The Plasmacytoma Growth Inhibitor Restrictin-P Is an Antagonist of Interleukin 6 and Interleukin 11

IDENTIFICATION AS A STROMA-DERIVED ACTIVIN A*

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A stromal protein, designated restrictin-P, that specifically kills plasma-like cells was purified to homogeneity and shown to be identical with activin A. The specificity to plasma-like cells stemmed from the ability of restrictin-P/activin A to competitively antagonize the proliferation-inducing effects of interleukin (IL) 6 and IL-11. Restrictin-P further interfered with the IL-6-induced secretion of acute phase proteins by HepG2 human hepatoma cells and with the IL-6-mediated differentiation of M1 myeloblasts. A competition binding assay indicated that restrictin-P did not interfere with the binding of IL-6 to its receptor on plasma-like cells, suggesting that it may act by intervening in the signal transduction pathway of the growth factor. Indeed, concomitant addition of restrictin-P and IL-6 to cytokine-deprived B9 hybridoma cells was followed by sustained overexpression of junB gene until cell death occurred, while IL-6 alone caused a transient increase only. This altered response to IL-6 stimulation was accompanied by a moderate increase in STAT protein activation. Thus, in this study, we identified the plasmacytoma growth inhibitor, restrictin-P, as being activin A of stromal origin. It is shown that activin A is an antagonist of IL-6-induced functions and that it modifies the IL-6 signaling pattern.

Regulation of hemopoiesis is mediated by cytokines that act through distinct mechanisms. Some, like colony-stimulating factors (CSFs)1 promote accumulation of hemopoietic cells by inducing proliferation coupled with differentiation (1). Others, like tumor necrosis factor, may cause cell cycle arrest and thus limit cell accumulation (2). The outcome of the interaction between the growth factor and the cell often depends on the nature of the target cell; as it is with transforming growth factor (TGF-β), the same cytokine may be stimulatory to one cell type and inhibitory to the other (reviewed in Ref. 3). Whereas some inhibitors operate by slowing down cell growth (4) or by induction of terminal differentiation (5), others cause cell death (6) by inducing apoptosis (7–10). Restrictin-P has formerly been described as an inhibitor of plasmacytoma cell growth (11–12). The biological activity of this factor was first noticed through the selective ability of primary stromal cells to slow down the proliferation of plasmacytoma cells (13, 14). A similar function was exhibited by trypsin-released proteins obtained by mild treatment of a bone marrow-derived stromal cell line of mouse origin (MBA-2.1) (12). The released crude protein mixture inhibited the growth of a series of plasmacytomas and hybridomas but did not have significant effects on the growth of a variety of other leukemia cell lines of lymphoid, erythroid, and myeloid origin (12). Similarly, no effect was observed on normal cell populations such as bone marrow cells responding to colony-stimulating factors or spleen cells induced by mitogens (12). This unique specificity prompted us to isolate the active component. However, factor(s) mediating the growth inhibition were found to be produced by the stroma cell line in minuscule amounts, and it was necessary to establish conditions for large scale production of the factor. We found that the producer cell line MBA-2.1 could be propagated on a three-dimensional carrier of nonwoven fabric of polyester loaded in a bioreactor system under complete protein-free conditions (15).

The study of such bioreactors showed that the cells could be maintained under protein-free conditions for up to 10 months while producing restrictin-P activity along with TGF-β, macrophage (M)-CSF, and IL-6. Restrictin-P obtained from the bioreactor system induced in its target cells early G1/G0 arrest, morphological changes, and signs of cell damage characteristic of apoptosis (11, 16) accompanied by intracellular ionic changes (17).

The present study was aimed at identifying restrictin-P by purifying it to homogeneity and at analyzing the mechanism by which restrictin-P exerts its specific inhibitory effect on plasma-like cells.

