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# Interleukin-9 induces 24P3 lipocalin gene expression in murine T cell lymphomas.

## Abstract

Interleukin-9 (IL-9) is a Th2 cytokine whose overexpression is associated with asthma and T cell lymphomagenesis. All the IL-9 activities studied so far are mediated by a specific hemopoietin receptor that activates a Jak/STAT pathway. Searching for genes specifically modulated by IL-9, we observed that the 24P3 mRNA is strongly upregulated in BW5147 T lymphoma cells upon IL-9 stimulation. 24P3 is a member of the lipocalin family, and has been reported to bind N-formyl-Met-Leu-Phe, a potent neutrophil chemoattractant, and possibly other

lipophylic mediators of inflammation. A similar 24P3 induction was observed in other T cell lymphomas (EL4 and TH201) in response to IL-9, as well as in EL4 cells stimulated with IL-6 or IL-1. By contrast, other IL-9-responsive cells such as mast cell line MC9 and B cell lymphoma A20 showed no 24P3 induction upon IL-9 stimulation. Experiments using IL-9R mutants indicated that STAT transcription factors, particularly STAT3, are involved in this process. However, 24P3 gene induction was slow, reaching a plateau from 36 to 72 hours after stimulation and was inhibited if cells were treated with cycloheximide during the first 8 hours of IL-9 stimulation, suggesting an indirect induction requiring new protein synthesis.

## Full text

### INTRODUCTION

Interleukin-9 (IL-9) is a pleiotropic cytokine secreted by activated Th2 lymphocytes and possibly involved in asthma pathogenesis. Both human and mouse genetic studies point to IL-9 and its receptor as candidate genes for asthma susceptibility [1-3]. IL-9 overproduction in lungs of transgenic mice leads to an asthmatic-like phenotype including broncho-hyperresponsiveness, increased IgE, mastocytosis, mucus production and lung eosinophilia [4-8]. In addition, IL-9 might play a role in T cell tumorigenesis. IL-9 transgenic mice show a high susceptibility to T cell lymphoma development [9], and constitutive IL-9 production was reported in human Hodgkin lymphoma [10]. Although freshly isolated normal T cells respond poorly to IL-9, this factor is much more potent for thymic lymphomas, particularly as an anti-apoptotic factor [11].

All the IL-9 activities studied so far are mediated by a receptor consisting of two chains. The IL-9Ralpha chain is sufficient to confer high affinity binding [12], but signaling requires its association with gammaC, a common component of the receptors for IL-2, IL-4, IL-7, IL-9 and IL-15. Upon IL-9 binding, Jak1 and Jak3 tyrosine kinases become activated and phosphorylate IL-9Ralpha on a single tyrosine residue [13]. This tyrosine is required for activation of STAT1, STAT3 and STAT5 transcription factors, and mutations at this position abolish all IL-9 activities tested, including growth regulation, apoptosis inhibition and cell differentiation [13-15].

Because STAT transcription factors seem to be required for the IL-9 response, we took advantage of a cDNA subtraction technique to identify the genes specifically regulated by this cytokine. Comparing T helper clones that proliferate either in IL-2 or in IL-9, we found that IL-9 specifically induces the expression of a series of proteases including granzyme A and B, as well as mast cell proteases of the MMCP family [16]. Further studies showed that IL-9 upregulates Bcl-3 expression in T cells and mast cells, thereby interfering with the NF-kappaB pathway [17], and the expression of M-Ras a new oncogenic member of the Ras superfamily [18]. In the BW5147 thymic lymphoma, we found that IL-9 induces the expression of a new cytokine, called IL-TIF (for IL-10-related T cell-derived inducible factor) [19].

Here, using the same lymphoma cells, we report that IL-9 upregulates the expression of 24P3, a member of the lipocalin family. Lipocalins are small extracellular proteins that display modest sequence homology (20-30%), but share a common structural feature, the lipocalin fold consisting of eight antiparallel beta strands. This structure forms a pocket for hydrophobic ligands, such as retinol, steroid hormones, pheromones and cholesterol. Beside their function as carrier proteins, some lipocalins such as prostaglandin D synthase exert enzymatic activities, and it is now clear that these proteins are involved in a variety of biological processes, including the regulation of cell homeostasis and modulation of the immune response. Our observation suggests that 24P3 might mediate some of the IL-9 activities.

## MATERIALS AND METHODS

### *Cell cultures, cytokines and other reagents*

BW5147 (ATCC, Rockville, MD, USA) and EL4, two murine T lymphoma cell lines, were cultured in Iscove-Dulbecco's medium supplemented with 10% FCS, L-asparagine 0.24 mM, L-glutamine 1.5 mM, L-arginine 0.55 mM and 2-mercaptoethanol 50 muM. TH201 (a gift from Dr. A. Van Pel, Ludwig Institute for Cancer Research, Bruxelles, Belgium), a tumorigenic murine T lymphoma clone, was grown in RPMI 1640 containing 10% FCS, L-asparagine 0.24 mM, L-glutamine 1.5 mM, L-arginine 0.55 mM and 2-mercaptoethanol 50 muM. T helper cell clones TS2 and TS3 [20], were grown in DMEM medium supplemented with 10% FCS, L-asparagine 0.24 mM, L-glutamine 1.5 mM, L-arginine 0.55 mM and 2-mercaptoethanol 50 muM. These factor-dependent cell lines were able to

grow in the presence of IL-2, IL-4 or IL-9 without antigen and feeder cells [16]. A20, a murine B lymphoma cell line, was cultured in Iscove-Dulbecco's medium containing 10% FCS, L-asparagine 0.24 mM, L-glutamine 1.5 mM, L-arginine 0.55 mM and 2-mercaptoethanol 50 muM. MC9 mast cell line was cultured in the same medium supplemented with mIL-3 (100 U/ml from CHO cell supernatants) or mIL-9 (200 U/ml).

