

BRIEF COMMUNICATION

Immunotherapy with plasmid DNA encoding mycobacterial hsp65 in association with chemotherapy is a more rapid and efficient form of treatment for tuberculosis in mice

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Tuberculosis (TB) remains a threat for public health, killing around 3 million people a year. Despite the fact that most cases can be cured with antibiotics, the treatment is long and patients relapse if chemotherapy is not continued for at least 6 months. Thus, a better characterization of the working principles of the immune system in TB and identification of new immunotherapeutic products for the development of shorter regimens of treatment are essential to achieve an effective management of this disease. In the present work, we demonstrate that immunotherapy with a plasmid DNA encoding the *Mycobacterium leprae* 65 kDa heat-shock protein (hsp65) in order to boost the efficiency of the

immune system, is a valuable adjunct to antibacterial chemotherapy to shorten the duration of treatment, improve the treatment of latent TB infection and be effective against multidrug-resistant bacilli (MDR-TB). We also showed that the use of DNA-hsp65 alone or in combination with other drugs influence the pathway of the immune response or other types of inflammatory responses and should augment our ability to alter the course of immune response/inflammation as needed, evidencing an important target for immunization or drug intervention.

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It is generally recognized that the most serious difficulty in chemotherapy of Tuberculosis (TB) is the necessity of continuous medication over a long period of time.^{1,2} Active TB requires a minimum of 6 months treatment with multiple drugs.^{1,2} Even latent infections require months of prophylactic therapy to reduce the risk of reactivation.³ Without close supervision, most patients are unwilling or unable to complete a full course of medication. Patients who default on therapy are in a severe risk of relapsing and acquiring drug resistance.^{4,5} The quest for better, faster and cheaper treatments will therefore require unconventional approaches to identify new and potentially more relevant targets. Most infected people never develop active disease. However, in approximately 10% of infected subjects, the reactivation of latent infection results in active TB.⁵ Thus, the immune response is adequate to control the infection in most people but apparently is not generally effective in destroying the bacteria.⁶ In these circumstances, immu-

notherapy to boost the efficiency of the immune system in infected patients could be a valuable adjunct to antibacterial chemotherapy.

We previously demonstrated that vaccination by intramuscular injection of naked plasmid DNA encoding hsp65 from *Mycobacterium leprae* protects mice against subsequent challenge with virulent *Mycobacterium tuberculosis* H37Rv strain.^{7,8} This protection is attributed to the establishment of a cellular immune response dominated by antigen-specific T lymphocytes that not only produce gamma interferon (IFN- γ) but are also cytotoxic towards infected cells (a type-1 cellular immune response).^{9,10} In addition, in heavily infected mice, the hsp65-encoding DNA vaccine can switch the immune response from one that is relatively inefficient and produce bacterial stasis to one that kills bacteria.¹¹ Thus, this DNA vaccine, initially designed to prevent infection by *M. tuberculosis*, can also have a pronounced therapeutic action in mice.^{11,12} We have also tested the therapeutic effect of a plasmid that expressed IL-12 instead of a mycobacterial antigen. This plasmid caused a significant reduction in bacterial numbers showing therapeutic activity in mice previously infected with *M. tuberculosis*.¹¹ Ha *et al*¹³ demonstrated that immunotherapy using a plasmid DNA encoding mycobacterial 85A antigen or IL-12N220L DNA vaccine combined with conventional

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chemotherapy was highly effective in mice for the prevention of tuberculosis reactivation. The therapeutic effects of DNA vaccination against *Mycoplasma pulmonis* have also been reported.¹⁴ Here, we show that immunotherapy with DNA-hsp65, in order to boost the efficiency of the immune system shortens the duration of treatment, improves the treatment of latent TB infection and is effective against MDR-TB.

After the initial infection, *M. tuberculosis* becomes into a latent phase, hiding inside macrophages.¹⁵ Both macrophage–lymphocyte interactions are likely to be important in protective immunity against TB.¹⁶ In this case, the challenge for the immunologist is to identify systems or antigens of the pathogens that allow lymphocytes to carry out their immunoprotective activities. We have therefore initiated our studies showing that the antigen-specific T cells that produce interleukin-4 (IL-4) which are not cytotoxic (a type-2 cellular immune response) are abundant during infection with *M. tuberculosis* in mice (Figure 1). The same was found in humans.¹⁶ Such type-2 responses do not contribute to protection and consequently a shift in the balance towards type-1 responses (T lymphocytes that produce IFN- γ and are cytotoxic towards infected cells) might be beneficial.^{10,11} Thus, we tested whether immunization with Bacillus Calmette–Guérin (BCG) or DNA-hsp65 could have a beneficial effect and enhance the type-1 response before or during the course of TB. In fact, these

