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BEHAVIORAL AND IMMUNE CHANGES IN v-Ha-*ras* TRANSGENIC MICE

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ABSTRACT

Transgenic mice (Oncomice) with an activated v-Ha-*ras* oncogene under the control of the mouse mammary tumor virus promoter develop mammary tumors. We wondered if the expression of the v-Ha-*ras* oncogene product would induce changes in mice behavioral activity, that could be associated with alterations in their immune system. Behavior was evaluated in an open field study considering line crossings and rears. Oncomice consistently showed less activity than FVB mice. Lieber-DeCarli diet decreased both types of activity in both strains. Cocaine treatment increased line crossings in both strains. Oncomice spleen and thymus cell supernatants contained higher levels of IL-2. Oncomice serum had higher levels of IL-1α. Our results suggest a direct association between higher levels of IL-1α and lower open field activity. Therefore, we can infer that the increased level of IL-1α found in Oncomice, could have a key role in oncogene induced immune and behavioral changes, and could be a requirement to facilitate its transforming activity.

INTRODUCTION

The presence of the v-Ha-*ras* oncogene and its product, the Ras protein, have been associated with several malignancies (1). In fact, it has been shown that Ras activation is an early event in tumor progression (2). The oncogene product is considered to be necessary but not sufficient to induce malignant transformation, since time to deregulate the cell and somatic events are still required (3,4). A transgenic mouse with an activated v-Ha-*ras* oncogene under the control of the mouse mammary tumor virus promoter has been described (1).

The v-Ha-*ras* oncogene mRNA was found in mammary, salivary and Harderian glands as well as in the spleen and thymus of female v-Ha-*ras* transgenic mice (1). Because the oncogene product was found expressed on lymphoid organs we considered the hypothesis that its presence could alter the immune response in transfected mice. Firstly, we demonstrated that splenocytes from female Oncomice produced less TNF-α than splenocytes from FVB mice (5). Secondly, we showed that Oncomice had smaller thymi with lower number of cells than FVB mice (6). Recently, we observed that IFN-γ secretion and sIL-2R release by Oncomice thymocytes after *in vivo* treatment with Lieber-DeCarli diet and *in vitro* culture, followed a nearly opposite pattern to the one shown by FVB mice thymocytes (Colombo, Lopez, Chen and Watson, unpublished data).

Recent data indicate that not only the neuroendocrine axis can alter the immune system, but that the opposite is also true. The best known example is interleukin 1 (IL-1), a cytokine secreted by macrophages, that increases in serum as body temperature increases and has been associated with depression (7). Taken into account that cancer patients suffer mood changes and depression, we wondered if the presence of the oncogene product could modulate nervous system activity and if such alteration could be associated with changes in the immune system. The objective of this paper was to study in mice, if the

expression of the v-Ha-*ras* oncogene product would induce changes in their behavioral activity, that could be directly related to alterations in their immune system.

MATERIALS AND METHODS

Animals and diets

Female and male Oncomice, 4 weeks old, were obtained from Du-Pont Company (Wilmington, DE), and female and male FVB mice were obtained from Taconic (Germantown, NY). Oncomice are v-Ha-*ras* transgenic mice in a FVB background. They were kept in the Animal Facility at the Arizona Health Sciences Center for 1 week and then according to the different protocols males and females were fed conventional chow diets, for behavioral and immunological studies. Meanwhile, female mice were fed the Lieber-DeCarli (LD) liquid diet alone -as control- or supplemented with 5% ethanol (v/v) for 14 weeks as described previously (6,8). Mice on liquid diets, for behavioral studies, were evaluated on week 3 of treatment. Mice were on a 12:12 hour light dark cycle. Four mice were caged together in all studies. Animals were cared for in accordance with the University of Arizona College of Medicine Committee on Animal Research.

Cocaine treatment

Cocaine was administered as cocaine hydrochloride in 0.9 % saline solution by intraperitoneal injection at a dose of 30 mg/kg body weight 5 minutes before

behavioral studies were performed. Mice were on the third week of cocaine treatment when the study was performed. All behavioral studies were performed between 2PM and 5PM. On the first week mice received daily injections of 10 mg/kg/day, and on the second week mice received 20 mg/kg/day. These mice received LD diet.