BW5147 cells were transfected with the wild-type human IL-9 receptor and its Phe116 mutant (mut1), as previously described [13] and cultured in the presence of 1.5 mug/ml puromycin. Two additional human IL-9 receptor mutants, mut6 (activating STAT1 and 3) and mut7 (activating STAT5), were similarly transfected in BW5147 [15].

Recombinant human IL-9 ($2 \times 10^7$ U/mg), mouse IL-4 ($3.8 \times 10^6$ U/mg), IL-6 ($10^9$ U/mg), and IL-9 ($5 \times 10^7$ U/mg) were produced in the baculovirus system in our laboratory and purified as previously described [21]. Human recombinant IL-1alpha was a gift from Dr. P. Lomedico (Hoffmann-La Roche, Nutley, NJ, USA). Human tumor necrosis factor-alpha (TNF-alpha) and mouse interferon-gamma (IFN-gamma) were kindly provided by Dr. W. Fiers (State University of Ghent, Ghent, Belgium). Cytokines were added to the cultures at the following concentration: 500 U/ml for human and mouse IL-9, 10,000 U/ml for mIL-6, 500 U/ml for mIL-4, 250 U/ml for IFN-gamma , 100 U/ml for IL-1alpha, 10 ng/ml for TNF-alpha. Actinomycin D (ICN, Irvine, CA, USA), protein synthesis inhibitor cycloheximide (Sigma, St. Louis, MO, USA), and proteasome inhibitor MG-132 (Biomol, Plymouth Meeting, PA, USA) were used at 5 mug/ml, 10 mug/ml and 50 muM, respectively.

### *Subtractive hybridization*

Total RNA was prepared from BW5147 cells either cultured in normal medium or stimulated with mIL-9 (500 U/ml) for 48 hours, using guanidium isothiocyanate lysis and CsCl gradient centrifugation [22]. Polyadenylated RNA was purified from total RNA with oligo(dT) cellulose columns. Double stranded cDNA was generated from 5 mug polyA$^+$ RNA using an oligo (dT) primer and the SuperScript Choice System for cDNA synthesis according to the manufacturer's recommendations (Gibco BRL). Representational difference analysis was performed as described by Hubank [23]. cDNA were digested with DpnII (New England Biolabs, Beverly, MA), ligated to R-BglI-12/24 adapters, and polymerase

chain reaction (PCR) amplified to generate representations. The R-Bgl oligonucleotides were removed by DpnII digestion, and J-Bgl-12/24 adapters were ligated to the cDNA from BW5147/IL-9 cells. For the first cycle of subtractive hybridization, 0.4 mug of J-Bgl-ligated BW5147/IL-9 cDNA was mixed with 40 mug BW5147 cDNA (1/100 ratio). After hybridization for 20 hours at 67° C, the ends of the resulting hybrids were filled in, and a PCR amplification was performed with the J-Bgl-24 oligonucleotide, which was further removed by DpnII digestion. The second and third cycles of subtraction were performed similarly with a ratio of 1/800 and 1/400,000, respectively.

After 3 rounds of subtraction, final difference product was digested with DpnII and cloned into the BamHI site of pTZ19R. Double stranded plasmid DNA was prepared and sequenced with a Thermo-sequenase Sequencing kit (Amersham, Arlington Heights, IL). Sequence comparisons with the GenBank and EMBL databases were performed with the BLAST search program. Oligo(dT)-primed cDNA libraries generated from IL-9-stimulated BW5147 cells were screened with the mouse lipocalin DpnII fragment as described [19].

### *RT-PCR analysis*

Reverse transcription was performed on 5 mug TriPure-purified (Boehringer-Mannheim) total RNA with an oligo(dT) primer. cDNA corresponding to 100 ng of total RNA was amplified for 27 cycles by PCR with specific primers for murine 24P3 as follows: sense 5'-GCACACATCAGACCTAGTAGC-3' (from position +21 of the mRNA sequence, GenBank accession number X81627) and antisens 5'-CTCACCACCCATTCAGTTGTC-3' (from position +748 of the mRNA sequence); for beta-actin: sense 5'-ATGGATGACGATATCGCTGC-3' and antisense 5'-GCTGGAAGGTGGACAGTGAG-3' (18 cycles). Post-PCR products were analyzed in ethidium bromide-stained 1% agarose gel. Signals were quantified by 1D Image Analysis Software Version 3.0 (KODAK Digital Science) and the 24P3/actin ratios were calculated.

### *Preparation of mRNA and Northern blot*

Total RNA was prepared using TriPure isolation method (Boehringer-Mannheim) from BW5147 cells. These cells were cultured in the presence of 500 U/ml mIL-9, at the following timing: 0, 12, 24, 36, 48, 60 and 72 hours. 10 mug of total cellular RNA was fractionated by electrophoresis in a 1.2% agarose gel containing 2.2 M

formaldehyde and was transferred onto a Hybond-C Extra nitrocellulose membrane (Amersham). cDNA inserts were labeled using the Rediprime DNA labeling kit from Amersham. Hybridizations and washes were performed as described [24]. The 24P3 probe was a 0.6 kb cDNA containing the coding sequence. After autoradiography, all blots were reprobed with a beta-actin probe to control each loading of RNA.