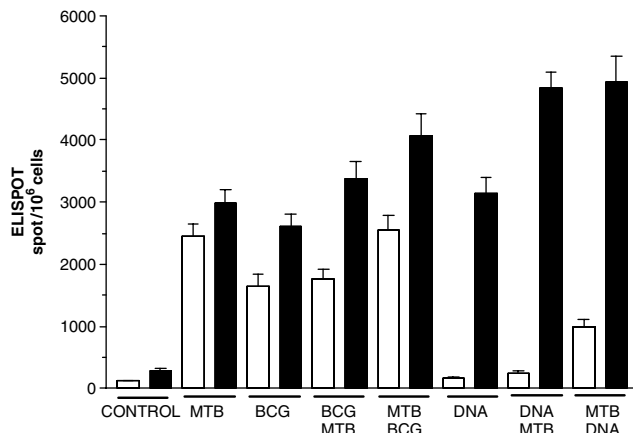


Figure 1 ELISPOT estimates of the frequencies of mice lymph node T cells that produced IL-4 (\square) or IFN- γ (\blacksquare). MTB, cells from mice infected with *M. tuberculosis* (intratracheal administration with 10^5 colony-forming units (CFU) of *M. tuberculosis* H37Rv); BCG, cells from mice vaccinated with BCG (intradermal injection of 1×10^5 CFU); BCG/MTB, cells from mice vaccinated with BCG and infected with *M. tuberculosis* 1 month later; MTB/BCG, cells from previously infected mice treated with BCG 1 month later; DNA, cells from mice vaccinated with DNA-hsp65 (four doses of plasmid DNA-hsp65 intramuscularly at 2-week intervals); DNA/MTB, cells from mice vaccinated with DNA-hsp65 and infected with *M. tuberculosis* 2 weeks after the last immunization; MTB/DNA, cells from mice previously infected with *M. tuberculosis* and treated with DNA-hsp65 (four doses of plasmid DNA-hsp65 intramuscularly at 2-week intervals) 1 month later. The lymph node T cells were assayed for the frequency of IFN- γ and IL-4-producing cells by ELISPOT as previously described,¹⁹ 4 weeks after infection or treatment. Statistically significant differences for IFN- γ and IL-4-producing cells from *M. tuberculosis* infected mice compared with either DNA-hsp65 immunized mice, vaccinated with DNA-hsp65 and postinfected with *M. tuberculosis*; and previously infected mice treated with DNA-hsp65, were observed (Student's *t*-tests, $P < 0.001$). These data are from a typical experiment repeated 3 times with similar results.

responses are particularly favoured by DNA-hsp65 and not by immunization or treatment with live BCG vaccine (Figure 1). Vaccination with DNA-hsp65 caused substantial increases in the frequency of IFN- γ -producing T lymphocytes that were not significantly altered even after infection with *M. tuberculosis*. The increase occurred equally in cells with CD4 and CD8 phenotypes (data not shown) and this result contrasted with the response to immunization with BCG, where the increase was equal for IFN- γ and IL-4 imitating the same pattern of immune response observed after infection with *M. tuberculosis*.¹⁷ The lymph node T cells from mice treated with DNA-hsp65, which were a month earlier infected with *M. tuberculosis*, produced more frequently IFN- γ than IL-4 in response to hsp65 antigen (Figure 1). Nevertheless, the present findings strengthen the case for evaluating immunotherapy as a complement to chemotherapy for the treatment of TB.

