Interleukin-9 stimulates the production of interleukin-5 in CD4+ T cells

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ABSTRACT. We recently showed that interleukin-9 (IL-9), a Th2 cytokine, promotes IL-5-mediated rejection of allografts in mice. This observation led us to investigate the functional link between IL-9 and IL-5 production during alloreactive T cell responses *in vitro* and *in vivo*. Firstly, we found that IL-9 was produced by alloreactive Th2 cells, and IL-9 mRNA was detected in skin allograft during Th2-type rejection. We then established that IL-5 production was impaired in alloreactive Th2 cells isolated from IL-9-deficient mice and that optimal IL-5 production after allogeneic stimulation requires a functional IL-9 receptor (IL-9R) on the responding cells. Finally, the production of IL-5 by anti-CD3-stimulated CD4+ T cells was abolished by neutralization of IL-9. Despite the fact that IL-9 promotes IL-5 production by alloreactive T cells, IL-9-deficient recipients of skin allografts still developed eosinophilic graft infiltrates and neither IL-9 nor IL-9R deficiency modified Th2-type allograft rejection.

Keywords: rodent, transgenic/knockout, transplantation, lymphokine, Th2 cells

Generating a Th2 alloresponse has been described as beneficial to the survival of the allograft. There is, however, a growing body of evidence showing that stimulating a Th2 alloresponse promotes alternate pathways of rejection. The main features of Th2-type rejection are its dependency on the secretion of Th2-type interleukins (IL)-4 and IL-5, the recruitment of eosinophils at the site of rejection, and its inhibition by alloreactive CD8+ T cells [1-6]. IL-9 is a multifunctional cytokine produced by activated Th2 clones *in vitro* and during Th2-like T cell responses *in vivo* [7, 8]. Initially described as a T cell growth factor [9], IL-9 has other biological targets such as B lymphocytes [10], epithelial cells [11], mast cells [12, 13] and hematopoietic progenitors [14]. In numerous studies, it has been associated with tissue eosinophilia [11, 15-18]. However, the mechanisms underlying the effect of IL-9 on eosinophils remain unclear. IL-9 could enhance directly the resistance of eosinophils to apoptosis [19], as well as acting indirectly on eosinophil recruitment by inducing the expression of chemotactic factors, such as CCL-11 [11]. IL-9 might also act synergistically with IL-5. *In vitro*, the combination of these two cytokines has been shown to induce eosinophil differentiation from human cord blood progenitors [19]. Moreover, IL-9 could potentiate eosinophil responses to IL-5 by stimulating the expression of IL-5R at the cell surface [19]. Recently, we have shown, in a class II, MHC-mismatched murine model, that heart allografts from IL-9 transgenic donors were acutely rejected, whereas grafts from wild-type donors were not [20]. Acute rejection of IL-9 transgenic hearts was associated with massive eosinophil infiltration that could be prevented by neutralization of either IL-4 or IL-5. The observation that neutralization of IL-5, a factor required for the development, activation and tissue recruitment of eosinophils [21], inhibits rejection induced by local IL-9 over-expression, supports the idea that IL-9 could regulate directly the production of IL-5.

METHODS

Mice and grafting

C57BL/6 (B6) (H-2^b) and BALB/c (H-2^d) mice were purchased from Harlan Netherland (Horst, The Netherlands). C57BL/6.CH2bm12 (bm12) (H-2^bm12) mice were obtained from the Jackson Laboratory (Bar Harbor, USA). bm12.CD95^lpr/lpr Fas-deficient mice [22], B6.IL-9^-/- [23] and B6.IL-9R^-/- (Steenwinckel *et al*., manuscript in preparation) mice have been described previously. FVB (H-2^q) mice expressing transgenic mouse IL-9, and wild type, control littermates have also been described [24]. All animals were bred in conventional animal housing facilities. Grafting tail skin onto the left flank of recipients was performed as previously reported [3]. All *in vivo* experiments were performed in compliance with the relevant laws and institutional guidelines.

Mixed lymphocyte reaction

Lymph node or spleen cells were stimulated with irradiated allogeneic or syngeneic splenic stimulators as

previously described [3]. After three days (for primed responding cells) or five days (for unprimed responding cells), culture supernatants were harvested and levels of IL-2, IL-4, IL-5, IL-9, IL-13 and IFN-γ were assessed by ELISA (R&D Systems, Abingdon, UK and ref. 25].