MATERIALS AND METHODS

Cell Cultures—The MBA-2.1 stromal cell line (18, 19) was grown in 100-mm plates ( Falcon, Oxnard, CA) and maintained by weekly passages in growth medium composed of Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Biokal, Jerusalem, Israel). IAM-1.4 macrophages (18) were grown in the above-mentioned medium, with the addition of L-cell or MBA-2.1-conditioned medium as sources of M-CSF, MIPC-11 (20), SP-2, N50, X-24, X-63, and P3.1 (21) plasmacytoma cell lines were grown in Roswell Park Memorial Institute (RPMI) 1640 (Life Technologies, Inc.) with 10% FCS. ABL-S8 (22) and AVRIJ-1 (23) pre-B lymphoma cells and the T lymphomas BW-517 (24), the myeloid tumor...
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Restrictin-P was monitored using either MPC-11 plasmacytoma or B9 hybridoma cells. MPC-11 cells were seeded at 8 x 10^5 cells/ml in 96-well microtiter plates (100 μl/well) (Coscar, Cambridge, MA) in RPMI supplemented with 7.5% FCS in the presence of serial dilutions of the restrictin-P-containing samples or buffer (20 nm Tris-HCI, pH 7.8, or 20 nm Hepes, pH 7.8). Cell viability was determined following 4 days of incubation using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay, which measures cell viability via mitochondrial activity (30). One unit of activity was designated as the amount of protein which, when incubated with Eagle’s medium containing 0.1% tritiated thymidine, caused 50% growth inhibition relative to the control. The assay was essentially the same using B9 cells except that the culture conditions were as indicated above for the B9 hybridoma. The latter was also used to titrate IL-6 levels. Briefly, B9 cells (5 x 10^5 cells) were cultured in 96-well plates (200 μl/well) in the presence of test samples and recombinant human IL-6 controls. At 64 h of incubation, the cells were washed with [3H]thymidine (Rotem Industries, Israel, 1 μCi/well) for 16 h. Unit IL-6 was determined by relating IL-6 to the 1st international standard (code 89/548). The biological effect of IL-6 was monitored in two additional assays. HepG2 cells were seeded at 10^3/ml in 24-well plates (Coscar, Cambridge, MA), washed twice in modified Eagle’s medium, and incubated with IL-6, at the concentrations indicated. The acute phase proteins α-acid glycoprotein and haptoglobin were monitored in the conditioned medium by Western blotting using corresponding polyclonal antibodies (Sigma, Israel). The ECL Western blotting kit (Amersham International plc, United Kingdom) was used according to the recommended instructions. M1 myeloid leukemia cells were seeded at 2 x 10^5/ml in microtiter plates (100 μl/well) and were induced to differentiate with the indicated concentration of IL-6. Mitochondrial activity was assayed by MTT as above.

Cytokines and Corresponding Neutralizing Antibodies—The antibodies to TGF-β that were used were rabbit anti-native porcine platelet TGF-β1, neutralizing for both TGF-β1 and TGF-β2. These antibodies were purchased from British Biotechnology Ltd. (Abingdon, Oxon, UK). Human TGF-β1 was obtained from the same source. Hamster anti-murine neutralizing antibodies were developed in our laboratory. IL-6 was purchased from Peprotec (Rocky Hill, NJ). Recombinant human and monoclonal rat anti-mouse IL-6 neutralizing antibodies were purchased from Genzyme Corp. Recombinant, N-terminally truncated, human IL-6 (mutan) (31) and basic fibroblast growth factor were kindly provided by Pharmacia Biocenter (Nerviano, Italy). Claude concentrated murine IL-6 was kindly provided by Dr. J. Lotem, Weizmann Institute, and murine IL-6 was obtained from Dr. J. Van Snick, Ludwig Institute for Cancer Research, Brussels. Goat anti-M-CSF neutralizing anti-serum was kindly provided by Dr. R. E. Stanley from the Albert Einstein University, Recombinant mouse IL-1α, IL-2, IL-4, and IL-7 and recombinant human IL-10, IL-11, and platelet-derived growth factor were kindly provided by Genzyme Corp. Recombinant human G-CSF was kindly provided by Dr. S. Gillis of Immunix Corp., Seattle. Recombinant bovine activin A was purchased from I nnogenetics (Belgium).

IL-6 Competition Binding on B9 Cells—IL-6 labeling and competition binding were performed as described previously (32). The amount of r-murine (Mu)-[3H]-IL-6 or r-human (Hu)-[3H]-IL-6 used was 30–40% of the saturation binding. Under these conditions, 2,070 ± 530 high affinity binding sites and the Mu-IL-6 and Hu-IL-6 bound with apparent K_a values of 5.5 x 10^-10 and 1.1 x 10^-10 M, respectively, as determined by the LIGAND program (33).