Meanwhile, we have been testing whether the application of immunotherapy with DNA-hsp65 in conjunction with conventional chemotherapeutic antibacterial drugs resulted in faster and more accurate cure of the disease including latent infection and MDR-TB. An infection initiated in mice by an intratracheal administration of *M. tuberculosis* H37Rv or MDR-TB was allowed to develop for 4 weeks, during this period the number of bacteria in the lungs increased by about 100-fold. The mice were then separated in several groups and treated with BCG; DNA-hsp65; DNAv (plasmid DNA without the hsp65 gene); drugs (combination of isoniazid and pyrazinamide); or with a combination of treatments containing drugs with either BCG, DNA-hsp65 or DNAv as stated above. The percentage of live H37Rv bacteria in lungs of mice declined rapidly on the 1st and 3rd month after the administration of the first dose of the combined treatment with DNA-hsp65 and drugs (Table 1). Although significant effects ($P < 0.001$) were obtained with the treatment with either DNA-hsp65 or drugs alone, as compared to control group, they were able to reduce 85 and 77% in CFU, respectively, only 6 months after the beginning of treatment. The experiments clearly show that the treatment with drugs and DNAhsp65 induce to a significant reduction in the number of bacilli immediately after the first month of treatment. Besides, 6 months after the beginning of the treatment with drugs and DNAhsp65, bacilli in the lungs of the animals submitted to treatment were not detected; therefore, showing that this strategy can reduce the length of treatment and is effective in eliminating residual bacteria. In general, in DNA vaccine model, the control is always the DNA vector.¹⁸ Therefore, we believe that the effect in hsp65 is specific, since we did not have the same results using the vector DNA. Moreover, in previous works we demonstrated that DNA vaccines encoding mycobacterial hsp70 or ESAT 6 antigens or injecting the protein hsp65 in Freund's incomplete adjuvant were unable to present therapeutic effects against an established *M. tuberculosis* H37Rv infection when compared to DNA-hsp65.^{11,19} Although the specific types of responses elicited following DNA immunization are determined by the encoded antigen, the data presented here as well as our previous results demonstrate that the efficacy of DNAhsp65 vaccination for tuberculosis varies with the method of DNA introduction *in vivo*.²⁰ DNA-hsp65 therapy alone was equally effective against an established infection in

Table 1 Combination approach of using immunotherapy plus chemotherapy was highly effective to control TB and avoid reactivation after treatment with corticoid

Infection with ^a	Treatment ^b	Number of CFU ($\times 10^5$) in lungs after infection ^c			Number of mice with reactivation of TB/number of mice treated with dexamethasone ^d
		1 month	3 months	6 months	
H37Rv	Untreated	9.81 \pm 0.70	16.40 \pm 2.30	28.90 \pm 3.43	— ^e
	DNA-hsp65	6.48 \pm 0.49	4.62 \pm 0.84*	0.86 \pm 0.06*	4/21
	DNAv	9.39 \pm 0.68	15.20 \pm 3.12	21.12 \pm 2.74	— ^e
	BCG	12.30 \pm 0.84	19.28 \pm 2.65	34.20 \pm 3.89	15/15
	Drug	7.18 \pm 0.55	5.45 \pm 0.84*	0.92 \pm 0.08*	15/18
	Drug+DNA-hsp65	3.64 \pm 0.36**	0.21 \pm 0.01**	Zero ^f	0/22
	Drug+DNAv	8.23 \pm 0.89	3.84 \pm 0.50*	0.29 \pm 0.05*	18/20
	Drug+BCG	7.35 \pm 0.42	3.86 \pm 0.13*	0.12 \pm 0.02*	7/15
MDR-TB	Untreated	5.58 \pm 1.98	8.70 \pm 2.14	13.45 \pm 2.42	— ^e
	DNA-hsp65	2.62 \pm 0.72	1.57 \pm 0.11*	0.09 \pm 0.01*	2/16
	DNAv	5.12 \pm 1.58	7.15 \pm 1.77	11.90 \pm 3.08	— ^e
	BCG	5.24 \pm 1.22	9.18 \pm 1.18	16.50 \pm 1.57	10/10
	Drug	6.05 \pm 1.45	8.29 \pm 2.32	13.55 \pm 3.49	— ^e
	Drug+DNA-hsp65	2.28 \pm 0.51	0.61 \pm 0.08*	0.08 \pm 0.01*	3/11
	Drug+DNAv	4.67 \pm 1.38	7.17 \pm 1.29	9.83 \pm 2.15	12/14
	Drug+BCG	5.31 \pm 1.39	7.58 \pm 1.29	12.6 \pm 1.75	11/13

^aBALB/c mice aged 6–8 weeks were infected through an intratracheal administration with 10^5 colony forming units (CFU) of *M. tuberculosis* H37Rv or with 10^5 CFU of a clinical isolate of MDR-TB.

^bThe treatment was done after four weeks of infection by the following schedule: untreated mice; DNA-hsp65 (four intramuscular injection of 100 μ g DNA plasmid encoding *M. leprae* hsp65 gene at 2-week intervals); DNAv (plasmid DNA without the hsp65 gene administered at the same scheme for DNA-hsp65); BCG (single intradermic injection of about 10^5 live bacteria in 50 μ l saline); Drug (treatment with a standard diet supplemented with isoniazid (25 mg/kg) and pyrazinamide (1000 mg/kg) for 24 weeks); a combination of treatments containing drugs with either DNA-hsp65, DNAv or BCG as stated above, beginning 4 weeks after infection.

^cMice were killed at intervals (0, 1, 3, and 6 months after the beginning of treatment) and bacteria in lungs were counted as CFU on 7H11 medium. Results are expressed as CFU/g tissue and represent mean estimates (\pm s.d.) from a typical experiment of five mice per group, repeated three times.