Activity determination

Open field activity was determined using a 42.5 x 35 x 15 cm plastic box, that was divided with a 1.7 cm wide tape, into 4 rectangles of 21.0 x 16.8 cm. An individual mouse at each time was placed in the center of the box, the number of times it crossed a line and the number of times it stood erect on its hind legs (rears) in the next 5 minutes were recorded. In general, mice ran near the side walls of the box. When they crossed in diagonal two or more lines, near the center, only one was recorded, in order to avoid an artificially increased measurement of activity. The number of fecal boli deposited was also recorded. All procedures were done under biosafety hood.

Spleen and thymus cell culture

Mice were always sacrified in the morning using ether anesthesia. Spleen and thymus cell suspensions were prepared by teasing the tissues with forceps in RPMI 1640 supplemented with 10% fetal calf serum and antibiotics (penicillin and streptomycin). Spleen red cells were lysed using ammonium chloride buffered solution. Splenocytes and thymocytes ($1x10^6$ cells/well) were cultured in the presence of 5 μg/ml of Concanavalin A (Con A) for 24 hr at 37°C, 5 %

CO_2 to measure IL-2 and for 72 hr in the presence of 2 μg/ml of Con A to measure sIL-2R and IFN-γ. After incubation the plates were spun down at 1100 rpm for 10 minutes, supernatants were collected and stored at -70°C until cytokines were measured.

ELISA for IL-1α

Ninetysix well ELISA plates (Dynatech Immunolon 2) were coated with 50 μl of anti-mouse IL-1α monoclonal antibody (kind gift from Dr. Chaplin, Washington University) in a concentration 0.1 μg/well in carbonate buffer pH 9.6, at 4°C overnight. Plates were washed with (PBS/Tween) once, and 50 μl of recombinant murine IL-1α standards (Genzyme, Boston, MA) prepared in RPMI 1640 containing 10% FCS or 50 μl of sample were added. Plates were incubated at 37°C for 1.5 h, and washed with PBS/Tween once. Fifty μl of rabbit anti-mouse IL-1α polyclonal antibody (Genzyme; diluted 1:500) were added, and plates were incubated for a further 1.5 h at 37°C. Plates were washed once, and 50μl of goat anti-rabbit IgG HRP conjugate (American Qualex, La Mirada, CA) diluted 1:5000 in PBS/Tween were added. Plates were incubated at 37°C for another hour. Then, plates were washed three times with PBS/Tween and once with PBS, and 100 μl of ABTS were added to each well. After 30 minutes the colored product was measured on a Titertek Multiskan Plus at 405nm. The standard range was 7.8 pg/ml-1ng/ml, detection limit was 0.78 pg/well.

ELISA for IFN-γ, IL-2 and sIL-2R

IFN-γ and IL-2 were measured as previously described using a sandwich ELISA (4,5). Briefly, the capture antibodies were rat anti-mouse IFN-γ (Lee

Molecular Lab, San Diego, CA) or rat anti-mouse IL-2 (Genzyme). The standards were recombinant IFN-γ (Genzyme) or recombinant IL-2 (Collaborative Research Incorporated, Bedford, MA). The revealing antibodies were rabbit anti-mouse IFN-γ (kind gift of Dr. Philip Scuderi) or rabbit anti-mouse IL-2 (Collaborative Research Incorporated). sIL-2R was measured as previously described in detail (9).

Statistical analysis

Student's *t* test and Chi^2 test were used.

RESULTS

Basic behavioral activity in Oncomice and FVB mice

The evaluation of basic behavioral differences in both strains was performed in mice of both sexes receiving conventional chow diets. Experiments were repeated several times and a typical experiment is presented in table 1. Two types of activities were evaluated in order to assess the basic level of activity (line crossings and rearing). Open field activity was determined considering the number of lines crossed in 5 minutes. Mice were divided into two groups, according to the number of lines crossed in 5 minutes: more than 60 or more than 75 lines. The results clearly showed that Oncomice were much less active than FVB mice. The level of anxiety was evaluated considering the number of rears made in 5 minutes, once again Oncomice proved to be much less active

TABLE 1

Changes in Behavioral Parameters Due to the Presence of the v-Ha-*ras* Oncogene

Activity	FVB	ONCO	[a]p<
Mice that crossed more than 60 lines in 5'	10/10	4/10	0.003
Mice that crossed more than 75 lines in 5'	5/10	0/10	0.009
Mice that made more than 66 rears in 5'	8/10	0/10	0.0002
Mice that deposited defecation pellets in 5'	10/10	3/10	0.001

[a]Chi-square test

(table 1). We also took into account the number of mice that deposited defecation pellets during the study since this is a normal response to stress after facing a new environment. Once again Oncomice responded less than FVB mice.

