MLR supernate was also incubated in the absence

of MLR-Ts and stimulator (+) as control. Supernates from these MLR-TsF production or

control cultures were harvested at 48 h and assayed for MLR-TsF content at concentrations

shown in BALB anti-SJL MLR. Data from a representative experiment are expressed as

percent MLR suppression.
B6 MLR largely restored MLR-TsF production by MLR-Ts co-cultured with fixed stimulators. In the absence of the fixed allogeneic stimulator population, primed MLR-Ts were unaffected by the supernate, suggesting that MLR supernate activity was not independently stimulatory. Moreover, MLR supernate did not directly suppress the assay MLR. MLR-TsF was produced in dose-related response to MLR supernate in the Ts costimulator culture and demonstrated production kinetics similar to those of standard MLR-TsF cultures (data not shown). Thus, a function required for the activation of primed MLR-Ts and prevented directly or indirectly by stimulator cell fixation appeared to be substantially reconstituted by a cofactor present in a primary MLR supernate.

Specificity Characterization of Ts Costimulator Activity in Primary MLR Supernates. To characterize antigen specificity of Ts costimulator activity, supernates were harvested from primary MLR composed of various responder and stimulator strains or of stimulator cells alone and examined for costimulator activity in relation to MLR-Ts stimulator alloantigens (Table I). BALB/B6 MLR-Ts cultured with fixed B6 stimulator cells produced substantial MLR-TsF with the addition of BALB anti-B6 MLR supernate but not with supernates of unstimulated BALB splenocytes or of B6 stimulators cultured alone. Primary MLR supernates prepared in response to H-2 and minor alloantigens identical to or distinct from those used to prime and restimulate MLR-Ts each expressed effective Ts costimulator activity with B6-primed BALB MLR-Ts. Similarly, MLR supernates prepared with responder cells identical to or dissimilar from the MLR-Ts strain with regard to H-2 or non-H-2 (including Igh) genotype were also equally effective. Thus, neither strain restriction nor alloantigen specificity limit the Ts costimulator property. MLR-Ts-stimulator strain combinations other than BALB anti-B6 provided similar results (data not shown). Again, none of the primary MLR supernates were directly suppressive of assay MLR. In addition, MLR-Ts precursors from unprimed rather than primed BALB spleens were not induced to significant TsF production within the 48-h culture period in the presence of either syngeneic or allogeneic fixed stimulators and Ts costimulator-containing MLR supernate. Together these findings differ from characteristics reported for Ts inducer or first-order Ts factors, including antigen specificity, V_h restriction, and the induction of Ts from unprimed precursors (34). Instead, Ts costimulator characteristics suggest a closer alignment with the lymphocyte- and monocyte-derived family of interleukins.

MLR-Ts Population Requirements for Expression of Ts Costimulator Activity. Ts costimulator activity results in the release of MLR-TsF, a product of Thy-1^+, Ly-2^+, I-J^+C^+ lymphocytes (35, 36). To determine if Ts costimulator expression directly or indirectly requires an intermediary Ly-1^+2^- cell, primed MLR-Ts-containing spleen cells were depleted of cell subsets by antibody and complement lysis and remaining viable cells were cultured for MLR-TsF production with fixed stimulators and primary MLR supernates (Table II). Absence of Thy-1^+, Ly-2^+, or I-J^+ cells from the MLR-Ts population prevented MLR-TsF production. In contrast, Ts costimulator-induced MLR-TsF production was unaffected by the depletion of cells bearing abundant Ly-1, suggesting that the expression of Ts costimulator activity does not require this population of Ly-1^+ cells or their soluble products.
Specificity Characterization of Ts Costimulator Activity in MLR Supernates