Purification of CD4⁺ T cells and anti-CD3 stimulation

Spleens were removed and prepared as a single cell suspension. CD4⁺ T cells were isolated using magnetic separation. Briefly, cells were incubated with FITC-conjugated rat anti-mouse CD4 monoclonal antibodies (GK1.5; BD Pharmingen, Erembodegem, Belgium]. Cells were then washed and incubated with anti-FITC microbeads (Miltenyi Biotech, Bergisch-Gladbach, Germany). Magnetic separation was performed on MACS^TM separation columns (Miltenyi) according to the suggested protocol (Miltenyi). The retained cells were eluted from the columns as CD4⁺ T cells. Purity of the cell preparation was more than 98% CD4⁺ T cells. Stimulation by immobilized anti-CD3 monoclonal antibodies was carried out as follows. Hamster anti-mouse CD3 antibodies (clone 145-2C11; BD Pharmingen) were incubated overnight at 4 °C in 100 μL of PBS per well, on flat-bottomed, 96-well plates. After three washes with PBS, plates were seeded with cells (1 x 10⁶ per well) and incubated for 72 hours with mouse anti-mouse IL-9 monoclonal antibodies (clone MM9C1 [25, 26]) or control isotype (IgG2a) monoclonal antibodies to trinitrophenol [20]. Culture supernatants were harvested and levels of IL-5, IL-13 and IFN-γ were assessed by ELISA kits (R&D Systems) using the manufacturer's protocol. In some of the experiments, recombinant IL-9 (rIL-9) [23] (100 ng/mL final concentration) were added to the cultures of immobilized anti-CD3-stimulated CD4⁺ T cells.

Quantification of IL-9 transcripts

Organs were frozen in liquid nitrogen after collection and total RNA was extracted by the Tripure® procedure (Roche Diagnostics, Brussels, Belgium). Samples were then treated with DNase (Promega Benelux, Leiden, The Netherlands) to remove any contaminating genomic DNA. After reverse transcription, quantitative polymerase chain reaction (PCR) was performed as previously described [27]. Briefly, amplification of cDNA was carried out with forward and reverse specific primers (Life Technologies). Fluorogenic probes were used for the detection of amplified products (Applied Biosystems, Forstercity, CA, USA). Amplification was carried out on a LightCycler® (Roche Diagnostics). A total of 45 cycles was performed. mRNA levels were expressed in absolute copy number, normalized against fixed numbers of copies of β-actin mRNA. Copy numbers were calculated for each sample from standard curves constructed from serial dilutions of purified plasmids for IL-9 [27] and β-actin (ATCC clone number 1150349) (ATCC, Manassas, VA, USA). The sequence of primers and probes for the real-time PCR reactions included: β-actin sense 5'-CTAAGGCCAACCGT GAAAAG-3', β-actin anti-sense 5'-AGCCTGGATGG CTACGTACAT-3', β-actin probe 5'-(6-Fam)TGACCC AGATCATGTTTGAGACCTTCA(Tamra)(phosphate)-3'; IL-9 sense 5'-GGCATCAGAGACACCAATTACCT-3',

IL-9 anti-sense 5'-TGGCATTGGTCAGCTGTAACA-3', IL-9 probe 5'-(6-Fam)CTCTCCGTCCCAACTGATGA TGATTGTACCAC(Tamra)(phosphate)-3'.

Histology

Organs were fixed by immersion for 24 h in 10% neutral formalin fixative before being embedding in paraffin. Two-micron sections were prepared and stained with hematoxylin-eosin [3]. Tissue sections were observed using an Axioscope light microscope from Carl Zeiss (Zaventem, Belgium). Eosinophilic infiltration was quantified by counting the number of cells present in five, distinct, high power fields (5 x 0.0025 mm²) across graft sections.

Statistical analysis

Results were compared by the Mann-Whitney non-parametric test. All comparisons were made two-tailed.

