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Interleukin-4 Up-Regulates Mouse Mammary Tumor Virus Expression yet Is Not Required for In Vivo Virus Spread

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The mouse mammary tumor virus (MMTV) superantigen induces T-cell production of cytokines, such as interleukin-4, which in turn increase MMTV transcription. However, interleukin-4 is not required for in vivo virus spread, because mice lacking interleukin-4 or the STAT6 transcription factor showed wild-type infection of lymphoid and mammary tissue. In spite of this, mammary tumor incidence was decreased in STAT6 null mice.

Virus infection in vivo leads to many interactions with cells of the immune system. In the case of mouse mammary tumor virus (MMTV), which is acquired neonatally through milk-borne transmission, the infection pathway directly involves lymphocytes. Initially, MMTV activates and infects B cells in the Peyer's patches of the small intestine, probably through interaction between the envelope (Env) protein and one or more molecules on the surfaces of these cells (2). One such molecule that interacts with the MMTV Env is Toll-like receptor 4 (TLR4), and this binding activates NF-κB transcription factors, followed by the production of cytokines such as interleukin-1 (IL-1) and IL-6 (J. C. Rassa, J. L. Meyers, Y. Zhang, R. Kudaravalli, and S. R. Ross, submitted for publication).

Subsequent to their initial activation, B cells become infected and present a viral superantigen (Sag) protein to cognate T cells. Presentation of viral Sag to cognate T cells results in their activation and the production of cytokines that stimulate these B cells (1, 35). At least in adult mice, B cells that receive this Sag-mediated T cell help differentiate into immunoglobulin G2a (IgG2a)-secreting plasma cells several days after injection of virus (11, 20, 22). Both B and T cells become infected in this process (8), and either cell type can transmit virus to uninfected wild-type (42) or SCID (36) mice.

T-cell help is provided to B cells through cell-cell interactions (CD40/CD40L; B7/CD28), many of which have been shown to be important for efficient MMTV infection in vivo (4, 5, 31). In adult mice that receive MMTV through a subcutaneous route, there is an initial production of predominantly type 1 cytokines, including gamma interferon and IL-2. There is B-cell production of IgG2a (21, 31), although it is not known whether milk-borne infection also induces IgG2a production. Later in the infection process, IL-4 is made (40), indicating that MMTV also results in the differentiation of T-helper 2 (Th2) cells, the subset that induces B-cell differentiation in response to cytokines such as IL-4 and IL-13 (6, 25, 28). Interaction of either of these cytokines with its receptor activates the signal transducer and activator of transcription factor 6 (STAT6) (33), and mice that have deletions in either the IL-4 or STAT6 genes have a paucity of Th2 cells (17, 38). Many cytokine and hormone receptors induce transcription through different Janus kinase (JAK)/STAT pathways (13, 37). In addition to the IL-4 receptor and STAT6, for example, activation of the receptors for prolactin and IL-2 results in signaling through the STAT5 transcription factors. There are two genes that encode STAT5, 5a and 5b, with distinct but overlapping functions in vivo (23, 39). We have shown previously that there is a STAT-like element in the long terminal repeat (LTR) of this virus that binds to STAT5 transcription factors and that prolactin induces virus transcription in mammary tissue culture cells (32). The IL-1 receptor, in contrast, belongs to the TLR/IL-1 receptor family, which signals through NF-κB transcription factors as well as other pathways (29). It has been shown that activation of TLR4 with bacterial lipopolysaccharide (LPS) induces MMTV transcription (3, 18). There is no consensus NF-κB site in the MMTV LTR, although a functional site in the *env* gene has been identified (I. Nepomnaschy and I. Piazzon, personal communication).

As MMTV is expressed both in T and B cells (8), and because virus infection results in cytokine production through the action of the Env and Sag proteins, we determined whether cytokines such as IL-1, IL-2, and IL-4 induce proviral transcription in lymphocytes. We first tested whether any of these cytokines induced transcription from endogenous MMTV loci in cultured primary splenocytes. Splenocytes were isolated from BALB/c mice and cultured in medium alone or in the presence of IL-1 (0.02 μg/ml; R&D Systems, Minneapolis, Minn.), IL-2 (50 U/ml), LPS (5 μg/ml), concanavalin A (ConA; 2 μg/ml; Sigma, St. Louis, Mo.) (Fig. 1), or IL-4 (100 U/ml; Gibco/BRL, Rockville, Md.) (Fig. 2) for 48 h (Fig. 1) or 20 h (Fig. 2). Total RNA was isolated using Trizol reagent (Gibco/BRL), and 40 μg was subjected to RNase protection using probes specific for the Mtv-9 and Mtv-6 proviruses, as previously described (9). Five micrograms of RNA from each sam-