| MLR-TsF production culture* | MLR assay† |
|-----------------------------|------------|
| MLR-Ts | Stimulator | MLR supernate | Δcpm | Percent suppression |
| --- | --- | --- | --- | |
| --- | --- | --- | 30210 | 0 |
| BALB | --- | --- | 29142 | 4 |
| BALBG | --- | --- | 11087 | 63 |
| BALB | BALB | --- | 28841 | 5 |
| --- | --- | 27793 | 8 |
| --- | B6 | 27189 | 10 |
| BALB | B6 | --- | 15619 | 48 |
| BALB | SJL | --- | 17242 | 48 |
| BALB | CBA | --- | 6927 | 77 |
| B10.D2 | B6 | --- | 17465 | 42 |
| DBA/1 | B6 | --- | 10742 | 64 |
| SJL | B6 | --- | 8604 | 71 |
| CBA | B6 | --- | 16145 | 47 |
| BALB | BALB | --- | 29303 | 3 |
| --- | B6 | --- | 24772 | 18 |
| --- | B6 | 27189 | 10 |
| --- | B6 | 30814 | -2 |
| --- | --- | 24772 | 18 |
| --- | --- | 27189 | 10 |
| --- | --- | 30540 | -1 |
| --- | --- | 30540 | -1 |

* MLR-TsF production cultures were prepared with B6-primed or unprimed BALB spleen cells, irradiated (BALB, B6) or glutaraldehyde-fixed (BALBG, B6G) stimulator splenocytes and control medium or supernate of a 48-h primary MLR at final concentration of 1:2. These were cultured for 48 h before harvest of supernates for MLR-TsF assay. To control for direct suppressive effects of primary MLR supernates, the MLR supernates were also incubated in the absence of MLR-Ts and stimulator populations and assayed as for MLR-Ts containing MLR-TsF production cultures.

† MLR-TsF-containing supernates were assayed in multiple concentrations in MLR of BALB responder and irradiated BALB or SJL stimulator spleen cells. Data represent MLR-TsF at 20% final volume and are from a representative experiment.

**Distinction Between Ts Costimulator Activity and IL-2.** Because supernates that contain IL-2 have also been reported to support Ts function (37, 38), we wished to determine if costimulator activity was directly associated with or distinct from the IL-2 that was present in the MLR supernates. In an initial approach, MLR supernates were depleted of IL-2 by adsorption with the cloned IL-2-dependent HT2 T cell line and then assayed for residual IL-2 activity by HT2 proliferation and for Ts costimulator activity by MLR-TsF production in the Ts costimulator.
TABLE II
MLR-Ts Population Requirements for Expression of Ts Costimulator Activity

| MLR-TsF production culture* | MLR assay* |
|-----------------------------|------------|
| MLR-Ts Treatment Stimulator | MLR assay* | Δcpm | Percent suppression |
| --- | --- | --- | --- |
| — | — | — | 26,600 | 0 |
| BALB | — | BALB | — | 21,546 | 19 |
| BALB<sub>B6</sub> | — | B6 | — | 8,512 | 68 |
| BALB<sub>B6</sub> | — | B6<sub>G</sub> | — | 22,344 | 16 |
| BALB<sub>B6</sub> | — | B6<sub>G</sub> | + | 15,960 | 40 |
| — | — | — | 24,472 | 8 |
| C only | B6<sub>G</sub> | + | 14,630 | 45 |
| α-Thy-1 + C | B6<sub>G</sub> | + | 23,541 | 11 |
| α-Ly-1 + C | B6<sub>G</sub> | + | 15,162 | 43 |
| α-Ly-2 + C | B6<sub>G</sub> | + | 21,546 | 19 |
| α-I<sup>d</sup> + C | B6<sub>G</sub> | + | 20,615 | 12 |

* B6-primed BALB spleen cells were untreated or treated with antibody and complement or complement alone. Viable cells were isolated, combined with irradiated (B6) or fixed (B6<sub>G</sub>) stimulator splenocytes and BALB anti-B6 48-h primary MLR supernate at 1:2 final dilution, cultured for 48 h, and supernates harvested. A control group consisted of MLR supernates incubated 48 h in the absence of MLR-Ts and stimulators.