RESULTS

### IL-9 is produced by alloreactive T cells during graft rejection

In the present study, we examined the specific secretion of IL-9 by animals rejecting MHC class II molecule-disparate bm12 skin grafts. Lymph node cells isolated from B6 recipients were stimulated *in vitro* by irradiated, bm12 spleen cells *(figure 1A)*. In addition to IFN-γ and the prototypic Th2 cytokines IL-4 and IL-5, B6 lymph node cells stimulated by bm12 cells also produced IL-9. Conversely, this production was not observed after stimulation by irradiated syngeneic cells. The results presented in *figure 1B* also show that mRNAs for IL-9 were present in rejected bm12 skin, whereas they were not detected in syngeneic grafts or in normal skin. Thus, IL-9 is produced by bm12-specific, alloreactive T cells and IL-9 mRNA transcripts are found in rejected bm12 skin allografts.

Lack of IL-9 production reduces IL-5, but not IFN-γ, secretion in alloreactive T cells

A recent study published by Arendse *et al.* [28] has demonstrated that the neutralization of IL-9 has multiple effects on the Th2 type *in vivo* response. The reduction of susceptibility to *L. major* paralleled the dramatic reduction of Th2 type cytokine production, including IL-4 and IL-13. Therefore, experiments were set up to determine whether the lack of IL-9 could also modulate the production of IL-5 in alloreactive CD4⁺ T cells. As shown in *table 1*, after stimulation with donor alloantigens, lymphocytes from B6.IL-9⁻/⁻ recipients that rejected allogeneic bm12 skin, secreted 5-6 times less IL-5 than cells from control B6 recipients. Both types of cells, however, produced comparable amounts of IFN-γ after allogeneic stimulation.

Lack of IL-9R expression on alloreactive T cells results in impaired production of IL-5

In order to investigate further the effect of IL-9 on IL-5 secretion, naive spleen cells from IL-9 receptor-deficient B6 recipients (IL-9R⁻/⁻) were compared *in vitro* with nor-
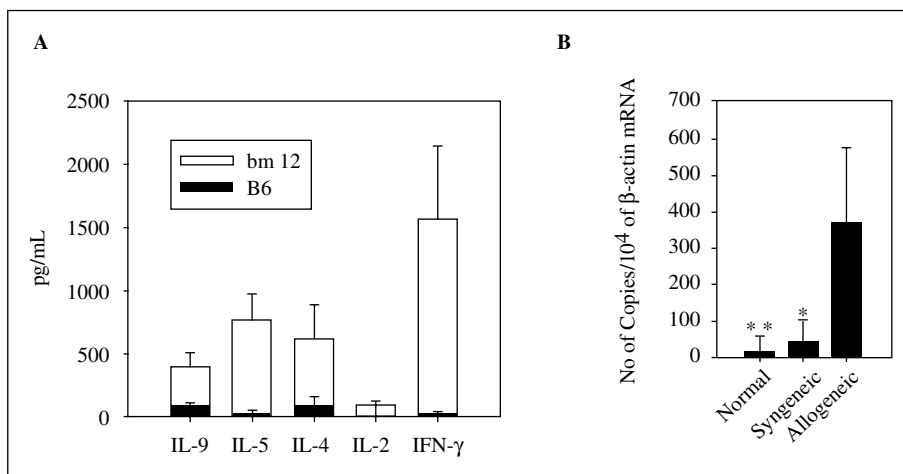
Figure 1
IL-9 is expressed during allograft rejection mediated by TH2 CD4^{+} T cells. **A**) The production of IL-9, IL-5, IL-4, IL-2 and IFN-γ was analyzed in the supernatants of cultures containing lymph node cells from B6 mice immunized by bm12 skin grafting, after stimulation with irradiated bm12 or B6 spleen cells. The data presented in the figure are representative of two independent experiments involving 3-5 mice per group. **B**) Real time detection and amplification of IL-9 mRNA in allogeneic, syngeneic and normal bm12 skin. Number of copies (± SD) of IL-9 mRNA is given per 1×10^4 copies of housekeeping gene (β-actin) mRNA present in the tested samples. The experiment involved groups of 4 individual mice. ** $p < 0.01$; * $p < 0.05$ compared with allogeneic skin grafts.