## RESULTS

### *IL-9 induces the 24P3 gene in BW5147 lymphoma*

To characterize the activity of IL-9 on T lymphocytes at the molecular level, we performed a representational difference analysis of gene expression on BW5147 lymphoma cells incubated with or without IL-9 for 48 hours. After three rounds of subtractive hybridization, the third difference product was cloned and 24 clones were sequenced. Three independent IL-9-specific cDNA sequences were identified, including Bcl-3 and IL-TIF. The third IL-9-induced transcript was found to match the sequence of 24P3, a gene upregulated in SV40-infected kidney cells [25]. To confirm 24P3 induction by IL-9, we performed a Northern blot hybridization with RNA from BW5147 cells cultured with or without IL-9, using the 24P3 cDNA as a probe. As shown in Figure 1, a strong signal was detected after IL-9 stimulation.

In order to study the kinetics of 24P3 induction, we stimulated BW5147 cells with IL-9 for different periods of time (12, 24, 36, 48, 60 and 72 hours) and Northern blots were hybridized with the 24P3 probe. As shown in Figure 2, the 24P3 message was upregulated only after 36 hours of IL-9 stimulation, peaked at 48 hours, and slightly decreased but remained detectable until at least 72 hours.

### *24P3 induction in thymic lymphomas stimulated with different cytokines*

In order to know if 24P3 induction in BW5147 cells is restricted to IL-9, we stimulated cells with different cytokines, including IL-6, IL-4, IFN-gamma, TNF-alpha and IL-1alpha for 48 hours, and analyzed 24P3 expression by RT-PCR. As shown in Figure 3A, IL-6 and IL-1alpha modestly induced 24P3 expression in BW5147 but failed to reproduce the effect of IL-9. There was no induction after stimulation of BW5147 cells with IL-4, IFN-gamma, TNF-alpha. We further studied 24P3 induction in two additional thymic lymphomas, EL4 and TH201. IL-9 clearly

upregulated 24P3 in these cell lines (Figure 3A). IL-6 had a similar effect in EL4 but not in TH201 cells. IL-1alpha induced a dramatic increase of 24P3 expression in EL4 cells only (Figure 3), exceeding the effect of IL-9 in these cells. As IL-1alpha and IL-9 activate distinct signal transduction pathways, we analyzed the kinetics of induction of 24P3 by IL-6, IL-9 and IL-1alpha. As shown in Figure 3B, these cytokines show the same delayed induction of this gene. Because corticoids have been reported to induce 24P3 in murine L cells [26], we studied 24P3 expression in BW5147 cells stimulated with IL-9 and dexamethasone. In the absence of IL-9, dexamethasone induced apoptosis in BW5147 cells. However, in the presence of IL-9, cells survived and 24P3 induction was further increased by dexamethasone (Figure 4). We analyzed 24P3 induction by RT-PCR in other IL-9-responsive cells such as T-helper clones (TS2, TS3), a mast cell line (MC9) and a B lymphoma cell line (A20). In A.20, MC9, TS2 and TS3 cells, a weak constitutive expression of 24P3 was detectable. Although all these cell lines responded to IL-9 by induction of the Bcl-3 gene, IL-9 stimulation did not significantly affect 24P3 expression in these cells (Figure 5), suggesting a tissue-specific regulation.

### *Mechanisms of 24P3 induction by IL-9*

The upregulation of the 24P3 message could result either from a transcriptional mechanism or from stabilization of this mRNA. To test the latter hypothesis, we stimulated BW5147 cells with IL-9 for 48 hours before incubation with 5 mug/ml of actinomycin D, an inhibitor of RNA synthesis, and with or without IL-9. We analyzed 24P3 expression by Northern blot, after different periods of incubation (Figure 6). In the presence of actinomycin D for up to 8 hours, the 24P3 mRNA decrease did not seem to be affected by IL-9, suggesting that this factor does not modulate the mRNA stability. Longer incubation could not be tested because of the toxicity of actinomycin D to BW5147. In addition, even without actinomycin D, cells that were starved of IL-9 for 8 hours still showed high levels of 24P3 expression, suggesting that the half-life of the 24P3 mRNA in BW5147 cells is longer than 8 hours, as previously observed in L cells [25]. This observation makes it unlikely that IL-9 induction is due to stabilization of 24P3 mRNA.

To analyze further the mechanisms by which IL-9 induces 24P3 expression, we took advantage of BW5147 transfectant cells, expressing mutated forms of the human IL-9 receptor (hIL-9R). Human IL-9 does not bind to the murine IL-9 receptor and therefore fails to induce 24P3 in BW5147 parental cells (data not

shown). When cells expressed the wild type hIL-9R, 24P3 was induced by human IL-9, as it was by murine IL-9 in the parental BW5147 cells (Figure 7). By contrast, human IL-9 failed to induce 24P3 in cells expressing a mutated form of hIL-9R (mut1). In this mutated receptor, a single tyrosine residue of the cytoplasmic part was changed into phenylalanine, resulting in the inability to activate STAT factors in response to IL-9 [13].

