

Endogenous Interleukin-4 Downregulates the Type 1 CD4 T Cell-Mediated Immune Response Induced by Intramuscular DNA Immunization

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ABSTRACT

Intramuscular (i.m.) administration of eukaryotic plasmid vectors containing foreign genes is a general immunization strategy capable of inducing protective type 1 immune responses against viral, bacterial, fungal, and parasitic infections. We have described that immunization with a plasmid containing a gene encoding a parasite antigen elicits specific type 1 protective immune responses against experimental infection with the human protozoan parasite *Trypanosoma cruzi*. However, we had evidence suggesting that DNA immunization concomitantly activated specific type 2 immune responses. To determine precisely the influence of the type 2 cytokine interleukin-4 (IL-4) during DNA immunization, we compared the immune responses of genetically modified IL-4-deficient or wild-type (wt) BALB/c mice. IL-4-deficient mice had a significantly lower ratio of specific serum IgG1/IgG2a, and on *in vitro* restimulation with antigen, their spleen cells secreted significantly higher amounts of interferon- γ (IFN- γ). In contrast, absence of IL-4 did not affect total serum antibody response, T cell proliferative responses, or activation of IFN- γ -producing CD8⁺ T cells. Our results suggested that in contrast to conventional adjuvants, such as alum and complete Freund's adjuvant, specific IgG1 in DNA-immunized BALB/c mice was highly dependent on IL-4. To our knowledge, our study provides the first evidence that endogenous IL-4 selectively downregulates the type 1 CD4⁺ T cell-mediated immune response induced by i.m. genetic immunization, a fact that may have implications for the design of certain DNA vaccines.

DNA IMMUNIZATION WITH PLASMIDS containing foreign genes can be used successfully to elicit immune responses to viral, bacterial, fungal, and parasitic antigens. This type of immunization has a series of advantages over other strategies because it is relatively simple and generates long-lived immune responses mediated by MHC class I and class II-restricted T cells. In addition, genetic vaccination has the advantage of inducing predominantly type 1 immune responses.⁽¹⁾

We have performed detailed studies on the immunogenic properties of a plasmid encoding an antigen expressed on the surface of infective forms of *Trypanosoma cruzi*. This protozoan parasite causes Chagas' disease, an acute and chronic illness that afflicts 16–18 million people in Latin America. Initially, we reported that intramuscular (i.m.) immunization of BALB/c mice with plasmid p154/13, which contains the gene encoding the catalytic domain of *T. cruzi* trans-sialidase (TS), elicited remarkable protective immunity against lethal infection with *T. cruzi*.⁽²⁾ A detailed characterization of the immune re-

sponses generated by vaccination with p154/13 revealed that bulk cells from DNA-immunized mice had CD4⁺ and CD8⁺ T cells that produced interferon- γ (IFN- γ) but not interleukin-4 (IL-4) or IL-10. The TS-specific T cells were characterized at the clonal level. From DNA-vaccinated mice, we obtained cytotoxic type 1 CD4⁺ or CD8⁺ T cell clones that displayed remarkable antiparasitic activities *in vitro*.^(3,4)

Unexpectedly, we also collected evidence suggesting that immunization with p154/13 activated specific type 2 CD4⁺ T cells. The first evidence was indirect and relied on the presence of specific IgG1 in the sera of immunized mice.⁽¹⁾ This evidence was corroborated by the fact that we isolated antigen-specific type 2 CD4⁺ T cell clones from DNA-immunized mice.^(3,4)

IL-4 has an important role in the generation of type 2 immune responses.⁽⁵⁾ To date, to our knowledge, no studies are available on the precise participation of IL-4 in the immune responses induced by genetic vaccination. To address this ques-

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TABLE 1. ANTIBODY IMMUNE RESPONSE OF IL-4^{+/+} AND IL-4^{-/-} BALB/c MICE IMMUNIZED WITH p154/13