Table 1
The production of IL-5 is impaired in alloreactive IL-9-deficient CD4^{+} T cells

Responder cells[a]	Stimulator cells	IL-5 (pg/mL)	IFN-γ (pg/mL)
Wild-type	B6	N.D.[b]	N.D.[b]
	bm12	275 ± 21[c,d]	548 ± 78[c]
IL-9^{-/-}	B6	N.D.[b]	N.D.[b]
	bm12	48 ± 42[c,d]	743 ± 739[c]

[a] Spleen cells from B6.IL-9^{-/-} and wild-type recipients were stimulated with irradiated syngeneic B6 or allogeneic bm12 cells. Three days later, culture supernatants were assayed by ELISA for the presence of IL-5 and IFN-γ.
[b] N.D.: not detected.
[c] pg/mL (mean ± SD).
[d] $p < 0.03$.

mal spleen cells for their ability to secrete IL-5 upon allogeneic stimulation. As shown in *table 2*, control B6 cells expressing functional IL-9R produced more IL-5 than IL-9R-deficient cells in response to irradiated bm12 stimulators. Both types of cells, however, produced low but equivalent amounts of IFN-γ after stimulation with irradiated allogeneic stimulators *(table 2)*. Thus, the presence of a functional IL-9R molecule at the surface of responding spleen cells is required for optimal IL-5 production.

Alloantigen-specific production of IL-5 induced by the expression of transgenic IL-9 requires the presence of functional IL-9R molecules at the surface of responding spleen cells

We also analysed the effect of IL-9 on IL-5 secretion in MLR of full MHC alloantigen disparity where transgenic IL-9 was expressed, in turn, by the responding cells or the stimulators. As seen in *figure 2A*, higher levels of IL-5 were produced in cultures of B6 (H-2[b]) spleen cells stimu-

Table 2
The production of IL-5 is impaired in alloreactive IL-9R-deficient CD4^{+} T cells

Responder cells[a]	Stimulator cells	IL-5 (pg/mL)	IFN-γ (pg/mL)
Wild-type	B6	N.D.[b]	N.D.[b]
	bm12	406 ± 138[c,d]	42 ± 8[c]
IL-9R^{-/-}	B6	N.D.[b]	N.D.[b]
	bm12	142 ± 38[c,d]	48 ± 29[c]

[a] Naive spleen cells from B6.IL-9R^{-/-} and wild-type recipients were stimulated with irradiated syngeneic B6 or allogeneic bm12 cells. Five days later, culture supernatants were assayed by ELISA for the presence of IL-5 and IFN-γ.
[b] N.D.: not detected.
[c] pg/mL (mean ± SD).
[d] $p < 0.03$.
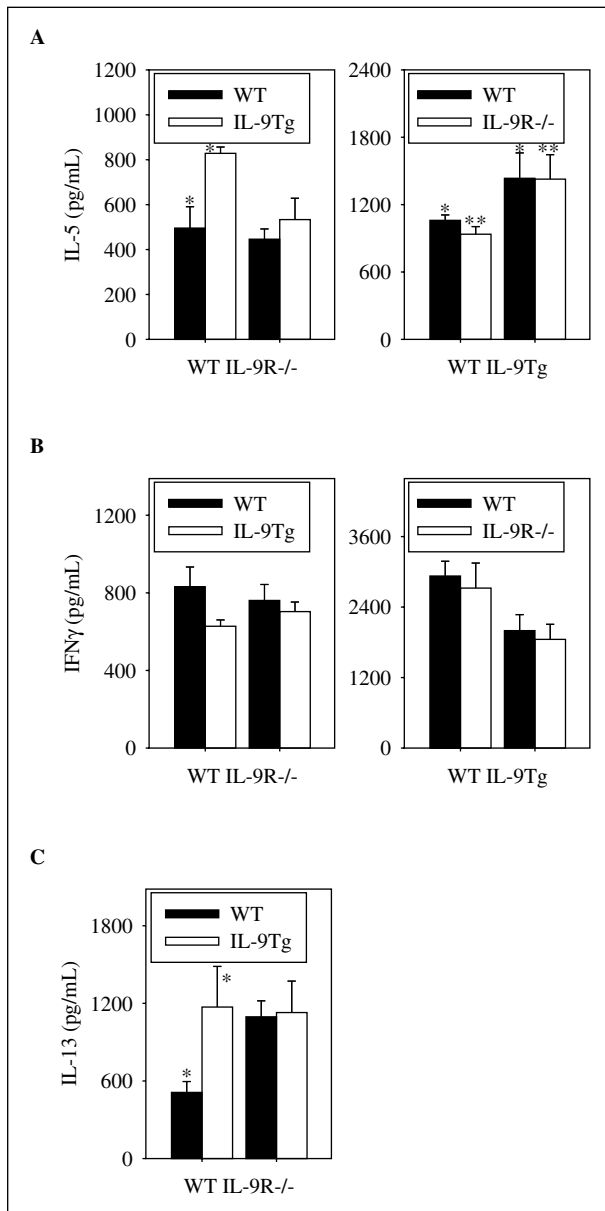
Figure 2