Protein Purification—Crude restrictin-P, 3.2 g of protein (2.5 x 10^6 units in 1.4 liters) in 1.4 liters) was prepared as previously reported (15). Aliquots (200 mg of protein) were further purified by ion exchange chromatography on Q-Sepharose, using a fast protein liquid chromatography (FPLC) system. After loading the sample and appropriate washing in buffer A (20 nm Tris-Cl, pH 7.8), restrictin-P was eluted with 0.05 M NaCl in buffer A. Elution of proteins was followed at 280 nm. The salt-eluted material was desalted and concentrated using reversed phase high performance liquid chromatography (RP-HPLC) on an Aquapore RP-300 column. Proteins were eluted with a nonlinear gradient of aqueous acetonitrile, 5–80%, in 0.1% trifluoroacetic acid. Elution of proteins was followed at 214 nm, protein content was determined according to Bradford (Bio-Rad, Munich), and restrictin-P content was assayed as described above. Fractions with restrictin-P activity (at -20 °C) were further purified on Superdex 75 (in batches of about 10 mg of protein each) in 2 x phosphate-buffered saline. Elution pattern was followed at 280 nm. Final purification of restrictin-P was achieved by RP-HPLC on RP-300 using a multistep linear gradient of aqueous acetonitrile in 0.1% trifluoroacetic acid. Elution fractions were pooled and rechromatographed (RP-HPLC) under essentially identical conditions. Fractions containing restrictin-P (eluted at 38% acetonitrile) were vacuum-dried and kept frozen at -20 °C. Polyacrylamide gel electrophoresis (PAGE) analyses of different protein fractions were performed using a Mini-PROTEAN II gel apparatus (Bio-Rad Laboratories). Silver staining was performed with a Quick-Silver kit (Amersham) or a Silver Stain Plus Kit (Bio-Rad Laboratories). Coomassie Brilliant Blue staining was performed using the Serva blue G stain. Sequencing of the purified protein was performed at Perkin-Elmer.

Northern Analysis—The JunB probe used, RSVJunB, was a 1.2-kilobase Xho-Smal fragment cloned into the pUC-18 vector (34). DNA probes labeled with a random primed DNA Labeling Kit (Boehringer Mannheim) were passed over a Sephadex G-50 minisep column. A minimum of 10^9 cpm/ml was used for hybridization. Signal intensities were measured by a 300A computing densitometer (Molecular Dynamics, Tampa, FL).

Gel Retardation Assay—HepG2 cells were treated with IL-6 and/or restrictin-P, and nuclei were isolated and extracted. Gel retardation analysis was carried out as described previously (35) except that 50 μg Tris, 41.5 μM boric acid, 0.5 μM EDTA, pH 8.3 was used as electrophoresis buffer. A DNA oligonucleotide containing mutant 67 of c-fos promoter sis-induced element (36) was labeled with [3P by filling in with Klenow and used as a probe.

RESULTS

The inhibitory activity of restrictin-P, as detected in conditioned media form MBA-2.1 cells, was specific to plasma-like tumor cell lines (Table I). A variety of other cell lines representing different hemopoietic lineages and stages of maturation were only slightly inhibited or were totally unaffected by this factor. To rule out the possibility that restrictin-P activity could be ascribed to one of the cytokines known to affect hemopoietic cells, we searched for factors that might have restrictin-P-like activity. IL-1, IL-2, IL-3, IL-4, IL-6, IL-7, IL-10, IL-11, M-CSF, G-CSF, TGF-β, platelet-derived growth factor, bovine fibroblast growth factor, interferon γ, and leukemia inhibitory factor were tested over a range of concentrations. These cytokines were found to be devoid of the ability to inhibit the growth of the MPC-11 plasmacytoma which is highly sensitive to restrictin-P (12). In addition, neutralizing antibodies to TGF-β1 and -2, tumor necrosis factor, IL-6, interferon γ, and M-CSF did not reduce restrictin-P-like activity in media conditioned by MBA-2.1 cells (results not shown).