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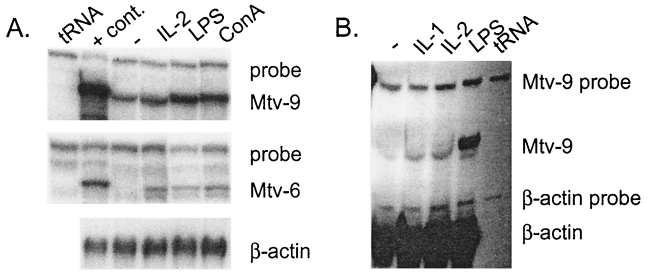

FIG. 1. Cytokine induction of endogenous MMTV transcripts. (A) RNase protection of RNA from BALB/c splenocytes cultured in medium alone (−) or with IL-2, LPS, or ConA. (B) RNase protection of RNA from BALB/c splenocytes cultured as for panel A in the presence of IL-2, IL-1, or LPS. + cont, positive control mammary gland RNA.

ple was also subjected to Northern blot analysis and hybridized with a probe for mouse β-actin (Fig. 1A). We found that IL-1, -2, and -4 all induced transcription of both the Mtv-6 and -9 proviruses in primary splenocytes cultured with these cytokines (Fig. 1 and 2). Similarly, LPS and ConA, a T-cell activator which has been shown to signal through multiple pathways, also induced transcription of Mtv-6 and -9. In contrast, IL-6, also induced by activation of TLR4, had no effect on endogenous MMTV transcription (data not shown).

We next tested whether splenocytes isolated from transgenic mice bearing the MMTV LTR linked to the bacterial chloramphenicol acetyltransferase (CAT) gene showed increased

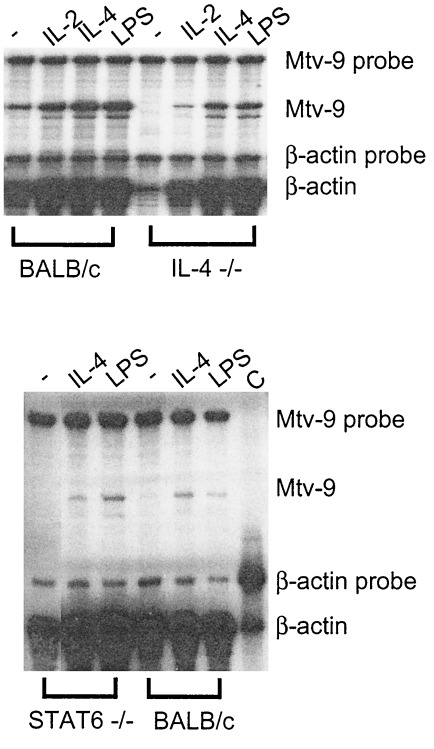

FIG. 2. IL-4 induction of MMTV RNA in BALB/c, IL-4$^{-/-}$ (top), and STAT6$^{-/-}$ (bottom) mice. Although the RNA sample for untreated IL-4$^{-/-}$ splenocytes (−) was slightly degraded, there is clear induction of Mtv-9 by IL-4 treatment (compare IL-2 and IL-4 treatment). C, BALB/c RNA control for the β-actin reaction.

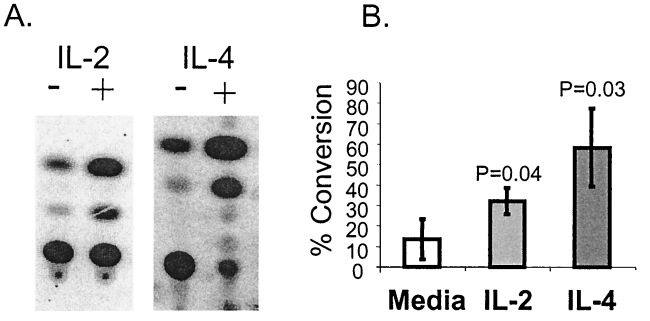

FIG. 3. IL-2 and IL-4 induction of LTR-CAT transgene in splenocytes. (A) Representative CAT assay from splenocytes isolated from one mouse. (B) Three LTR-CAT transgenic mice were sacrificed, and their splenocytes were individually cultured with medium alone, IL-2, or IL-4. The conversion of chloramphenicol to the acetylated form was averaged for each condition. *P* values were calculated using Student's *t* test, compared to controls with medium alone.

enzymatic activity in the presence of either IL-2 or IL-4 (32). Splenocytes from transgenic LTR1-CAT mice were cultured with medium alone or in the presence of IL-2 (300 U/ml) or IL-4 (100 U/ml) (Gibco/BRL) for 48 h. Extracts were prepared, and CAT assays were performed as previously described (32). The CAT activity in LTR1 mice was stimulated on average 3.5-fold by IL-2, while induction by IL-4 was 6.5-fold (Fig. 3). The relative induction by IL-2 and IL-4 was similar to that seen with the endogenous proviruses (Fig. 1 and 2) and showed that induction of transcription maps to the LTR.