The relationship of IL-2 and Ts costimulator activities was also characterized by gel chromatography (Fig. 4). Concentrated BALB anti-B6 MLR supernate was applied to a G100 column and collected fractions were dialyzed against culture medium and tested for IL-2 activity by HT2 proliferation at multiple dilutions and for MLR-Ts costimulator activity at a 1:2 final dilution. Major peaks of IL-2 and Ts costimulator activities were largely distinct. IL-2 activity
eluted in the 28–32,000 mol wt region while two peaks of 19,000–23,000 and 40,000–45,000 mol wt contained costimulator activity; a shoulder of costimulator activity at 28,000 mol wt was approximately coincident with the IL-2 peak. None of the MLR supernate fractions were suppressive when added directly to assay MLR, and G100-chromatographed control MLR culture medium containing 5% FCS neither contained Ts costimulator activity nor directly suppressed assay MLR (data not shown). Thus, Ts costimulating activity exists in two major peaks of approximately 21,000 and 43,000 mol wt that are largely independent of IL-2-containing fractions.

Expression of Ts Costimulator Activity in EL4 Supernates. The supernates of PMA-induced EL4 thymoma cells are a potent source of IL-2 activity, and distinct B cell reactive lymphokines have also been identified in these supernates (9, 39). Thus it was of interest to determine if standard EL4 supernates also contained Ts costimulator activity and if this activity could be differentiated from IL-2. MLR-Ts costimulator assays were established with MLR supernates or with dilutions of a standard EL4 supernate. After 48 h, the MLR-Ts culture supernates were harvested, adsorbed with HT2 cells to remove residual IL-2, and assayed for MLR-TsF activity (Fig. 5); as demonstrated in previous studies (31) and confirmed here, HT2 adsorption of conventional MLR-TsF does not inter-
Figure 3. Ts costimulator activity in MLR supernates prepared with CsA. BALB anti-B6 MLR supernates were prepared in the absence (A, x) or presence (Δ, *) of 1 μg/ml CsA, dialyzed, added to BALB<sup>B6</sup> MLR-Ts and fixed stimulators, and assayed for activation of MLR-TsF production as in previous figures. Inset shows IL-2 activity, measured by HT2 proliferation, of an IL-2 standard as in Fig. 2 (○), untreated MLR supernate (A), and CsA-treated supernate (x).

Figure 4. Ts costimulator and IL-2 activities in G100 fractions of primary MLR supernates. Primary BALB anti-B6 MLR supernates were G100 chromatographed and aliquots of dialyzed fractions were added to BALB<sup>B6</sup> MLR-Ts and irradiated (B6) or fixed (B6<sub>c</sub>) stimulators at a 1:2 final concentration. Resultant MLR-TsF (20% final concentration) was assayed in BALB anti-SJL MLR; data are provided as the percent MLR suppression (○). Fraction aliquots were also added to HT2-proliferative assays at multiple dilutions; maximal proliferation, in all cases observed at 1:2 final concentration, is provided as cpm of [<sup>3</sup>H]TdR incorporated by HT2 (○).

fere with TsF function. MLR-Ts costimulator cultures that received EL4 supernate, in addition to those receiving MLR supernate, were activated in a dose-related fashion to MLR-TsF production, suggesting that EL4 supernates also contain an activity effective in MLR-Ts activation. In this and the following
Ts costimulator activity in EL4 supernates. EL4 supernate, prepared as described in Materials and Methods, at 1:100 (□) or 1:200 (△) final concentration or BALB anti-B6 MLR supernate at 1:2 (▲) final concentration were added to BALB<sup>B6</sup> MLR-Ts and fixed (B6<sub>G</sub>) stimulators. Except for the positive control group designated with an asterisk (*BALB<sup>B6</sup>), MLR-TsF from all cultures or EL4 and MLR supernates cultured alone (data not shown) were adsorbed with HT2 cells to remove residual IL-2 before assay in BALB anti-SJL MLR. In this and Fig. 6, higher EL4 concentrations did not have greater activity.

Studies, HT2-adsorbed EL4 supernates were not suppressive when added directly to assay MLR (data not shown).

To determine if the Ts costimulator activity in EL4 supernates was the result of IL-2 or of a distinct material, EL4 supernates were adsorbed with HT2 cells to remove IL-2 before their addition to the MLR-Ts costimulator assay (Fig. 6). As with HT2-adsorbed MLR supernates, IL-2-depleted EL4 preparations clearly expressed substantial Ts costimulator activity. Although these experiments do not address the role that IL-2 may also play, it appears evident that a material expressing MLR-Ts costimulator activity and distinct from IL-2 exists in EL4 supernates.