To determine the respective role of different STAT factors activated by IL-9 (STAT1, 3, and 5), we used other hIL-9R mutants [15] that specifically activate STAT5 (mut7) or STAT1 and 3 (mut6). As shown in Figure 7, STAT5 was not necessary for 24P3 induction, because this gene was still induced in mut6 cells, in which STAT5 is not activated by IL-9. In addition, STAT5 alone was not sufficient to induce the 24P3 gene, as there was no induction upon hIL-9 stimulation of mut7 cells (Figure 7). In mut6 cells, IL-9 activated both STAT1 and STAT3. Because IFN-gamma did not induce 24P3 (Figure 3) but activated STAT1 and other genes such as Ly6A2 [15], it could be speculated that STAT3, or STAT1-3 heterodimers, mediate the activity of IL-9 on 24P3 expression.

To determine whether IL-9 directly induces 24P3 gene expression or whether this process requires new protein synthesis, BW5147 cells were stimulated with IL-9 in the presence of cycloheximide (CHX), an inhibitor of protein synthesis. Because cells could not be cultured in the presence of CHX for 48 hours, cells were washed after 8 hours and further stimulated for 40 hours, before RNA extraction and RT-PCR analysis. As shown in Figure 8, inhibition of protein synthesis by CHX during the first 8 hours of IL-9 stimulation, dramatically decreased 24P3 induction, indicating an indirect process. Under the same conditions, Bcl-3 induction by IL-9 was not affected, which is compatible with its direct transcriptional regulation [17].

## DISCUSSION

In the present report, we describe the upregulation of the 24P3 gene by IL-9 in murine T lymphoma cells, but not in mast cells nor B cells. This gene is induced upon 36 hours of IL-9 stimulation through an indirect mechanism involving STAT transcription factors. 24P3 is a member of the lipocalin family, which includes small secreted proteins characterized by a conserved folding pattern, with a single eight-stranded antiparallel beta-sheet closed back on itself to form a continuously hydrogen-bonded beta-barrel [27]. This beta-barrel defines a cup-

shaped structure that encloses an internal ligand binding site for small, principally hydrophobic molecules such as retinoids, arachidonic acid, steroids and pheromones. Little is known about the endogenous 24P3 ligand. Its human counterpart called NGAL (neutrophil gelatinase associated lipocalin) was originally isolated from neutrophil granules [28] and was found to be associated with N-formyl-methionyl-leucyl-phenylalanine (fMLP) [29]. However, the size, shape and character of the NGAL calyx, based on crystal structure, suggest that small molecules such as fMLP, LTB4 or PAF would not complement or fill the calyx of the protein, which is large enough to accommodate macromolecules, such as small proteins on the order of the size of chemokines [30].

Lipocalins have, in general, been classified as extracellular transport proteins, as typified by RBP, the retinol transporter in plasma. Accumulating data suggest that these proteins fulfil many different and potentially significant biological functions, including roles in olfaction, enzymatic synthesis, immunoregulation and regulation of cell homeostasis [27]. The murine cDNA coding for 24P3 was initially isolated in kidney cells, in which the gene was induced upon SV40 virus infection, raising the possibility that 24P3 may contribute to the SV40-induced transition from quiescent to a proliferative state [25].

A role for IL-9 in tumorigenesis has been suggested because of the high susceptibility to thymic lymphomas observed in IL-9-transgenic mice [9]. *In vitro*, IL-9 protects thymic lymphoma cells against dexamethasone-induced apoptosis [11]. Because lipocalins were described as potential carriers for corticosteroids and the 24P3 gene was overexpressed in BW5147 cells costimulated with IL-9 and DEX (Figure 4), we tested whether this protein was involved in the anti-apoptotic effect of IL-9. However, BW5147 lymphoma cells transfected with the 24P3 cDNA were still sensitive to dexamethasone-induced apoptosis (data not shown). This observation indicates that 24P3 does not antagonize dexamethasone activity, although it does not exclude the possibility that 24P3 could operate *in concertum* with other genes induced by IL-9. A role of 24P3 in cell proliferation is also unlikely, as we did not observe any difference of growth rate in 24P3-transfected cells (data not shown).

Several lipocalins have been shown to possess immunosuppressive properties *in vitro*. For instance, alpha1- microglobulin (A1M) suppresses antigen-induced polyclonal proliferation of cultured lymphocytes [27]. We tested this possibility for 24P3, using supernatants from transiently transfected cells, but we failed to

observe any effect of 24P3 on polyclonal activation of spleen cells, or on NK cell proliferation (data not shown). However, we can not rule out the possibility that a post-translational modification or association with hydrophobic molecules is required for 24P3 to exhibit its full biological activities. A role for this lipocalin in the inflammatory reaction is suggested because of its production by LPS-stimulated macrophages [31], and in liver during an acute phase response induced by turpentine injection or in response to TNF [32]. Moreover, the 24P3 human homologue, NGAL, was found in the granules of human neutrophils, in association with gelatinase [28]. In this respect, it should be stressed that a series of proteases such as granzymes have been shown to be induced by IL-9 [16]. It is therefore possible that 24P3 induction takes place within a proteolytic pathway, by playing the role of cofactor or chaperone for some of these proteases. The same hypothesis might be raised for chemotactic factors that can be induced by IL-9 and were proposed to bind to 24P3 [7, 30].