Since IL-2 and IL-4 both induced MMTV transcription, we tested whether mice that lacked STAT5a, IL-4, or STAT6 were susceptible to MMTV infection. IL-4 and STAT6 knockout mice as well as the BALB/cJ controls were obtained from The Jackson Laboratory; the STAT5a mice, initially obtained from L. Hennighausen (19), were bred at the University of Pennsylvania. First we examined whether the initial activation of B cells by virus was altered after subcutaneous injection of MMTV(LA). Virus was isolated from mammary tumor tissue of MMTV(LA)-infected mice and footpad injections were performed, as previously described (8). At 20 and 96 h following MMTV(LA) injection into the right footpad, lymphocytes were harvested from the draining lymph node and from the contralateral nondraining lymph node as a control. Lymphocytes were stained with fluorescein isothiocyanate-conjugated anti-B220, anti-Vβ6 [for MMTV(LA) Sag cognate T cells], and anti-Vβ10 (for noncognate T cells) antibodies and phycoerythrin-conjugated anti-CD69 or anti-CD4 antibodies (Pharmingen, San Diego, Calif.). As expected, the ability of MMTV to induce B-cell activation, as measured by expression of CD69, was not affected by the mutations, since this is the result of direct virus–B-cell interaction (Table 1) (2; Rassa et al., submitted). The difference between the STAT5a$^{-/-}$ and other mice in this assay probably reflects the influence of strain background (mixed C57BL/6 and 129 crossed to C3H/HeN for two generations for the STAT5a$^{-/-}$ mice versus BALB/c for the IL-4$^{-/-}$ and STAT6$^{-/-}$ mice).

We next determined whether Sag presentation in the knockout mice occurred by measuring the response of both T and B lymphocytes at 96 h after subcutaneous injection of MMTV. No significant differences were seen in any of animals in the

TABLE 1. B- and T-cell responses to MMTV (LA) in vivo in STAT6$^{-/-}$ and IL-4$^{-/-}$ mice

Mouse strain (*n*)	% Positive cells (avg ± SD)[a]							
	20 h, CD69/B220		96 h					
			CD69/B220		Vβ6/CD4		Vβ10/CD4	
	D	ND	D	ND	D	ND	D	ND
BALB/c (3)	29.3 ± 5.4	9.3 ± 4.2	50.8 ± 3.0	5.4 ± 0.7	36.0 ± 4.4	17.0 ± 0.8	3.6 ± 0.4	7.8 ± 0.4
STAT5a$^{-/-}$ (3)	72.3[b]	11[b]	46.5 ± 5.0	12[b]	47.3 ± 1.8	16.9 ± 0.1	2.6 ± 0.5	5.7[b]
IL-4$^{-/-}$ (2)	53.4	9.9	51.3	13.9	38.2	20.3	4.5	10.1
STAT6$^{-/-}$ (3)	23.6 ± 6.3	7.5 ± 0.5	40.1 ± 7.3	6.0 ± 3.1	31.5 ± 1.4	16.9 ± 0.7	4.4 ± 0.7	7.4 ± 1.2

[a] D, draining lymph node; ND, nondraining (contralateral) lymph node.
[b] $n = 2$.

Sag-mediated Vβ6-bearing T-cell activation or the T-cell-mediated B-cell stimulation (Table 1).