We used the above-conditioned media in an attempt to obtain some clue as to the mechanism by which the inhibition of plasma-like cells is mediated. The MBA-2.1 cell-conditioned medium inhibited the growth of the MPC-11 plasmacytoma and, as detailed below, it also interfered with the growth-promoting effect of IL-6 on B9 cells. To determine whether these two functions were mediated by the same molecule, it was necessary to purify restrictin-P to homogeneity. We therefore constructed a bioreactor production system wherein restrictin-P activity could be observed in media conditioned by the cells in absolute protein-free conditions (15). A batch of 600 liters of conditioned medium was concentrated by diafiltration and subjected to a further step of concentration by Amicon ultrafiltration followed by fractionation using an automated FPLC column. The fractionation included anion exchange chro-
The various cell lines were grown as indicated under "Materials and Methods." Cells were seeded in 96-well microtiter plates at 400 cells/well. Partially purified conditioned medium from MBA-2.1 cells was added in the concentration indicated. Cultures were incubated for 4 days, and viability was measured by the MTT colorimetric assay. Values represent the mean of triplicates ± S.D.

| Cell type                  | Name     | 0.6 μg/ml | 5 μg/ml | 40 μg/ml |
|----------------------------|----------|-----------|---------|----------|
| Plasmacytoma               | MPC-11   | 1.7 ± 0.04| 0       | 0        |
| Plasmacytoma               | SP2      | 96.9 ± 0.049| 29.7 ± 0.019| 0.2 ± 0  |
| Plasmacytoma               | X24      | 109.6 ± 0.005| 65.3 ± 0.004| 44.0 ± 0.014 |
| Plasmacytoma               | X63      | 113.6 ± 0.045| 35.9 ± 0.015| 10.2 ± 0.011 |
| Plasmacytoma               | NSO      | 34.9 ± 0.008| 0.4 ± 0   | 0.5 ± 0  |
| Plasmacytoma               | P3       | 47.0 ± 0.030| 0.2 ± 0   | 0.2 ± 0  |
| Erythroleukemia            | F4N      | 96.6 ± 0.07| 88.5 ± 0.01| 62.9 ± 0.015 |
| Myeloid cell line          | M1       | 83.7 ± 0.012| 80.2 ± 0.007| 94.4 ± 0.016 |
| Myeloid cell line          | WEHI-265.1| 163.7 ± 0.05| 115.6 ± 0.09| 148.2 ± 0.06 |
| Pre-B lymphoma             | AVL1     | 108.9 ± 0.03| 116.9 ± 0.08| 97.2 ± 0.03 |
| T lymphoma                 | BW-5147  | 95.6 ± 0.02| 96.3 ± 0.01| 63.1 ± 0.015 |
| Macrophage cell line       | Pu-5-1R  | 114.0 ± 0.02| 146.4 ± 0.02| 146.5 ± 0.01 |

**TABLE I**

Target cell range of restrictin-P activity in conditioned media of the MBA-2.1 stromal cell line

**FIG. 1. Purification of restrictin-P from stromal cell conditioned medium.** A, Q-Sepharose anion exchange chromatography. Processed conditioned medium of MBA-2.1 cell line (1.5 × 10⁶ units) was loaded onto a Q-Sepharose column (100 ml, XK 50). The column was washed with 1200 ml of the initial buffer (20 mM Tris-Cl, pH 7.8, 8 ml/min), and restrictin-P was eluted with 400 ml of 0.05 M NaCl in the initial buffer. B, Superdex 75 gel filtration. Fractions containing restrictin-P from the Q-Sepharose column were concentrated, dissolved in 20 mM Hepes (pH 7.8), and loaded onto a Superdex 75 column (20×60, Pharmacia). The column was washed with 2 × phosphate-buffered saline (2 ml/min). C, C-8 reversed-phase HPLC-I. Biologically active fractions from the Superdex 75 column were loaded onto an Aquapore RP-300 column (7 μm, 4.6 × 100 mm) with a mobile gradient of aqueous acetonitrile in 0.1% trifluoroacetic acid (0.5 ml/min). D, C-8 reversed-phase HPLC-II. Partially purified restrictin-P was rechromatographed (RP-HPLC) under essentially identical conditions as in C above, except for an additional isocratic step at the elution time of the protein peak (at approximately 36% acetonitrile). Broken lines indicate the absorption at 280 nm (A and B) or 214 nm (C and D); open circles and lines show the biological activity of restrictin-P as measured by the MPC-11 assay, and lines indicate percentage of acetonitrile (C and D).