A striking feature concerning the 24P3 gene is its inducibility that led to its independent identification as a gene induced by SV40 virus infection in kidney cells [25], turpentine injection in liver [32], dexamethasone or retinoic acid in fibroblasts [26], LPS stimulation in macrophages [31], and now IL-9 in thymic lymphomas. The mechanisms underlying this regulation remain poorly understood. Garay-Rojas and colleagues identified putative regulatory elements in the 5'-flanking region of the 24P3 gene, including a TATA-like box and two glucocorticoid responsive core elements (GRE), and concluded that dexamethasone induces 24P3 both directly and indirectly, through a putative autocrine mechanism [26]. Interestingly, we found that IL-9 and dexamethasone synergised for 24P3 gene induction in BW5147 cells, indicating that distinct activation pathways cooperate to regulate 24P3 expression. It was tempting to speculate that
IL-9 and DEX activate an autocrine loop in BW5147. Incidentally, we recently showed that Il-9 upregulates the expression of a new cytokine in BW5147 [19, 33]. However, we failed to detect any 24P3-inducing activity in the supernatant of IL-9 stimulated BW5147 cells (data not shown).

In our system, most of the IL-9-mediated induction appeared to be indirect because it is blocked by CHX during the first 8 hours of stimulation. A single point mutation of the IL-9R that abolishes STAT activation, also blocked 24P3 induction suggesting that a gene controlled by STAT transcription factors might be responsible for 24P3 expression. Bcl-3 was a potential candidate for this

activity because it is induced by IL-9 in BW5147, it modulates gene expression by interacting with NF-kappaB proteins, and several 24P3 inducers, such as IL-1, LPS and TNF, activate the NF-kappaB pathway. However, there was no correlation between 24P3 and Bcl-3 induction in IL-9 responsive cells (Figure 5), arguing against the involvement of Bcl-3 in this process. Other genes induced by IL-9 include SOCS-1, -2 and -3. However, so far, we could only ascribe inhibitory activities on IL-9 signaling to the SOCS-3 protein [34], making it unlikely that members of this family mediate 24P3 expression. Finally, the observation that Il-1 and IL-9 induce 24P3 expression with a similar delayed kinetics raises the possibility that this regulation is mediated by a factor controlled by distinct signal transduction pathways such as Jak/STAT or NF-kappaB.

CONCLUSION

In summary, we show here that the 24P3 lipocalin gene can be upregulated by IL-9 in T cell lymphomas through an indirect mechanism. Although the function of the 24P3 protein remains to be determined, its inducibility upon inflammatory stimuli in various cells suggests a role in inflammation. The identification of its endogenous ligand is definitely required to understand further its physiopathological function and its putative value as a target to antagonize the activities of cytokines in general and IL-9 in particular.

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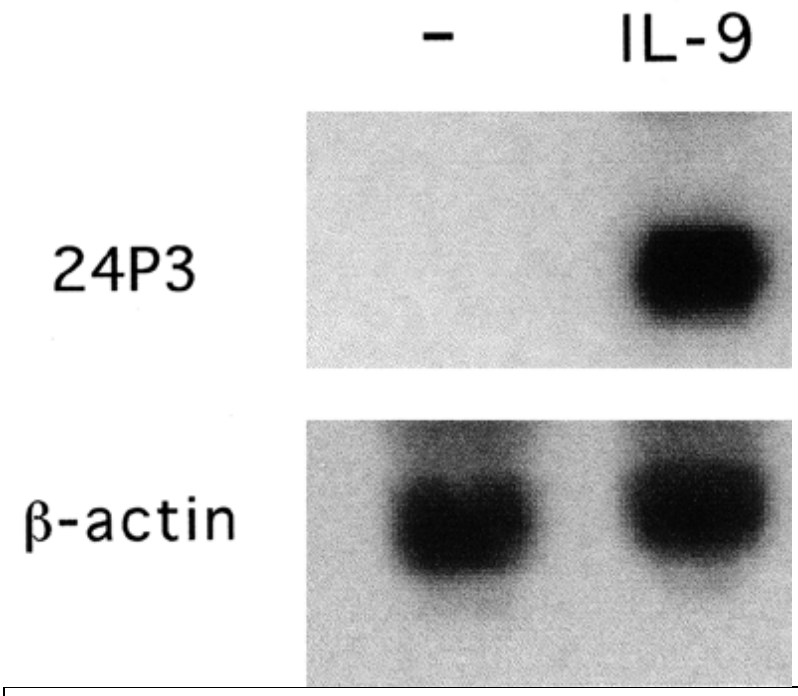
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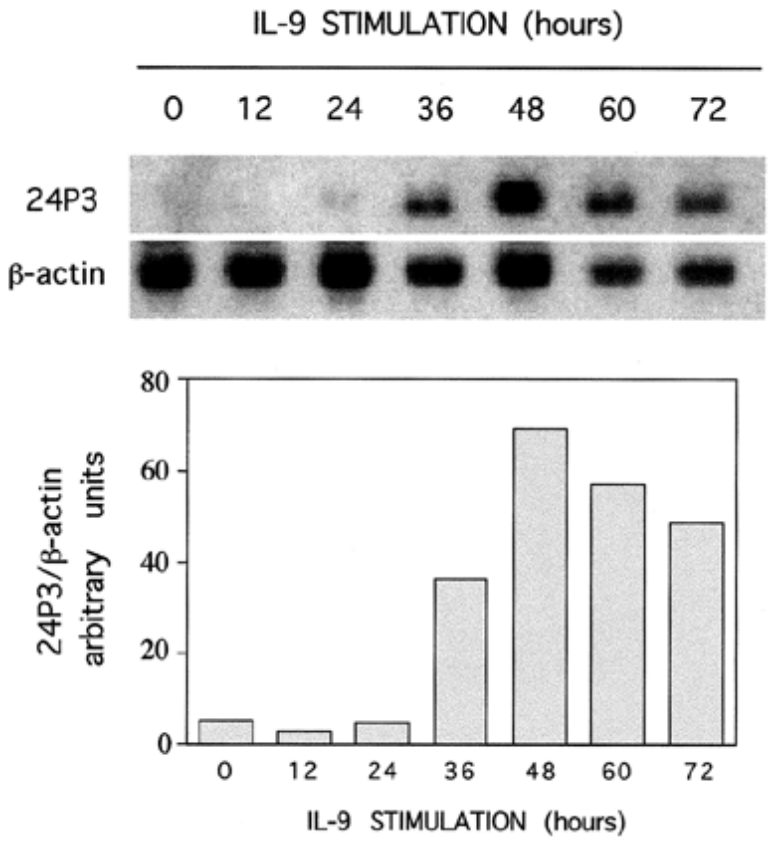
## Figures