Mouse strain	Immunogen ^a	Antibody titers ^b (log)	IgG subclasses antibody titers ^b (log)		
			IgG1	IgG2a	IgG2b
IL-4 ^{+/+}	pcDNA3	<2.00	NT ^c	NT	NT
	p154/13	5.72 ± 0.33 ^d	5.56 ± 0.12 ^e	4.5 ± 0.24 ^e	4.15 ± 0.35 ^d
IL-4 ^{-/-}	pcDNA3	<2.00	NT	NT	NT
	p154/13	5.61 ± 0.30 ^d	3.8 ± 0.62 ^e	5.16 ± 0.16 ^e	4.35 ± 0.12 ^d

^aIL-4^{+/+} or IL-4^{-/-} BALB/c mice were immunized i.m. with three doses of plasmid p154/13 or pcDNA3. Each dose consisted of 100 µg plasmid administered at 3-week intervals.

^bFourteen days after the last immunization, blood samples were collected, and each individual serum sample was assayed in serial dilutions for the presence of antibodies to recombinant TS by ELISA. The individual titers were considered as the highest dilution of serum that presented an OD at 492 nm > 0.1. Results represent the mean value obtained for 9 mice ± SD of the mean.

^cNT, not tested.

^dThe results obtained for groups of IL-4^{+/+} and IL-4^{-/-} mice immunized with p154/13 were compared by Student's *t*-test. The difference between them was not statistically significant ($p > 0.05$).

^eThe differences between groups of IL-4^{+/+} and IL-4^{-/-} BALB/c mice immunized with p154/13 were statistically significant by the Student's *t*-test. IgG1, $p < 0.0001$; IgG2a, $p = 0.0006$.

tion, we compared the immune responses elicited by DNA vaccination with p154/13 in wild-type (wt) and IL-4-deficient BALB/c mice.

Female 5-to-8-week-old wt (IL-4^{+/+}) or genetically modified IL-4-deficient (IL-4^{-/-}(6)) BALB/c mice were kindly provided by the department of immunology of the University of São Paulo.

Purification and i.m. immunization with plasmid p154/13 were carried out exactly as described earlier.^(2,3) Production of the recombinant TS protein and detection of specific serum antibodies were achieved as previously described.^(2,3) ELISA for the detection of mouse IgG subclasses was performed with peroxidase-labeled secondary antibodies specific for mouse IgG1, IgG2a, and IgG2b (all obtained from ICN Biochemicals, Irvine, CA) diluted 1:1000.

Two to five weeks after the third and last dose of plasmid, spleens from DNA-immunized BALB/c mice (3 animals per group) were removed to prepare splenocyte suspensions used

in the different immunologic assays. The T cell proliferation assay and determination of IFN-γ secretion by spleen cells stimulated with A20J cells coated with the TS peptide IYNVGQVSI were performed as described earlier.^(3,7) For IFN-γ determination, 4 × 10⁶ spleen cells were cultivated in triplicate in flat-bottom 96-well plates in a final volume of 200 µl. The recombinant protein was added to the cultures at a final concentration of 10 µg/ml. After 5 days, the supernatants were collected for cytokine determination. IFN-γ was estimated by capture ELISA using antibodies and recombinant cytokines purchased from PharMingen (San Diego, CA).^(3,7) The number of IFN-γ-secreting cells specific for the peptide IYNVGQVSI was determined by the ELISPOT assay, as originally described.^(8,9)

Antibody titers similar to those of recombinant TS were detected in the sera of IL-4^{+/+} and IL-4^{-/-} mice immunized with p154/13 (Table 1). When antibody titers were compared after each plasmid immunization, no significant differences were observed between them (data not shown). Control mice of both

TABLE 2. IFN-γ SECRETION BY SPLEEN CELLS FROM IL-4^{+/+} AND IL-4^{-/-} BALB/c MICE IMMUNIZED WITH p154/13

Mouse strain	Immunogen ^a	Number of experiments	IFN-γ concentration median ng/ml (range)	
			No antigen	Recombinant TS ^b
IL-4 ^{+/+}	pcDNA3	2	<0.2	<0.2
	p154/13	5	<0.2	2.0 (1.8–2.7) ^c
IL-4 ^{-/-}	pcDNA3	2	<0.2	<0.2
	p154/13	5	<0.2	9.8 (4.8–12.8) ^c

^aIL-4^{+/+} and IL-4^{-/-} BALB/c mice were immunized as described in Table 1. Two to five weeks after the last immunization, pooled spleen cells were collected from 3 mice immunized with the indicated plasmid.