Effect of alcohol and cocaine on behavior

. The effect of alcohol and cocaine on behavior was evaluated only in female mice, since our model of long term alcohol and cocaine administration always employed female mice mainly to avoid the casualties due to fighting among males (10). Four female mice were always caged together. In this study we considered how many lines were crossed in 5 minutes and how many rears were made in the same time. As mentioned for chow fed mice, the basic activity of

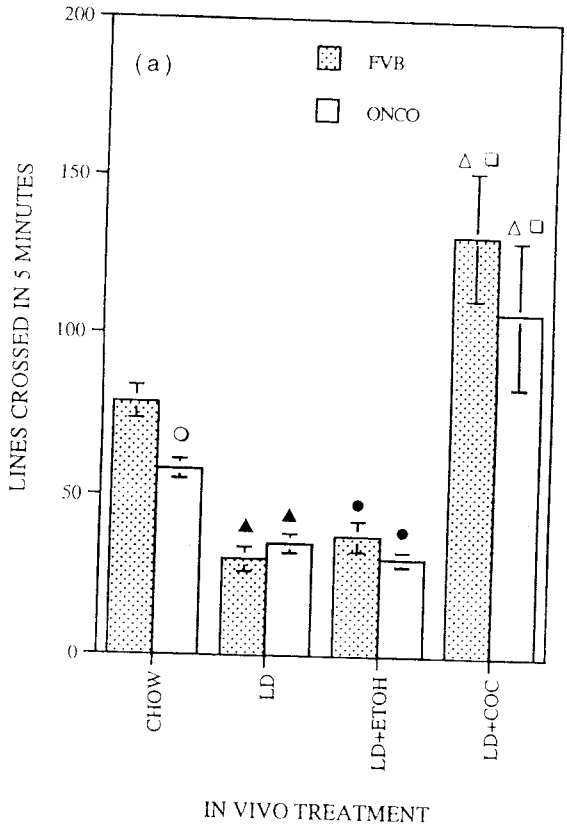

FIG. 1. Changes in behavioural parameters due to Lieber-DeCarli diet, ethanol and cocaine. Mice activity was measured as lines crossed in 5 minutes (a) and rears in 5 minutes (b). Data are presented as mean ± SEM. Statistical differences: $^{O}p< 0.005$ Oncomice vs. FVB; $^{\bullet}p< 0.001$ ethanol vs. chow fed control; $^{\Delta}p< 0.05$ cocaine vs. chow fed control; $^{\blacktriangle}p< 0.005$ Lieber-DeCarli vs. chow fed control; $^{\square}p< 0.01$ cocaine vs. Lieber-DeCarli.

Oncomice was lower than that of FVB mice measured as lines crossed and as rears in 5 minutes (Fig. 1). LD liquid diet administered for 3 weeks decreased both types of activities in both strains of mice abolishing strain differences. Ethanol intake did not modify mice behavior when compared with liquid diet