## Figure 1

**IL-9 induces 24P3 lipocalin expression.**

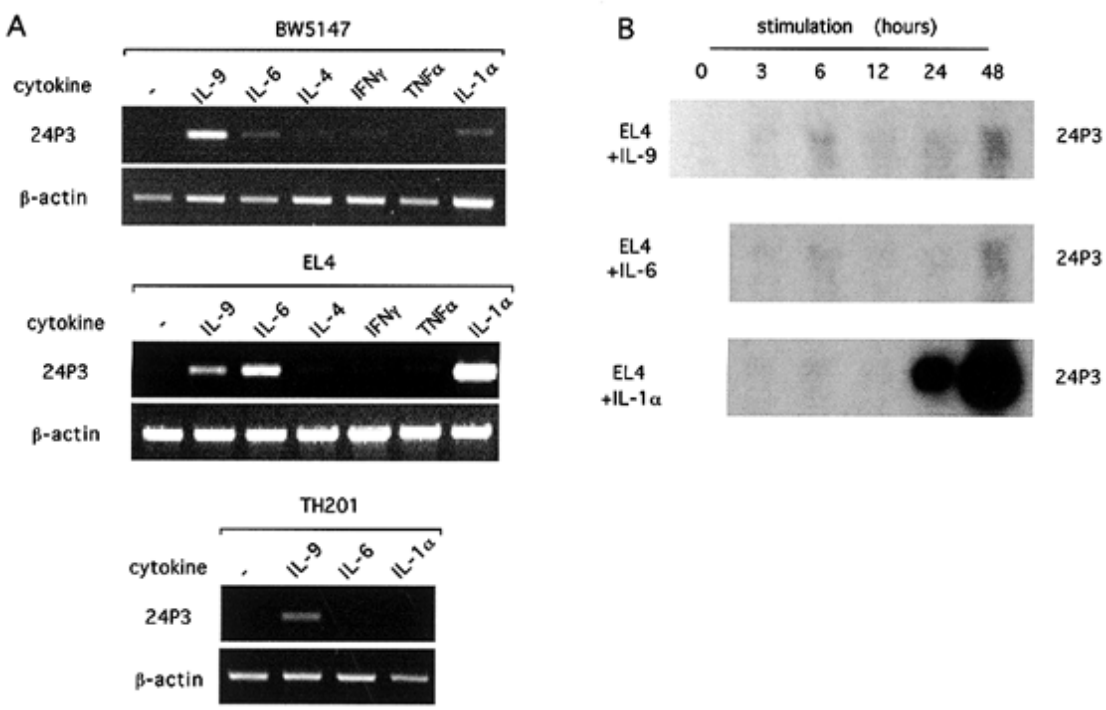
BW5147 lymphoma cells were stimulated with 500 U/ml of murine IL-9 for 48 hours. Total RNA from unstimulated or stimulated cells was fractionated by electrophoresis and transferred to nitrocellulose before hybridization with a $^{32}$P-labeled 24P3 probe. Hybridization with beta-actin probe confirmed that comparable amounts of RNA had been loaded in each lane.




## Figure 2

**Kinetics of the expression of 24P3 mRNA.**

Total RNA from BW5147 cells stimulated with IL-9 for the indicated periods of time was fractionated by electrophoresis and transferred to nitrocellulose before hybridization with a $^{32}$P-labeled 24P3 probe. Hybridization with beta-actin probe confirmed that comparable amounts of RNA had been loaded in each lane. After autoradiography the film was analyzed by 1D Analysis Software (Kodak) and ratio 24P3/beta-actin was quantified.




## Figure 3

**24P3 induction in thymic lymphomas stimulated with different cytokines.**

**A:** BW5147, EL4 and TH201 cells were stimulated for 48 hours with the indicated cytokines. Total RNA was extracted and RT-PCR was performed with specific primers for murine 24P3 and beta-actin as described in material and methods. **B:** total RNA from EL4 stimulated

with IL-9, IL-6 or IL-1alpha for the indicated periods of time, was fractionated by electrophoresis and transferred to nitrocellulose before hybridization with a $^{32}$P-labeled 24P3 probe.