Ts Costimulator Activity in the Absence of MLR-Ts Proliferation. In an initial approach we wished to examine the nature of the Ts costimulator function(s), i.e., proliferative or differentiative, that supports activation of MLR-Ts. If the primary function is proliferation of the MLR-TsF-producing cell pool, it would be anticipated that the expression of Ts costimulator activity would be inhibited by prevention of response to proliferative signals. Therefore MLR supernates were added to Ts costimulator assays in which the BALB<sup>B6</sup> MLR-Ts population was either irradiated (1,500 rad) or untreated (Fig. 7). Ts costimulator activity was assayed by MLR-TsF production, and MLR-Ts proliferation was assessed by [<sup>3</sup>H]TdR incorporation in aliquots of the various MLR-Ts cultures. MLR-TsF
production by irradiated MLR-Ts was decreased but still clearly present in positive control cultures with irradiated stimulator cells. Significantly, the capacity of Ts costimulator to induce TsF production was unaltered by irradiation of the MLR-Ts population. These data imply a costimulator function that does not act via proliferative stimulus, but rather appears to reflect the induction of a differentiative process required for TsF secretion. For simplicity of reference, the MLR-Ts costimulating activity represented in primary MLR supernates has been provisionally termed Ts differentiation factor (TsDF).

Discussion

In this report we describe a Ts costimulating factor that synergizes with alloantigens on fixed stimulator cells to induce MLR-TsF production by alloantigen-primed MLR-Ts. Based on several functional criteria the Ts costimulating property appears to be distinct from the class of soluble factors with antigen-specific, Ts-inducing qualities, and instead to be most closely associated with the regulatory interleukins. Functional and molecular weight characteristics differentiate Ts costimulator in both primary MLR and induced EL4 supernates from the T cell lymphokine IL-2, and indirect evidence discussed below suggests distinction from IL-1 and gamma interferon (IFN-γ) as well. Thus the Ts costimulator factor(s) described here, and specifically the differentiative activity
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Figure 7. Expression of Ts constimulator activity in the absence of MLR-Ts proliferation. BALB anti-B6 MLR supernates were added to untreated (△-△) or irradiated (△-△) BALB\(^6\) MLR-Ts and fixed (B6G) stimulators. In addition, untreated (●) or irradiated (●) BALB\(^6\) MLR-Ts were also cultured with irradiated B6 stimulators. Resultant MLR-TsF was assayed in BALB anti-SJL MLR; proliferation was assessed by \[^{3}H\]TdR incorporation into aliquots of MLR-Ts cultures as described in Materials and Methods.

termed TsDF, may constitute distinctive members of the family of interleukins required in the antigen-driven activation of Ts to effector function.

In several murine and human suppressor T cell systems, primed and restimulated T cells termed, alternatively, suppressor inducers (Tsi) or first-order suppressor cells (Ts1), or their soluble products, induce a subset of T cells to effector suppressor activity from unprimed precursors (40-48). In so doing they impart antigen specificity to the induced Ts, either for the antigen used to stimulate the Tsi (40, 41) or for determinants serologically described as idiotypic and assumed to exist on the Tsi antigen-binding receptor (42-44). Additional specificity of Ts inducer–Ts precursor interaction may be imposed by \(V_n\) restrictions (40). Soluble products of murine Tsi express antigen-binding sites, idiotypic or \(V_n\) determinants, and in most cases 1–J determinants (34); from affinity column analyses these determinants exist on a molecule(s) both required and sufficient to initiate Ts function (41, 45-47). These inducer molecules may provide the antigenic and perhaps the major histocompatibility complex-encoded elements required to initiate Ts precursor activation; clearly, they are distinct from the antigen-nonspecific, nonrestricted, hormone-like factors classified as interleukins. Similarly, the Ts costimulator preparations described here are also distinct from Ts-inducing factors in several respects, including lack of antigen specificity and genetic restriction, production early in a primary response rather than after priming and restimulation, and failure to activate naive Ts. BALB/
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MLR-Ts activation was achieved with Ts costimulator preparations that violate both H-2 (CBA, SJL, DBA/1 costimulator sources) and IgH (B10.D2, DBA/1, SJL sources) restrictions. In addition, although the alloantigenic stimuli of MLR-Ts and of Ts costimulator production are each potentially complex, no general strain-associated alloantigen specificity of Ts costimulator effect was apparent. Moreover, Ts costimulator promotes activation of primed but not naive Ts within the limited time frame analyzed, while Ts-inducing factors generally fail to affect primed Ts (44, 49, 50). These qualities thus appear to characterize Ts costimulator activity as a function of an interleukin-like material and distinguish it from the antigen-specific Ts inducers.