 secretions of both α- and β-casein proteins, and haptoglobin induced in HepG2 cells by IL-6 was markedly reduced by addition of restrictin-P (Fig. 5). M1 (clone 11) myeloblastic cells differentiate into adherent monocytes under the influence of IL-6 (28, 29). Following IL-6 induction, M1 cells exhibit high mitochondrial activity and growth inhibition. As shown in Table II, restrictin-P abolished the IL-6-induced effect.

It was concluded, therefore, that IL-6 and restrictin-P are...
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Restrictin-P/Stromal Activin A Antagonizes IL-6 and IL-11

**Restrictin-P antagonizes IL-6- and IL-11-induced proliferation of B9 cells.** Growth factor-deprived B9 cells were washed (3 times) with growth medium (RPMI + 5% FCS) and were seeded in 96-well Falcon microtiter plates (5 x 10^3 cells/200 μl/well) in growth medium supplemented with the indicated dilutions of recombinant human IL-6 (A, open symbols) or with the same IL-6 dilutions and 1.5 units/well of purified restrictin-P (A, closed symbols). A similar experiment was set with serial dilutions of recombinant human IL-11 (B, open symbols) or with IL-11 and 0.018 unit/well of restrictin-P (B, closed symbols). Cells were incubated for 48 h and then pulsed with [3H]thymidine (1 μCi/well) for 2 h. Values represent the mean of triplicate determinations ± S.D.

**Competing on some target machinery used to generate a signaling pathway in at least 3 completely different target cell types.** A candidate target molecule for restrictin-P action was the IL-6 receptor complex. We studied the possibility that restrictin-P is a receptor antagonist by testing its ability to compete with radiolabeled IL-6 for binding to its receptor on the surface of B9 cells. Fig. 6 shows that "cold" IL-6 competed out the binding of radiolabeled IL-6 to its receptor as expected. On the other hand, restrictin-P, at a concentration that would completely abolish the growth-stimulating effect of IL-6, failed to reduce the binding of radiolabeled IL-6 to its receptor. Thus, restrictin-P does not seem to interfere with ligand binding and may therefore interfere with postreceptor event(s) within the IL-6 signaling pathway. B9 cells stimulated by IL-6 following a period of cytokine deprivation showed a transient increase in expression of the early response gene junB (Fig. 7). Restrictin-P added to such cells caused increased and sustained expression of the junB gene until 24 h post-treatment at the time cell death already occurs. A similar augmented expression of junB mRNA was observed in the MPC-11 cell line. The expression of another early response gene TIS11 was increased in B9 cells incubated with restrictin-P but was unaffected in MPC-11 cells. The effect of restrictin-P on junB expression was also observed in HepG2 hepatoma cells (not shown). In these cells, restrictin-P did not interfere with the AK/STAT pathway (42) (Fig. 8). The data further suggest that restrictin-P moderately increased STAT activation (Fig. 8). This is surprising in view of the fact
that restrictin-P abrogated the IL-6-induced secretion of acute phase proteins by HepG2 hepatoma (Fig. 5). It is implied therefore that a separate, possibly unknown pathway exists which allows cross-talk between the restrictin-P and IL-6 signaling cascades.

**FIG. 6.** Restrictin-P does not interfere with the binding of IL-6 to B9 cells. B9 cells were weaned from IL-6 in the growth medium 24 h before binding assay. A, specific high affinity binding of a constant amount of r-Mu-\(^{125}\text{I}\)-IL-6 competed with varying amounts of crude concentrated mouse IL-6 (○) or partially purified restrictin-P (●). Mean total r-Mu-IL-6 bound was 1120 ± 48 cpm. B, specific high affinity binding of a constant amount of r-Hu-\(^{125}\text{I}\)-IL-6 mutein (○) or purified restrictin-P (●). The mean total r-Hu-IL-6 binding was 2350 ± 66 cpm which was competed to 123 ± 30 with a 200-fold excess of crude concentrated murine IL-6. Results of the competition binding studies are plotted as a function of fold excess where 1 unit of restrictin-P inhibits 1 unit of IL-6 by 50% on B9 cells. Error bars indicate standard deviation of replicates.