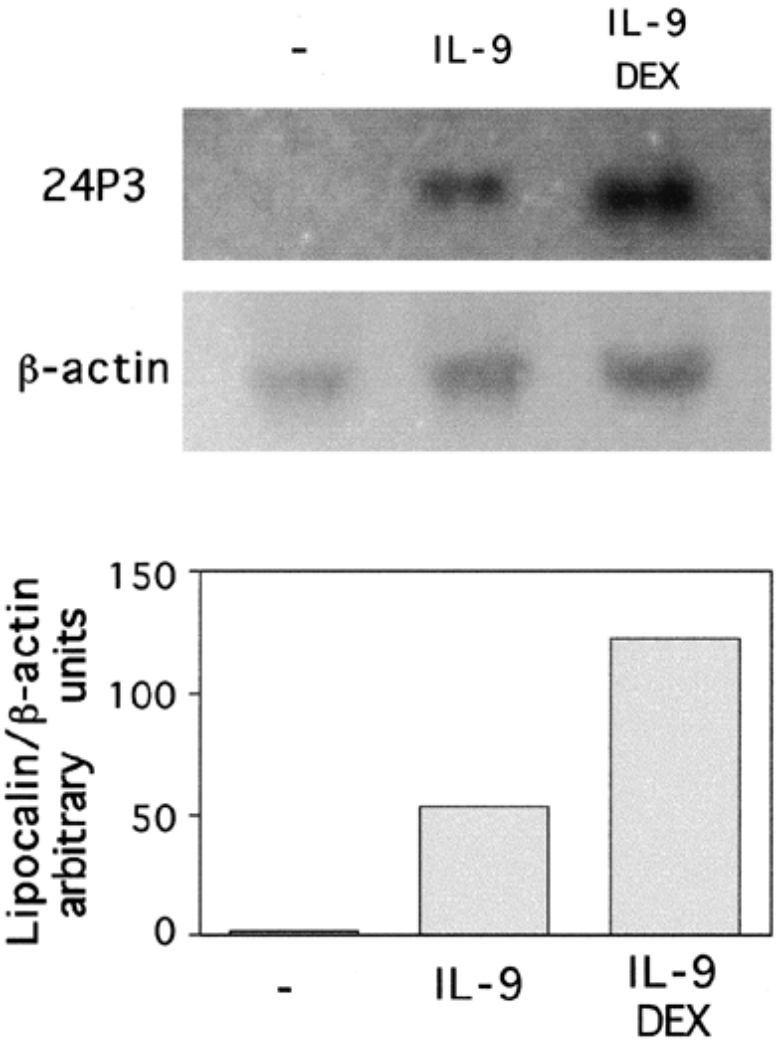



**Figure 4**

**24P3 induction after DEX stimulation.**

BW5147 cells were stimulated with IL-9 ± dexamethasone (DEX) for 48 hours. Total RNA was blotted to nitrocellulose and hybridized with $^{32}$P-labeled 24P3 or beta-actin probes. The autoradiography was analyzed by 1D Analysis

Software (Kodak) and ratio 24P3/beta-actin was quantified.

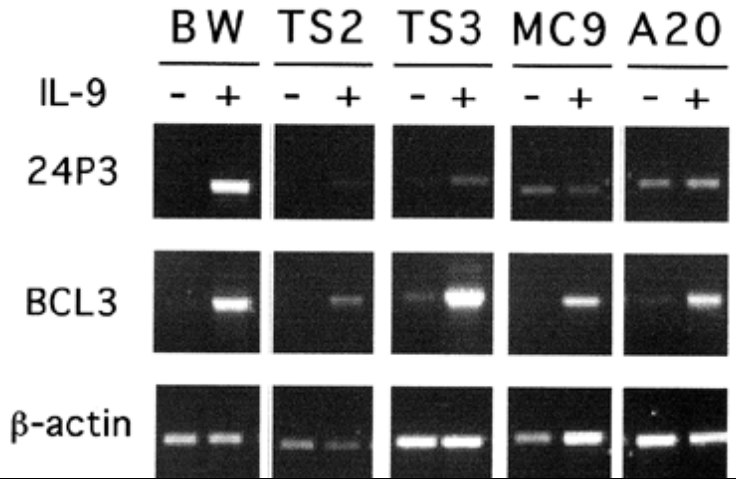
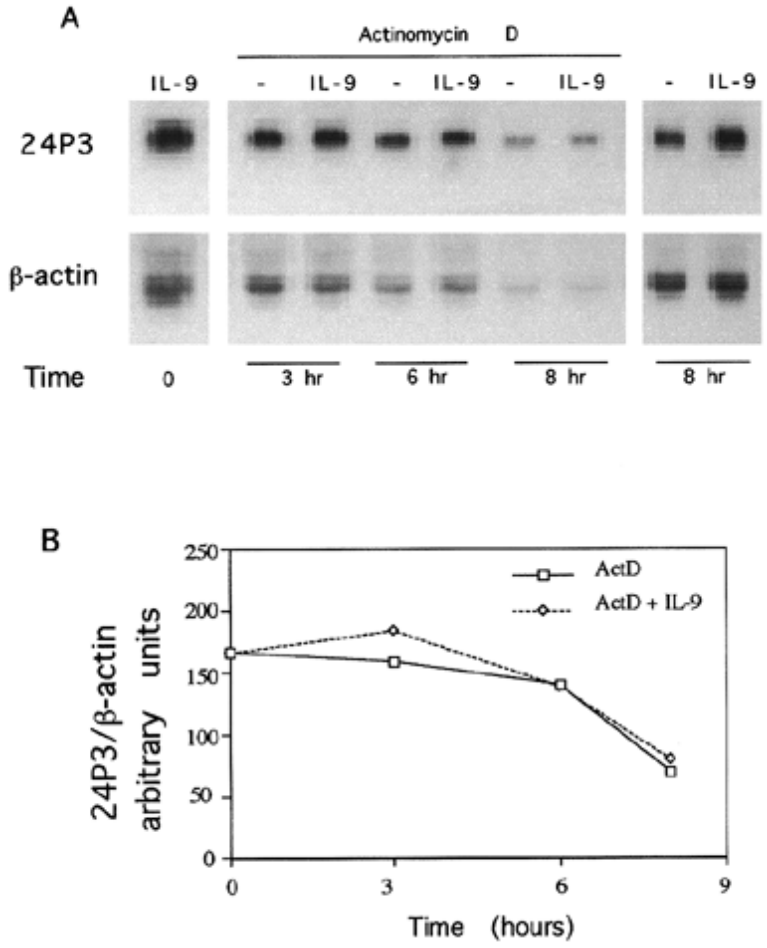