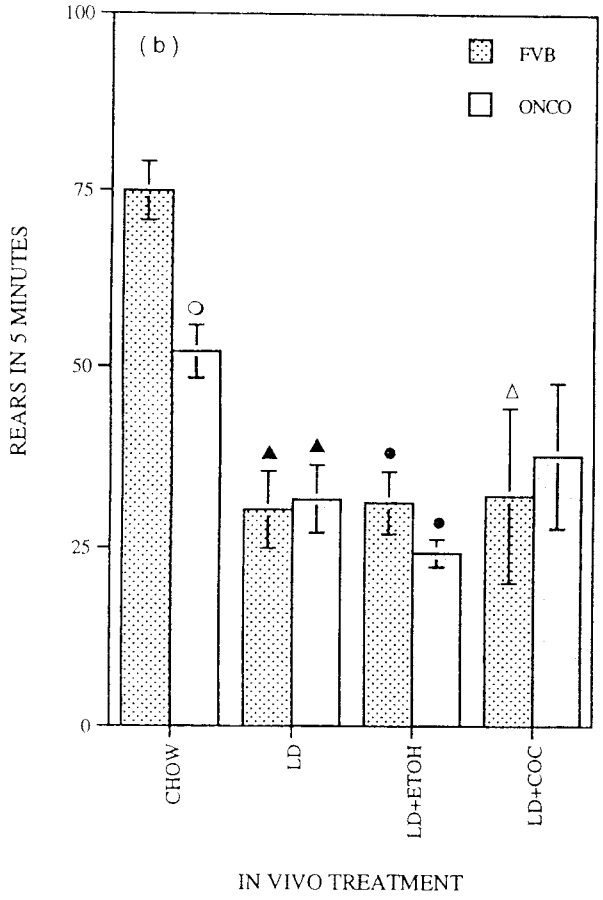

FIG. 1. Continued

controls. Cocaine increased line crossings in Oncomice and FVB mice receiving LD diet (Fig. 1a). This increase was significant when the open field activity was compared with control mice receiving either the LD diet or conventional chow diets. When compared with chow fed mice, cocaine decreased in a significant fashion, the number of rears, only in FVB mice, although no strain difference could be detected (Fig. 1b).

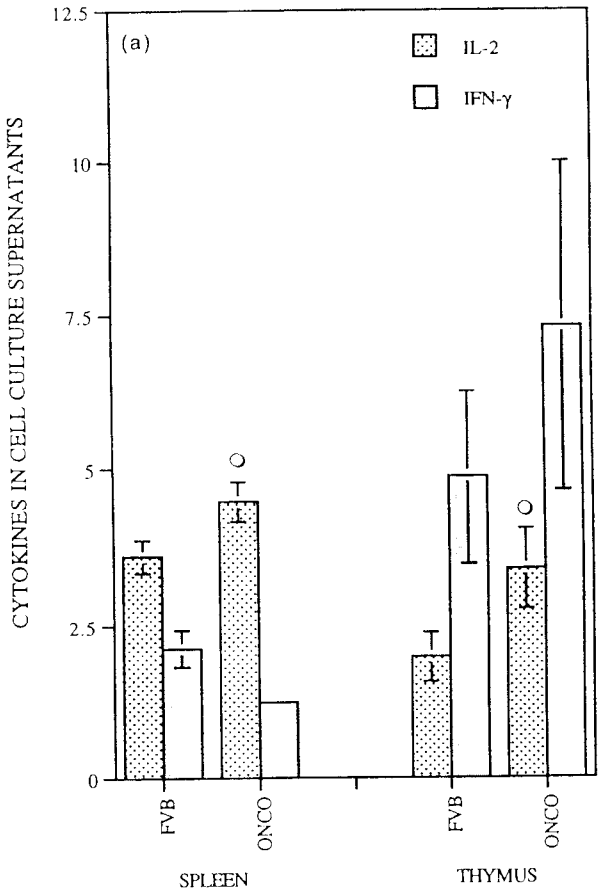

FIG. 2. Changes in immune parameters in v-Ha-*ras* transgenic mice. IL-2 and IFN-γ were measured in spleen and thymus cell culture supernatants (a). IL-1α was measured in serum (b). sIL-2R was measured in serum and in spleen and thymus cell culture supernatants (c). Data are presented as mean ± SEM. Statistical differences: $^{o}p< 0.05$ FVB vs. Oncomice.

Immune parameters and strain difference

The assessment of immune parameters was done using chow fed mice of both sexes. Spleen and thymus cell culture supernatants were used to determine the levels of IL-2, and IFN-γ (Fig. 2a). Only IL-2 secretion was higher in Oncomice