After the initial Sag stimulation of cognate T cells, there is a gradual deletion of these cells in MMTV-infected mice (12). The kinetics of deletion can reflect the level of virus infection, since mice that are not highly infected delete their cognate T cells more slowly than those that are highly infected (10). We therefore examined Sag-mediated deletion of Vβ6-bearing T cells. BALB/c, IL-4$^{-/-}$, and STAT6$^{-/-}$ mice were foster nursed on C3H/HeN MMTV(LA)$^{+}$ mothers and bled at the indicated times (Fig. 4). Peripheral blood lymphocytes were stained with fluorescein isothiocyanate-conjugated anti-Vβ6 and phycoerythrin-conjugated anti-CD4 antibodies (Pharmingen). We found that Sag-mediated deletion occurred with similar kinetics in MMTV(LA)-infected BALB/c, IL-4$^{-/-}$, and STAT6$^{-/-}$ mice (Fig. 4). Although not statistically significant, deletion appeared to occur somewhat earlier in the mutant mice but reached the same ultimate level. We also found that STAT5a$^{-/-}$ mice infected with MMTV(C3H) showed deletion kinetics of the Sag-cognate Vβ14 T-cell population similar to those of C3H/HeN mice (not shown). Thus, the lack of cytokine-mediated stimulation in the null mice did not affect MMTV-mediated activation of B or T cells, nor did it affect virus infection of lymphocytes.

We then used RNase protection analysis of viral RNA in milk to examine infection of mammary tissue in all three strains of mice. RNA was isolated from the milk of MMTV (LA)-infected mice at the second pregnancy, and 10 μg was subjected to an RNase protection assay using probes specific to MMTV(LA) and mouse β-actin. Not surprisingly, STAT5a null mice showed no defects in infection by MMTV (data not shown). It has been reported that STAT5a null mice have defects in their T-cell compartment (16, 26) and show some alterations in their mammary gland biology (19). However, in most cases the STAT5b protein has been shown to compensate for these defects (39). Additionally, although the β-actin levels were variable in milk RNA, we found that both the IL-4 and STAT6 knockout mice had wild-type infection of their mammary tissue (Fig. 5). Thus, although the cytokines produced by the Sag-activated T cells stimulate MMTV transcription, loss of any single cytokine response was not sufficient to decrease mammary gland infection.

STAT6$^{-/-}$ mice lack a major signaling pathway for the type 2 cytokines IL-4 and IL-13 and show defects in the expression of a number of genes that are regulated by these cytokines, such as major histocompatibility complex, Thy-1, and CD23 (38). However other pathways for these cytokines exist, such as phosphorylation of the insulin receptor substrate followed by activation of phosphatidylinositol 3-kinase (14). To determine whether other pathways operated in the IL-4-mediated induction of MMTV transcription in the STAT6$^{-/-}$ mice, we performed RNase protection assays using probes specific for the Mtv-6 and -9 loci on RNA isolated from splenocytes cultured in medium alone or in the presence of IL-2 (50 U/ml), IL-4 (100 U/ml; Gibco/BRL), and LPS (5 μg/ml; Sigma) for 20 h. As a control, these experiments were also performed with the IL-4$^{-/-}$ mice that retain IL-4 receptors and are thus fully responsive to this cytokine. The STAT6$^{-/-}$ mice showed nor-

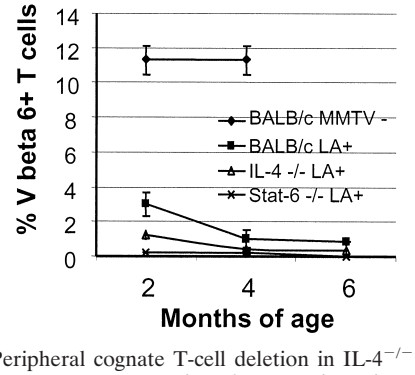

FIG. 4. Peripheral cognate T-cell deletion in IL-4$^{-/-}$ and STAT-6$^{-/-}$ mice. Data are representative of two to nine mice per group.

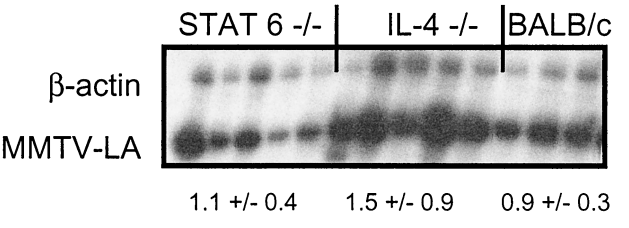

FIG. 5. MMTV infection of the mammary gland in STAT-6$^{-/-}$ and IL-4$^{-/-}$ mice. RNase protection was performed on RNA isolated from the milk of five STAT6$^{-/-}$, five IL-4$^{-/-}$, and three BALB/cJ mice foster nursed on MMTV(LA)-infected C3H/HeN mothers. Densitometry analysis was performed on the autoradiographs using Gel Doc 1000 with a conversion screen (Bio-Rad Laboratories, Hercules, Calif.), and the MMTV signal was normalized to the β-actin signal. Shown below each group are average normalized signals with standard deviations.

mal induction of Mtv-9 (Fig. 2) and Mtv-6 (data not shown) transcription. Thus, IL-4 must initiate signaling through other pathways in the null mice that result in MMTV transcription. Other functions of IL-4 have been shown to be independent of STAT6 in null mice, including the death of resting T cells, the ability to control Friend virus infection, and the induction of IgE production and murine AIDS by the LP-BM5 murine leukemia virus (7, 24, 41).