In MLR, transgenic IL-9 stimulates the production of IL-5 when responding cells express a functional IL-9R. IL-5 (**A**), IFN-γ (**B**) and IL-13 (**C**) production (pg/mL ± SD) was assessed in full allogeneic primary MLR crisscross experiments where transgenic IL-9 (IL-9Tg) overexpression or the absence of functional IL-9 receptor (IL-9R$^{-/-}$) was featured by stimulator or responder spleen cells. Results are representative of two separate experiments.
* p < 0.03; ** p < 0.03.

lated by irradiated, IL-9-transgenic, FVB (H-2^q) allogeneic spleen cells than in cultures containing non-transgenic H-2^q stimulators. This increase of IL-5 production depended on the presence of a functional IL-9R at the surface of the responding cells since stimulating IL-9R-deficient splenocytes by irradiated IL-9-transgenic cells did not result in the production of higher levels of IL-5. IL-9 overexpression by responding H-2^q spleen cells also increased the capacity of alloreactive T cells to produce IL-5 in response to allogeneic stimulators *(figure 2A)*. Conversely, neither the transgenic expression of IL-9 nor the lack of a functional IL-9R on responding T cells had an effect on the production of IFN-γ *(figure 2B)*. Because

IL-9 was recently shown to modulate the production of other Th2 cytokines [28], we analyzed the effect of IL-9 on the production of IL-4 and IL-13 by alloreactive T cells. We did not detect the presence of IL-4 in this study (data not shown). The reason might be that the low level of IL-4 produced in primary T cell responses is usually consumed by resident cells and is no longer available in the supernatant for detection [29]. As depicted in *figure 2C*, the level of IL-13 produced by alloreactive T cells was increased in the presence of transgenic IL-9 in the culture supernatant. This effect was mediated by the activity of IL-9 since the presence of IL-9 did not stimulate the production of IL-13 in IL-9-R-deficient cells.
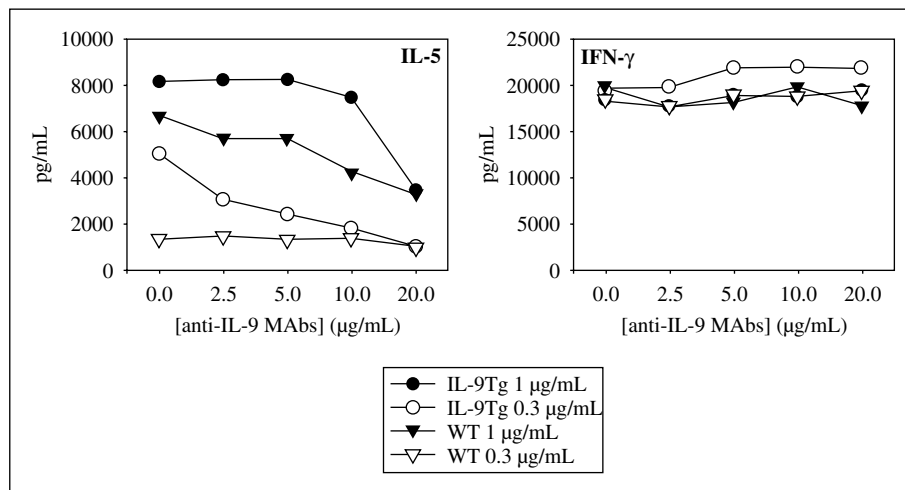
IL-9 acts directly on CD4$^+$ T cells to stimulate the production of IL-5