The interpretation that Ts costimulator activity is mediated by molecules distinct from IL-2 is supported by each of the approaches to determining the relationship of these two activities. Thus Ts costimulator function is retained in HT2-adsorbed, IL-2-deficient supernates, is generated in cultures in which IL-2 production is blocked by CsA, and demonstrates distinct molecular weight characteristics. Moreover, proliferation is not required for Ts costimulator expression. The presence of Ts costimulator activity as a component of EL4 supernate distinct from IL-2 is additionally interesting in light of the recent identification of an 18,000 mol wt fraction containing a B cell growth factor (BCGF) (39) and a late-acting B cell differentiative cofactor termed EL4-TRF (9) in PMA-induced supernates of the same EL4 subline. The relationship of Ts costimulator to these B cell-related cofactors, or to other characterized T cell and macrophage-derived factors has not yet been directly examined, although the reported susceptibility of IL-1 (17, 19) and IFN-\(\gamma\) (51) production to CsA suggest that these factors are not mediators of the Ts costimulation described in this report. In addition, the present studies do not yet allow the conclusion that the material in EL4 supernates and that characterized in primary MLR supernates are biochemically or functionally identical. Each demonstrates Ts costimulator activity but may achieve that activity by distinct mechanisms. Moreover, it is not clear if the MLR supernate-derived 43,000 and 21,000 mol wt fractions expressing costimulator activity represent distinct species or complexed forms of a single molecule. Finally, although this Ts costimulator activity is not apparently mediated by IL-2, additional roles of IL-2 or other lymphokines in support of the process of MLR-Ts activation to TsF production have not been directly approached in these studies and are clearly possible. Complex IL-2-rich culture supernates have been used in the promotion of Ts activity (37, 38) and maintenance of long-term Ts lines (11, 15–16); however, Ts utilization of IL-2 per se as a proliferative trigger has not yet been formally demonstrated.

The production of Ts costimulator in the presence of CsA has interesting implications concerning interleukin stimuli that contribute to Ts function. Although CsA effects appear to be complex and somewhat controversial, it is generally appreciated that CsA prohibits IL-2 production by alloantigen-activated T cells (17–20); as previously stated, it appears that the generation of IFN-\(\gamma\) (51) and IL-1 (17, 19) are also interrupted. In contrast, Ts costimulator is produced in primary MLR cultures in the presence of CsA, and in the absence of IL-2. Activation of Ts costimulator-producing cells thus appears to be CsA resistant and to occur in the absence of both IL-1 and IL-2 differentiative and proliferative
support. Hess et al. (52) and more recently Mohagheghpour et al. (53) have demonstrated T4⁺ or Leu-3⁺8⁺ cells, respectively, in CsA-suppressed MLR of human cells, which appear to be required for expression of T8⁺ suppressor cell function. Such cells thus may be the CsA-resistant producers of Ts-associated factors such as the Ts costimulator described here.