**Figure 5**

**24P3 expression in different cell lines.**

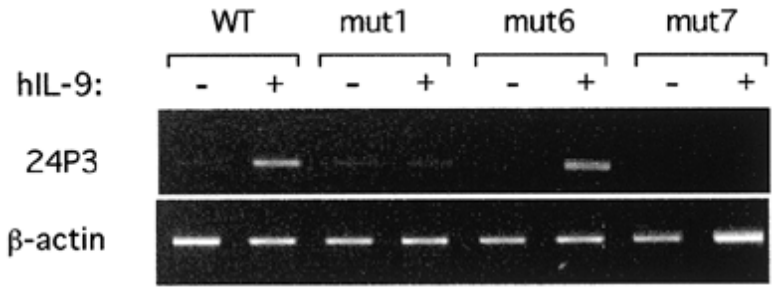
TS2 and TS3 T helper clones, MC9 mast cell line and A20 B lymphoma were stimulated with 500 U/ml murine IL-9 for 48 hours. Total RNA was extracted and RT-PCR was performed with specific primers for 24P3, Bcl-3 or beta-actin. PCR products were analyzed by ethidium bromide-stained agarose gel electrophoresis.




## Figure 6

**IL-9 does not increase 24P3 mRNA stability.**

BW5147 cells were pre-incubated with mIL-9 (500 U/ml) for 48 hours, then washed and further incubated in the presence of actinomycin D (5 mug/ml) with or without IL-9, for different times. Total RNA was blotted to nitrocellulose and hybridized with $^{32}$P-labeled 24P3 or beta-actin probe. Signals were analyzed by autoradiography **(A)** and ratio 24P3/beta-actin was quantified using a Phosphorimager (Molecular Dynamies) **(B)**.




## Figure 7

**24P3 upregulation in cells expressing mutated hIL-9R.**

BW5147 cells transfected with wild type (WT) or the mutated form of hIL-9R (mut 1, mut 6, mut 7) were stimulated with human IL-9 500 U/ml for 48 hours. Total RNA was extracted and RT-PCR was performed with specific primers for 24P3 and beta-actin.



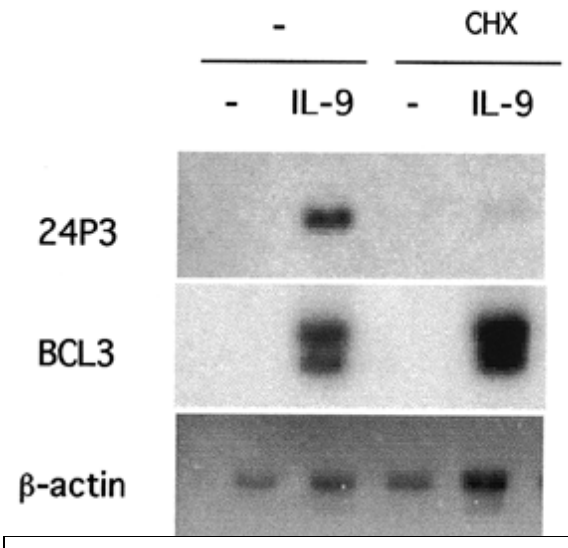



Figure 8

**New protein synthesis is required in 24P3 upregulation by IL-9.**

BW5147 cells were stimulated with 500 U/ml of mIL-9 for 48 hours, with or without the combination of cycloheximide 10 mug/ml and MG-132 50 muM during the first 8 hours of stimulation. Total RNA was blotted to nitrocellulose and hybridized with $^{32}$P-labeled 24P3, Bcl-3 or beta-actin probe.

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## In this issue

Regulation of interferon-g receptor (INF-gR) chains: a peculiar way to rule the life and death of human lymphocytes.

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The interleukin-6 cytokine system in embryonic development, embryo-maternal interactions and cardiogenesis.

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## Role of cytokines in the modulation of nitric oxide production by cyclic AMP.

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TNF, cytokine, LPS.

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MRC5, fibroblasts, TNF receptor, RT-PCR, binding, flow cytometry.

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C. Orabona, L. Dumoutier, J.C. Renauld
cytokines, lipocalin, IL-9, 24P3.

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L. Escoubet-Lozach, C.F. M'Rini, A. Rey, M. Béraud, J.C. Lepert, J. Bernard, J.R. Mathieu, B. Pipy
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A. Hatzfeld, B. Panterne, N.O. Fortunel, M.N. Monier, J.A. Hatzfeld
human CD34+ cells, fibrinogen, competence factor, IL-6 receptor.

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P. Bossù, E. del Grosso, M.P. Cesaroni, G. Maurizi, N. Balestro, A. Stoppacciaro, E. del Giudice, P. Ruggiero, D. Boraschi
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