To complement our observation that IL-9 regulated the secretion of IL-5 in alloreactive T cells, we examined the effect of neutralization of IL-9 on the production of IL-5 by CD4$^+$ T cells after polyclonal activation. Highly purified CD4$^+$ T cells from IL-9 transgenic or wild-type mice were stimulated by immobilized anti-CD3 antibodies. As depicted in *figure 3*, anti-CD3 stimulation induced the secretion of IL-5 in a dose-dependent manner in both types of cell. As expected, IL-9-transgenic, responding CD4$^+$ T cells produced more IL-5 than wild-type counterparts. Neutralization of IL-9 activity by specific monoclonal antibodies however, inhibited the secretion of IL-5. Moreover, high doses of neutralizing anti-IL-9 antibodies fully abolished the capacity of both IL-9-transgenic and normal CD4$^+$ T cells to produce IL-5. Conversely, neutralization of IL-9 did not affect the secretion of IFN-γ in IL-9-transgenic or wild-type CD4$^+$ T cells stimulated by immobilized anti-CD3 antibodies. The effect IL-9 neutralization on IL-5 secretion had to have been specific because the addition of control isotype-matched monoclonal antibodies did not modify the capacity of IL-9-transgenic or wild-type CD4$^+$ T cells to produce IL-5 after anti-CD3 stimulation (data not shown). Taken together, these results demonstrated that secretion of IL-9 is required for the synthesis and production of IL-5 in activated CD4$^+$ T cells.

Effect of IL-9 on IL-5-mediated eosinophilic rejection of skin allograft

We, and others, have shown that graft rejection mediated by Th2 CD4$^+$ T cells requires the activity of IL-5, and the presence of substantial eosinophilic infiltrates within the rejected grafts suggests that eosinophils represent the main effector cells of Th2-type rejection [1, 3-6]. We have also shown that transgenic expression of IL-9 within cardiac allografts induces IL-5-dependent eosinophilic rejection [20]. It is not known however, whether IL-9-mediated control of IL-5 secretion in CD4$^+$ T cells could influence the outcome of Th2-dependent allograft rejection. The major role played by IL-5 and eosinophils in the rejection of bm12 skin by B6 recipients can only be demonstrated in the absence of Fas-mediated cytotoxicity [3]. Therefore, the role played by IL-9 in the process of eosinophilic rejection of non-transgenic graft was assessed by transplanting IL-9- or IL-9R-deficient mice with Fas-deficient *lpr* bm12 skin. All *lpr* allografts were acutely rejected by both B6.IL-9$^{-/-}$ (mst= 14 days) and B6.IL-9R$^{-/-}$ (mst= 13
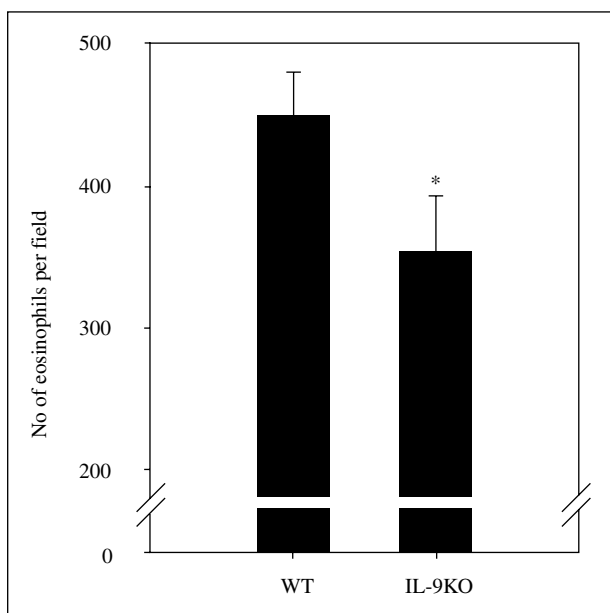
Figure 3
IL-9 acts directly on CD4+ T cells to stimulate IL-5 production. CD4+ T cells from normal (triangles) or IL-9 transgenic FVB mice (circles) were polyclonaly activated with either 1 (filled symbols) or 0.3 (open symbols) µg/mL of immobilized anti-CD3 antibodies for 72 hours. The effect on IL-5 and IFN-γ production of adding increasing concentrations of neutralizing anti-IL-9 antibodies to culture supernatants was determined by ELISA. The results are representative of two independent experiments.

days) recipients, and graft survival was not modified as compared to that of *lpr* bm12 skin transplanted onto control wild type, B6 recipients (mst= 14 days). Histological analysis revealed that the lack of a functional IL-9R on recipient cells did not modify the extent of eosinophil infiltration within rejected allografts (data not shown). Eosinophils were moderately less frequent in skin grafted onto B6.IL-9-/- mice than in that onto control B6 recipients producing functional IL-9 *(figure 4)*. Thus, although IL-9 promotes IL-5 production, the absence of IL-9 activity does not prevent the recruitment of eosinophils in skin allografts.