An alloantigen-primed MLR-Ts population and fixed stimulator cells bearing priming alloantigens are required in the Ts costimulator assay. It is clear that alloantigen recognition by primed cells is necessary for interaction with Ts costimulator and is achieved with glutaraldehyde-fixed stimulators. Thus antigen binding may trigger the expression of TsDF or other Ts costimulator-specific receptors on primed MLR-Ts, allowing subsequent cofactor interaction. In the absence of Ts costimulator, activation to MLR-TsF production fails regardless of stimulator cell concentration, indicating that fixation has impaired an additional and requisite stimulator cell function distinct from surface antigen display. MLR-Ts typically interact with stimulator cells of novel I-C⁺ or I-J⁺C⁺ phenotypes (54). Whether Ts costimulator directly represents an activity of such a stimulator cell or rather the product of a secondary cell that requires stimulator cell interaction is presently unclear. Furthermore, the immediate target of Ts costimulator in the primed MLR-Ts-containing population has not been directly established in these studies. It is evident that T cells bearing abundant Ly-1 alloantigen and routinely removed by anti-Ly-1 antibody-mediated lysis are not target cell candidates. In addition, under usual conditions of MLR-Ts reactivation, MLR-TsF production is insensitive to rigorous macrophage depletion of both MLR-Ts and stimulator populations (52), suggesting indirectly that these cells are also unlikely Ts costimulator targets. Similarly, based on depletion protocols, B cells play no requisite role in MLR-Ts activation (S. Rich and C. Arhelger, unpublished observations). Thus the most likely although unproven target exists in the Ly-2⁺, I-J⁺C⁺ MLR-Ts population. The observation that primed but not unprimed cells are responsive to Ts costimulator in concert with alloantigen may reflect the expression of a late-appearing cofactor requirement, presumably differentiative, for the final expression of Ts effector function. Alternatively, this cofactor requirement may be more generally expressed after antigen recognition and simply require extended culture of unprimed cells for measurable effect.

Production of MLR-TsF is the endpoint of Ts cofactor activity defined by this costimulator assay. An ordered and complex series of interleukin-mediated differentiative and proliferative events have been described after initial antigen-receptor interaction in the functional development of IL-2-producing helper T cells, cytotoxic T lymphocytes, and antibody-producing B cells. It is likely that Ts will demonstrate a similar scheme of activation. In this initial characterization, unfractionated MLR supernates minimally contain a Ts cofactor that fulfills a differentiative requirement of antigen-activated MLR-Ts. It will now be of substantial interest to examine the contributions of purified TsDF, IL-2, and other differentiative and proliferative cofactors in control of Ts activation to effector function.
Summary

This report describes a Ts costimulator assay and its use to analyze cofactors required for the expression of suppressor T cell function. Activation of primed MLR-Ts (alloantigen-activated suppressor T cells suppressive of mixed leukocyte reaction) to suppressor T cell factor (TsF) production typically fails in the presence of glutaraldehyde-fixed rather than irradiated allogeneic stimulator cells. However, MLR-TsF production was restored by the addition of 48-h primary MLR supernates; MLR-derived Ts costimulator neither activated primed MLR-Ts in the absence of fixed allogeneic stimulators nor directly suppressed assay MLR. Lack of antigen specificity or genetic restriction and failure to activate unprimed MLR-Ts precursors suggested that Ts costimulator activity differed from previously described Ts inducer functions and was more closely aligned with the lymphocyte- or monocyte-derived interleukins (IL). Three findings distinguished Ts costimulator from IL-2. Depletion of IL-2 activity from MLR supernates by HT2 adsorption failed to affect Ts costimulator function. In addition, MLR supernates prepared in the presence of cyclosporin A contained no IL-2 but expressed Ts costimulator activity. Finally, gel chromatography demonstrated Ts costimulator in peaks of 21,000 and 43,000 mol wt that were largely distinct from the IL-2-containing fractions. Ts costimulator activity was also identified in phorbol myristate acetate (PMA)-induced EL4 supernates and was retained in those supernates after IL-2 depletion by HT2 adsorption. In preliminary functional characterization, MLR supernate-derived Ts costimulator triggered MLR-TsF production from irradiated MLR-Ts in the absence of proliferation. Thus a differentiative rather than proliferative stimulus required for primed MLR-TsF production from irradiated MLR-Ts in the absence of proliferation. Thus a differentiative rather than proliferative stimulus required for primed MLR-TsF function appears to be provided by this Ts costimulator and has been provisionally termed Ts differentiative factor (TsDF). This initial characterization may thus identify one of a possibly distinctive family of interleukins required in the alloantigen-driven activation of suppressor T cells to effector function.

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