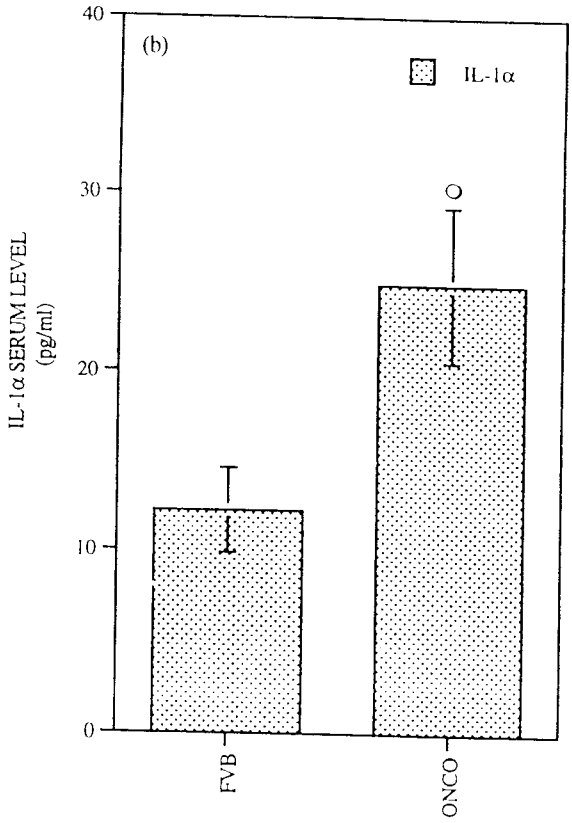

FIG. 2. Continued

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than in FVB mice. Mice serum was used to evaluate the levels of IL-1α (Fig 2b). Oncomice presented higher levels of serum IL-1α when compared with FVB mice. The levels of sIL-2R in spleen and thymus cell culture supernatants and in serum did not differ between strains (Fig. 2c).

DISCUSSION

Oncomice are v-Ha-*ras* transgenic mice expressing the oncogene under the

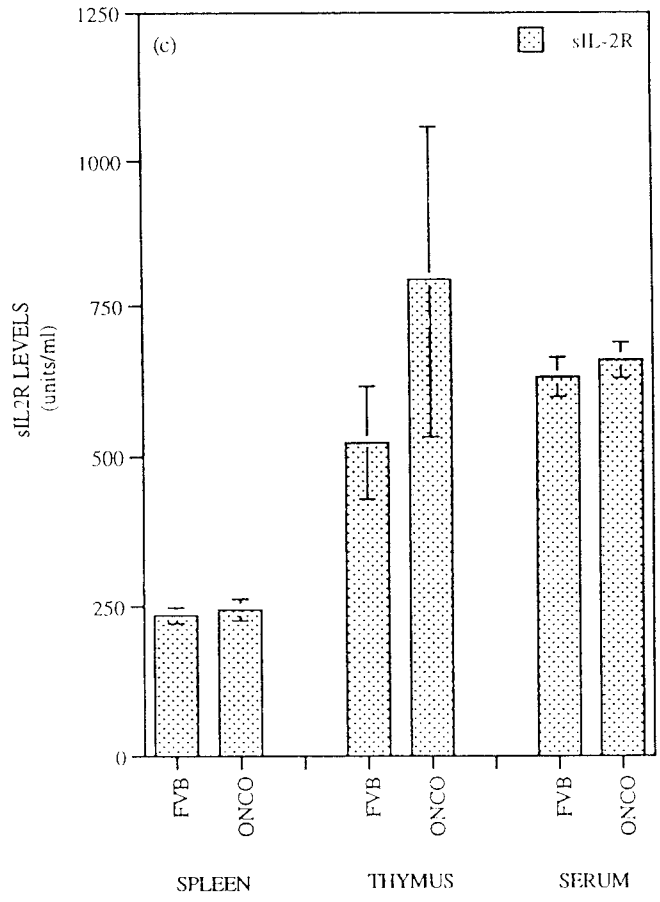

FIG. 2. Continued

control of the mouse mammary tumor virus promoter (1). These mice develop mammary gland malignancies and Harderian and parotid gland hyperplasia. Interestingly, the v-Ha-*ras* oncogene mRNA is also expressed in lymphoid tissues as thymus and spleen (1), letting us speculate on a possible abnormal regulation of the immune system.