^bSpleen cells (2 × 10⁷/ml) were stimulated *in vitro* with 10 µg/ml recombinant TS protein. After 5 days in culture, the supernatants were collected, and the concentration of IFN-γ was estimated. Results are expressed as median of the indicated number of experiments.

^cThe results obtained for groups of IL-4^{+/+} and IL-4^{-/-} mice were immunized with p154/13 were compared statistically using the Mann-Whitney test. The difference between them was significant ($p = 0.006$).

TABLE 3. CD4 DEPENDENCE OF IFN- γ SECRETION BY SPLEEN CELLS FROM IL-4^{+/+} AND IL-4^{-/-} BALB/c MICE IMMUNIZED WITH p154/13

Mouse strain ^a	Experimental group no.	No antigen	IFN- γ concentration (ng/ml) ^b Recombinant TS			
			Rat IgG	α CD4	α CD8	α CD4 + α CD8
IL-4 ^{+/+}	1	<0.2	1.7 \pm 0.2	0.3 \pm 0.1	1.3 \pm 0.1	0.3 \pm 0.1
	2	<0.2	1.5 \pm 0.1	0.1 \pm 0.0	1.5 \pm 0.1	0.2 \pm 0.0
IL-4 ^{-/-}	1	<0.2	4.9 \pm 0.2	<0.2	5.4 \pm 0.4	<0.2
	2	<0.2	6.0 \pm 0.2	0.4 \pm 0.1	6.1 \pm 0.2	0.5 \pm 0.1

^aIL-4^{+/+} or IL-4^{-/-} BALB/c mice were immunized with plasmid p154/13 as described in Table 1.

^bFor IFN- γ detection, spleen cells were stimulated *in vitro* with 10 μ g/ml recombinant TS in the presence of 10 μ g/ml rat IgG or mAb α CD4, α CD8, or α CD4 + α CD8. Results are expressed as average of triplicate cultures \pm SD.

strains immunized with pcDNA3 did not present specific antibodies.

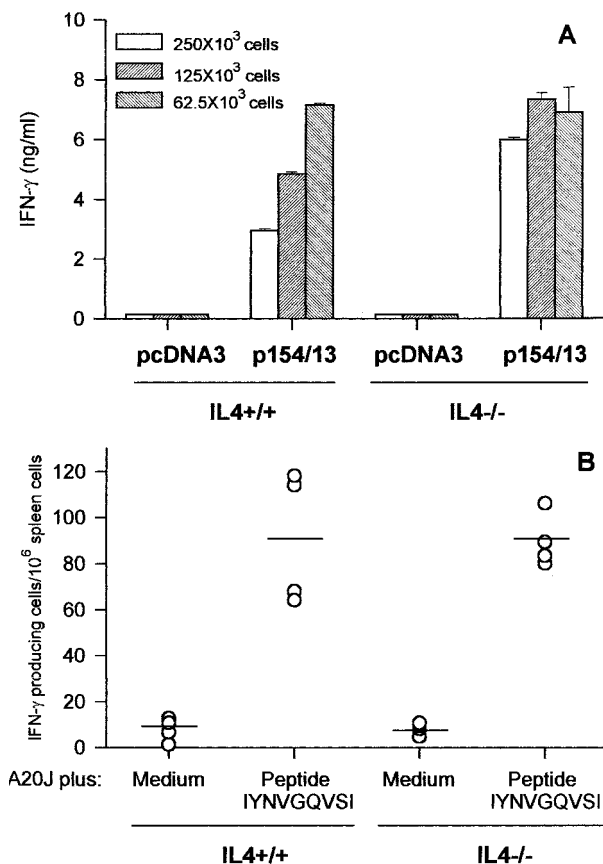
A significant difference between IL-4^{+/+} and IL-4^{-/-} mice immunized with p154/13 was detected when the titers of specific antibodies of the different IgG subclasses were estimated. As noted earlier, IL-4^{+/+} mice showed a predominance of specific IgG1. In contrast, IL-4^{-/-} mice had significantly lower titers of specific antibodies of this IgG subclass and higher titers of IgG2a (Table 1). The IgG1/IgG2a ratio changed dramatically from 10.35 in IL-4^{+/+} mice to 0.09 in IL-4^{-/-} mice.