The STAT6$^{-/-}$ and IL-4$^{-/-}$ mice were generated in the BALB/c background, a strain that does not normally generate good Th1 cellular immune responses to pathogens such as *Leishmania* (34). In adult mice, infection by MMTV has been shown to predominantly generate a Th1 immune response, characterized by the production of IgG2a (21), although whether this occurs neonatally during natural milk-borne infection is not known. When the STAT6 mutation is introduced into the BALB/c background, in the absence of a Th2 response the default becomes a stronger Th1 response, with increased levels of IgG2a and other type 1 antibodies (17, 38). Thus, both the IL-4 and STAT6 null mice, both of which also have increased cellular immunity due to this profound shift to the Th1 type of T cell, might be expected to have decreased infection of mammary tissue if a stronger Th1 response eliminated virus-infected cells more effectively. If, however, there was a strong antiviral response that required Th2 cells, these mice should have shown higher levels of infection. Since there was no apparent change in the virus load in the null mice, the change in the balance of Th1 and Th2 cells apparently did not affect antiviral immune responses.

Interestingly, the one difference between the wild-type BALB/c and STAT6 knockout mice was that the mutant mice had an increased latency of mammary tumorigenesis. BALB/c, STAT6$^{-/-}$, and IL-4$^{-/-}$ mice were foster nursed on C3H/HeN MMTV(LA)$^{+}$ mothers and monitored for tumor development. Both the mean and median times to tumor development were significantly longer for MMTV-infected STAT6$^{-/-}$ mice (mean of 287.5 days; median of 271 days) than for BALB/c mice (mean of 232.3 days; median of 215 days) (Fig. 6). IL-4$^{-/-}$ mice also showed a delay in tumor development (mean of 263.1 days; median of 255 days), although the difference was not statistically significant compared to BALB/c mice.

It has been observed previously that BALB/c mice, which have poor Th1 responses, are more susceptible to MMTV(C3H)-induced mammary tumors than are C3H/HeN mice, which have normal Th1 responses (average mean latency of tumor incidence of about 7 months versus 9 months for the two strains) (8, 27). The increased susceptibility in BALB/c mice is not the result of increased lymphocyte infection; it was shown previously that the lymphoid tissues of BALB/c mice have lower virus levels than C3H/HeN mice (8). Moreover, there seems to be no correlation between tumor latency and the infection levels in mammary tissue, as shown here, since there was no difference in the virus load in STAT6$^{-/-}$ and BALB/c mice (Fig. 5). One possible explanation for the increased latency in STAT6$^{-/-}$ mice is increased immune recognition of tumor cells in these mice. Recent work from other labs has shown that antitumor immunity is increased in STAT6 null mice, resulting in the decreased growth of transplanted tumor cells (15, 30). The natural lack of a good Th1 antitumor response in the wild-type BALB/c background may therefore contribute to the increased susceptibility of these inbred mice to MMTV-induced mammary tumors compared to strains like C3H/HeN.

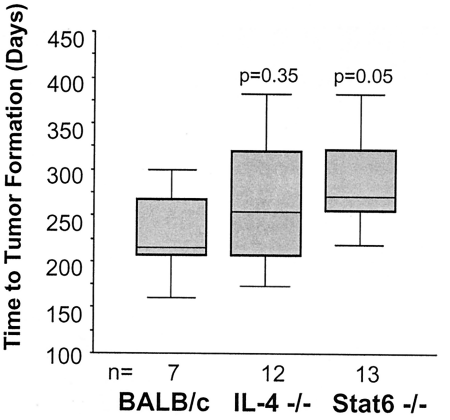

FIG. 6. Mammary gland tumor incidence in BALB/c, IL-4$^{-/-}$, and STAT6$^{-/-}$ mice. Box plots of the time to tumor development are shown. Middle bars show medians, the top and bottom edges of the boxes show 75th and 25th percentiles, and the top and bottom bars show minimum and maximum values. A nonparametric Mann-Whitney test was used to generate the statistics using SPSS (SPSS, Inc., Chicago, Ill.). The *P* values are two-sided compared to BALB/c.

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