**FIG. 7.** Restrictin-P causes augmented expression of junB mRNA in plasma-like cells. The IL-6-dependent B9 cells were incubated overnight without IL-6 and were then induced by 2 units/ml of this cytokine for 20 min before addition of restrictin-P (RP) (200 units/ml) or 10 mM Tris-HCl (0 min) (A). MPC-11 plasmacytoma cells were seeded with or without IL-6 and with restrictin-P as above (B). Following the time point indicated, RNA was extracted and examined by Northern blotting for junB and TIS11 transcripts.

**FIG. 8.** Effect of restrictin-P on STAT activation. HepG2 cells were incubated with human recombinant IL-6 (10 units/ml) and restrictin-P as indicated. Either IL-6 and restrictin-P were added simultaneously or restrictin-P was added 30 min or 16 h prior to IL-6. 15 min after addition of IL-6 to the medium, the cells were harvested and nuclear extracts were prepared. 10 μg of protein was then analyzed in a gel retardation assay using a \(^{32}\text{P}\)-labeled oligonucleotide probe that contained a high affinity mutant of the c-fos promoter sis-induced element (SIE). The positions of DNA-protein complexes containing either Stat3 and Stat1α homodimers or Stat3/Stat1α heterodimers are indicated.

**DISCUSSION**

We investigated the nature of the activity, designated as restrictin-P (11), found in media conditioned by stromal cells (12), which causes growth arrest and subsequent cell death of mouse plasmacytomas and hybridomas. This activity was mediated by a protein that was purified to homogeneity from medium conditioned by the stromal cell line MBA-2.1 and was found to have an N-terminal amino acid sequence indistinguishable from that of activin A (37–39) which is also known as follicle-stimulating hormone releasing protein or erythroid differentiation factor (39, 43–46). Activin A was found to be expressed by stromal cells (47). The molecular mass of monomeric restrictin-P, as deduced from PAGE, was 15 kDa, a size similar to that of monomeric activin A (βA-inhibin). Like activin A, restrictin-P is a dimer of 25 kDa under nonreducing conditions and loses its biological activity upon reduction (not shown). Furthermore, recombinant activin A was inhibitory to the MPC-11 plasmacytoma to the same extent as was restrictin-P. It is therefore concluded that these two molecules are identical.

Restrictin-P in its purified form killed the factor-dependent hybridoma cell line B9 by competing with externally added IL-6 or IL-11. On the basis of the inability of a 270-fold excess of partially purified restrictin-P to compete with r-Mu-\(^{125}\text{I}\)-IL-6 binding (Fig. 6A) or a 340-fold excess of highly purified restrictin-P to compete with r-Hu-\(^{125}\text{I}\)-IL-6 (mutein) (Fig. 6B) binding, it is concluded that restrictin-P does not exert its effect by competing with IL-6 for high affinity IL-6 ligand binding sites.
The antagonistic effect of restrictin-P is specific to IL-6 and IL-11 since restrictin-P did not affect the growth of other cytokine-dependent cell lines such as 14M1.4 macrophages that depend on M-CSF for growth, MC/9 mastocytoma which are IL-3-dependent or NFS-60, GM-CSF-dependent cells (Fig. 3B).

The strict specificity of killing by restrictin-P of plasmacytomas and hybridomas suggested that the factor detects some molecular machinery characteristic to this cell type. The growth dependence on IL-6 is common to many plasmacytomas and hybridomas. We show here that restrictin-P inhibits the growth of B9 cells by competing with the growth factors obligatory for the survival of the hybridoma. However, some cells, like the MHC-1 clone, are cytokine-independent, but are nonetheless growth-inhibited by restrictin-P. The question raised is whether the mechanism of action of restrictin-P in the case of IL-6-dependent B9 cells is different from that in MHC-1 cells.

An alternative possibility is that the restrictin-P/activin A receptor complex is specific for the growth factor and through this property it would not be restrictive to other cells that depend on alternative growth factors.

In these different cell systems, the inducer molecule IL-6 has been shown to control the growth of B9 cells (65). This activity was not further noted an increase in STAT activation.

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