The presence of IFN- γ in supernatants of spleen cells from

DNA-immunized mice was determined after *in vitro* restimulation with recombinant TS. In five independent experiments, the median concentration of IFN- γ in the supernatant of cells obtained from IL-4^{-/-} mice immunized with p154/13 was significantly higher than that in cultures of cells from IL-4^{+/+} mice injected with p154/13 ($p = 0.006$, Mann-Whitney test) (Table 2).

IFN- γ secretion was dependent on the activation of CD4⁺ T cells, as the addition of the monoclonal antibody (mAb) α CD4 (GK1.5) drastically reduced its concentration in the supernatant of spleen cells stimulated with the antigen. In contrast, under

FIG. 1. IFN- γ secretion by spleen cells specific for peptide IYNVGQVSI of IL-4^{+/+} or IL-4^{-/-} BALB/c mice immunized with p154/13. Mice were immunized as described in Table 1. (A) Two to five weeks after the last immunization, pooled spleen cells obtained from 3 mice were expanded for 6 days in the presence of irradiated A20J-TS cells.^(3,7) The cells were then washed, and the indicated number of cells were restimulated in cultures containing 10⁵ irradiated A20J cells pulsed with 1 μ M peptide IYNVGQVSI. IFN- γ was estimated in supernatants collected after 18 h. Results are expressed as average of triplicate cultures \pm SD. This experiment was reproduced three times, with similar results. (B) The ELISPOT assay was performed by adding 5 \times 10⁵ spleen cells from each individual mouse per well. To each well, we added 10⁵ irradiated A20J cells pulsed or not with 1 μ M peptide IYNVGQVSI. After 24 h, the cells were removed, and the ELISPOT assay was carried out as described earlier.^(8,9) Each circle represents the result of averages of triplicate cultures of spleen cells from a single mouse. Bars represent the averages of 4 mice. When the results obtained for IL-4^{+/+} and IL-4^{-/-} mice immunized with p154/13 were compared by the Mann-Whitney test, the difference between them was not statistically significant ($p > 0.05$).



the same circumstances, the addition of mAb α CD8 (clone 2.43) had little or no impact on IFN- γ secretion by specific spleen cells (Table 3).

IFN- γ secretion by CD8⁺ spleen cells from DNA-immunized mice was evaluated after *in vitro* restimulation with A20J cells coated with peptide IYNVGQVSI.^(3,7) This peptide has been shown previously to be a CD8⁺ T cell epitope.⁽³⁾ We found that in the presence of peptide IYNVGQVSI, cells from IL-4^{+/+} and IL-4^{-/-} mice immunized with p154/13 secreted comparable amounts of IFN- γ (Fig. 1A). These results were further confirmed by the direct ELISPOT assay. The number of cells (per 10⁶ spleen cells) that secreted IFN- γ in the presence of peptide IYNVGQVSI was similar in IL-4^{+/+} and IL-4^{-/-} mice immunized with p154/13 (Fig. 1B). Control mice immunized with pcDNA3 did not present IFN- γ -secreting cells in the presence of peptide IYNVGQVSI (data not shown).

The present study addressed the question of whether endogenous IL-4 influenced the immune responses elicited by genetic vaccination with a plasmid harboring a parasite gene. The absence of IL-4 did not influence total serum antibody response, T cell proliferative responses (data not shown), or activation of IFN- γ -producing CD8⁺ T cells specific for the peptide IYNVGQVSI. Conversely, IL-4^{-/-} BALB/c mice had significantly lower titers of specific serum IgG1, accompanied by a significant increase in specific IgG2a. Also important was the observation that on *in vitro* restimulation with recombinant antigen, spleen cells from IL-4^{-/-} mice secreted significantly higher amounts of IFN- γ compared with cells from IL-4^{+/+} mice.