Figure 4
IL-9 and graft eosinophil infiltration. Eosinophil counts in bm12-lpr/lpr skins rejected by normal (WT) and IL-9-deficient B6 recipients (IL-9-/-). Number of cells in five, high power magnification fields/section (0.0025 mm^2) is given. Results are means ± SD of four mice per group.
* p < 0.03.

DISCUSSION

Our results highlight the role played by IL-9 in the production of IL-5 by Th2 CD4+ T cells. We consistently observed, in three different experimental settings, that IL-9 increased the capacity of alloreactive CD4+ T cells to produce IL-5. We also noticed that the effect of IL-9 on IL-5 production required a functional IL-9R expressed at the surface of responding cells. Finally, we observed that neutralization of IL-9 abolished the capacity of polyclonally activated CD4+ T cells to secrete IL-5. Taken together, our results demonstrate that the production of IL-5 by CD4+ T cells is promoted by IL-9.

The observation that transgenic expression of IL-9 increases Th2 cytokine and IL-5 secretion [15, 17] supports the concept that IL-9 controls the production of IL-5 in CD4+ T cells. However, it has been shown that, in the absence of IL-9, IL-5 expression still occurs during pulmonary Th2 inflammatory responses, and cells from draining mediastinal lymph nodes of IL-9-deficient mice infected with *S. mansoni* were found to produce IL-5 in response to parasite antigens [30]. Moreover, in the present study, whole spleen cells from IL-9- or IL-9R-deficient recipients were found to secrete substantial levels of IL-5 in response to allogeneic stimulators. These results, together with the observation that eosinophil infiltration was present in bm12 allografts rejected by IL-9- or IL-9R-deficient animals, demonstrates the existence of alternate, IL-9-independent pathways for the stimulation of IL-5 secretion in CD4+ T cells and for the recruitment of eosinophils in rejected allografts. These pathways could result from the functional redundancy that exists among TH2 cytokines, as demonstrated in other models of Th2 inflammatory diseases [30]. This also raise the possibility that IL-5 production in cultures of antigen-stimulated lymphoid cell populations is not restricted to CD4+ T cells, emphasizing the potency of other cell lineages to secrete IL-5 in the absence of IL-9. Cells that are known to produce IL-5 upon antigenic stimulation include type-2 CD8+ TcR α,β+ T cells, as well as TcR γ,δ+ T cells [31, 32] Whether IL-5 production by these cells requires the pres-

ence of IL-9 remains to be determined. It is also important to note that in our study, we investigated the IL-9-regulated IL-5 expression exclusively *in vitro*, but the *in vivo* effect may show major differences.

We have shown in previous work that the rejection of bm12 skin by B6 recipients does not occur in IL-5-deficient recipients if Fas/FasL-dependent cell death has also been disrupted [3]. The fact that rejection of normal bm12 skin still occurs in many IL-5-deficient mice may be due to the involvement of cytotoxic anti-MHC class II alloreactive CD4 T cells. Indeed, the cytotoxic activity developed by CD4 T cells results from the interactions between FasL and its counter-receptor Fas on allogeneic targets [33]. In the skin, keratinocytes are known to express Fas in the basal state [34] and may therefore become sensitive to FasL-mediated apoptosis induced by alloreactive CD4 cytotoxic T cells. The observation made in the present report that Fas-deficient bm12 skin grafts are rejected by both B6.IL-9$^{-/-}$ and B6.IL-9R$^{-/-}$ recipients also raises the possibility that IL-5 can be produced by the allograft itself, and have a non-T lymphocyte origin that would not be controlled by recipient IL-9. Mast cells are present in skin. They have also been reported to produce IL-5 once activated [35], as have lung and bronchial epithelial cells [36]. Comparable studies have not been reported for other cell types, but it is possible that other cell types may have the capacity to produce IL-5, for example, in two organs showing consistent, IL-5-producing activity, the normal mouse bladder and thymus [37], mast cells are not prominent. Whether cells of the skin can produce IL-5 has to be verified.

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