We took advantage of the availability of v-Ha-*ras* transgenic mice (Oncomice) to test the hypothesis that the presence of the oncogene product

could modulate the immune system, by modifying the secretion of certain cytokines. These cytokines, not only would downregulate the ability of the immune system to recognize tumor cells; but could also act at the level of the central nervous system altering behavior. In this study, we evaluated behavior using an open field study concluding that conventionally chow fed Oncomice were less active than their non transgenic FVB counterparts. Surprisingly, the administration of the Lieber-DeCarli liquid diet had a dramatic effect on both types of activity in both strains of mice. LD diet was designed to study the pathological changes induced in liver by high doses of ethanol (8). LD diet provides 35% of the total energy through plant fat containing a high percentage of monounsaturated fatty acid. It also contains several times more vitamins A, D and E and pyridoxine than recommended for mouse growth by the National Research Council (11). Although it is generally accepted that high levels of vitamins have immunostimulatory effects, this may not be true, if in association with monounsaturated fatty acids. It has been shown that long term calorie restriction can induce increased open field behavior in mice and in monkeys (12). Moreover, undernourished rats receiving oil enriched diets showed decreased locomotor activity and rearing when compared with undernourished rats on standard diets (13). Therefore, we can conclude that LD diet by itself modified mice behavior independently of the oncogene presence. Moreover, a higher tumor incidence was reported in v-Ha-*ras* transgenic mice fed diets that provided increasing number of calories from corn oil (14). Nevertheless, it is not possible yet to decide whether the high levels of fat favored tumorigenesis through an immune mediated mechanism, changes in sexual hormone levels, or by direct tumor promotion. Supporting the last option comes the work done by Fernandes et al showing that lipid and calorie restriction have protective effect on Oncomice (15). Food restriction increased survival, reduced tumor incidence, favored the expression of tumor suppressor genes and reduced the expression

levels of *ras* gene products. Taken together these observations could imply that diets with high fat content would favor more sedentary lifestyles, and accelerate the onset of malignancies in genetically prone individuals. Addition of ethanol did not further modulate activity in FVB or Oncomice. Nevertheless, other authors observed increased open field activity in rats after chronic ethanol ingestion (16). Cocaine injection to LD fed mice, increased line crossing over LD controls and chow fed controls. Rearing was not further modified by cocaine administration.

It is well known among clinical oncologists that cancer patients suffer depression, and it has been shown that cancer patients have a higher frequency of depressive mood than the general population (17-20). This is not an abrupt change in mood after realizing of the severity of their disease, but it is a change that can even appear a long time before the diagnosis. Some patients, even have changes in their senses of smell and taste, associated with changes in food preferences, these changes could be related to the central nervous system activity. These phenomena were generally attributed to substances released by tumor cells acting on the nervous system.

It has been shown that immunosuppressive drugs as cyclosporine A also induce behavioral changes including decreased ambulatory and rearing activity in the open field study (21). Furthermore, the activation of the immune system by pathogens induces the release of proinflammatory cytokines, including IL-1β and TNF-α. Other studies have also shown that IL-1β can alter brain function, resulting in a variety of responses resembling sickness that include: increased sleep, decreased food intake, and fever (22,23). These effects have been partially blocked by the intravenous administration of antibodies anti IL-1, soluble IL-1 receptor and large amounts of IL-1 receptor antagonist (24-26). Peripheral and central administration of recombinant rat IL-1β have been shown to decrease social exploration in rats (27). This is in agreement with the finding that

melancholics exhibited significantly more IL-1β accumulation in phytohemmagglutinin stimulated cell culture supernatant than healthy controls (28). Both IL-1α and IL-1β share similar immunological and endocrinological effects. Although much is known on IL-1β, we focused our interest on IL-1α. It has been shown that radiolabeled IL-1α can bind to its specific receptor, is endocytosed and translocated into the nucleus (29), suggesting that serum IL-1α could also act at distant effector sites. Our data indicated that Oncomice presented higher levels of IL-1α in serum. Therefore, we could partially explain the decreased activity in Oncomice, when compared with FVB mice, as a consequence of higher levels of serum IL-1α.

IL-1 administered *in vivo* stimulated IL-2 production in normal mice (30). Therefore, we could also propose that the higher secretion of IL-2 by Oncomice splenocytes and thymocytes had been conditioned *in vivo* by the higher levels of IL-1α. An *in vivo* IL-1α dependent increase in IL-2 secretion might imply stimulation of cytotoxic T cells and NK cells. Nonetheless, it has been shown that administration of recombinant murine IL-1α altered lymphocyte migration into inflammatory sites, either by inhibiting their homing or by accelerating their traffic through inflammed tissues (31). Therefore, we could conclude that the increased level of IL-1α found in Oncomice, could have a key role in oncogene induced immune and behavioral changes, and could be a requirement to facilitate its transforming effect on target tissues.

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FOOTNOTES

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