Our results confirm our initial hypothesis that vaccination of BALB/c mice with p154/13 concomitantly activates type 1 and type 2 immune responses. The presence of mixed type 1 and 2 immune responses had been noticed also in mice vaccinated i.m. with plasmids containing genes expressed in a variety of microorganisms (viruses, bacteria, fungi, and parasites) and tumor cells.⁽¹⁰⁻²⁰⁾ However, the role of IL-4 as a mediator of this type of immune response was not addressed in most of these studies.

In a single study, the predominance of IgG1 observed after genetic immunization with a plasmid expressing a secreted form of the influenza virus hemagglutinin was studied in IL-4^{-/-} BALB/c mice. Similar to our observation, the ratio of specific serum IgG1/IgG2a antibodies changed from 15 in IL-4^{+/+} mice to 0.4 in IL-4^{-/-} mice. However, in contrast to our results, the authors reported that the overall antibody titers were lower in IL-4^{-/-} BALB/c mice.⁽¹¹⁾

Few earlier publications addressed the participation of IL-4 and its receptor in the control of specific IgG1 generated after immunization with conventional adjuvants, such as alum and complete Freund's adjuvant. In these studies, the reduction of specific IgG1 in BALB/c IL-4^{-/-} mice was not as dramatic as we describe herein.^(21,22) These results led to the proposition that the type 2 immune response induced in the presence of alum was at least in part independent of IL-4 and its receptor.⁽²²⁾ Nevertheless, in the case of immunizations performed in the presence of alum, the absence of IL-4 led to a significant increase in the titers of IgG2a and in IFN- γ production by spleen cells.^(21,22) Therefore, similar to the observations described here, IL-4 downregulates adjuvant-assisted type 1 CD4 immune responses.

A possible participation for IL-13 in the switch of specific

IgG1 seems very limited because the average IgG1 titer in IL-4^{-/-} mice is reduced to only 1.75% of the average titer found in IL-4^{+/+} mice (Table 1). Whether IL-13 interferes with the activation of Th1 CD4⁺ T cells is unknown at present and remains to be investigated.

An important observation in our study was the fact that in the absence of IL-4, spleen cells secreted larger amounts of IFN- γ . Although it is well documented that IL-4 can inhibit the *in vivo* generation of type 1 immune responses, the precise molecular mechanism mediating this event is still a matter of intense study. Evidence indicates that IL-4-mediated activation of Stat6 leads to silencing of IFN- γ gene expression, therefore reducing type 1 immune responses.⁽²³⁾ Whether this or another mechanism operates during DNA immunization will require future studies.

The cells responsible for the *in vivo* production of IL-4 have not been characterized. It is plausible that CD4⁺ T cells specific for TS are at least in part responsible for IL-4 production. This hypothesis is based on the fact that we isolated IL-4-secreting CD4⁺ T cell clones from mice immunized with plasmid p154/13. Nevertheless, because IL-4 can be produced *in vivo* by a variety of cells, direct evidence should be obtained in the future by adoptive transfer of purified cells.

Recent studies have reported that IL-4 may be important for the development of immune responses mediated by CD8⁺ T cells. In these studies, IL-4^{-/-} mice have a drastically reduced priming of specific CD8⁺ T cells.^(24,25) The role of IL-4 in CD8⁺ T cell priming after DNA vaccination has never been directly addressed. We found that after DNA immunization with plasmid p154/13, priming of peptide-specific, IFN- γ -secreting CD8⁺ T cells could be achieved in the absence of IL-4. This result indicates either that IL-4 is not important for priming of CD8⁺ T cells after i.m. DNA vaccination or that other compensatory mechanism may substitute for IL-4 in our system.

Finally, from a practical point of view, it will be important to establish an experimental model that will allow determination of whether the neutralization of IL-4 during priming represents an alternative for the improvement of protective type 1 immune responses elicited by DNA vaccination.

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