^dFor reactivation evaluation, six subcutaneous injections of dexamethasone (6 mg/kg) were given 2 days separately, 6 months after the beginning of treatment and bacterial CFU in lungs were measured after a further 8 weeks.⁷

^eNot determined.

^fZero, indicate organs with counts below the level of detection. * Indicates statistically significant differences for CFU counts from treated mice in relation to untreated mice.

**Indicate that the effects of DNA-hs65 plus drugs were highly significant since the 1st month of the beginning of treatment compared with the treatment with drugs or DNA-hsp65 alone in mice infected with H37Rv (Student's *t*-tests, $P < 0.001$).

mice with a clinical isolate of MDR-TB (Table 1). The treatment with BCG in the animals previously infected with H37Rv or MDR-TB was not able to reduce the number of bacilli in the lungs of these animals, while the combined treatment with drugs and BCG enabled a significant CFU reduction in H37Rv-infected animals 6 months after the beginning of the treatment. A possible explanation for these findings could be related to a strong decline in the H37Rv bacilli number by the treatment with drugs. This fact allows a more BCG effective action which modulates the immune system for a Th1 response pattern. These findings are very important once the application of DNAhsp65 in conjunction with conventional chemotherapeutic antibacterial drugs results in a faster and more certain cure of the disease including MDR-TB (Table 1).

Conventional drugs for TB are highly effective against actively replicating cells, but they are far less potent against cells in stationary phase as those found in lungs of latent infected people.⁵ Most infected individuals develop a latent or persistent infection that can be reactivated at any time during the individuals lifetime.⁵ This is primarily attributed to an inadequate immune response towards infecting bacteria, which suffers growth inhibition rather than death and subsequently multiplies catastrophically.⁵ To study drugs or immu-

notherapy in TB latency in mice, variants of a so-called Cornell model are widely used.²¹ The original model is based upon intravenous challenge with *M. tuberculosis*, subsequent treatment of mice with antibiotics (until culturing of lung and spleen homogenates provide no mycobacterial CFUs after chemotherapy withdrawal), and resuscitation of infection later on, usually after the administration of immunosuppressive hormones. The relapse of overt TB under these conditions apparently reflects the return of mycobacteria from truly dormant to active metabolic state. Here, we tested whether the injection of immunosuppressive corticosteroid at the end of chemotherapy or immunotherapy or at the end of the combination approach of treatment could increase reactivation. Corticosteroid was unable to reactivate H37Rv bacterial growth in animals treated with the combination of DNA-hsp65 plus drugs (Table 1). The proportion of mice with lungs that appeared to be sterile decreased significantly from 15 of 18 mice which were treated only with drugs, to four in 21 for DNA-hsp65 and zero in 22 for the combination of drugs plus DNA-hsp65 (Table 1). We can speculate therefore that DNA-hsp65 used prophylactically and therapeutically might be able to both prevent the establishment of the persistent state and eliminate it faster if it is already established.

Table 2 DNA-hsp65 can switch the immune response from one that is type-2 producing IL-4 to one that is type-1 producing IFN- γ

Infection ^a	Treatment ^b	IFN- γ and IL-4 producing T-cell frequency ($\times 10^3$) ^c								Percent of T cells showing CD44hi fluorescence ^d		Percent of T cells showing CD62L fluorescence ^d	
		Time 0		1 month		3 months		6 months		Time 0	6 months	Time 0	6 months
		IFN- γ	IL-4	IFN- γ	IL-4	IFN- γ	IL-4	IFN- γ	IL-4				
H37Rv	Untreated	1.7 \pm 0.05	1.9 \pm 0.04	1.8 \pm 0.04	1.7 \pm 0.03	1.2 \pm 0.05	1.7 \pm 0.04	0.9 \pm 0.03	1.7 \pm 0.03	17 \pm 2	39 \pm 4	58 \pm 3	28 \pm 4
	DNA-hsp65	1.8 \pm 0.04	2.0 \pm 0.05	5.6 \pm 0.09*	0.4 \pm 0.02*	12.5 \pm 0.15*	0.3 \pm 0.01*	10.4 \pm 0.16*	0.2 \pm 0.01*	19 \pm 2	86 \pm 5*	48 \pm 3	12 \pm 2*
	DNAv	1.7 \pm 0.05	1.9 \pm 0.06	1.5 \pm 0.04	1.3 \pm 0.03	1.8 \pm 0.06	1.1 \pm 0.04	1.3 \pm 0.03	1.6 \pm 0.03	21 \pm 3	37 \pm 4	39 \pm 2	32 \pm 3
	BCG	1.9 \pm 0.06	2.0 \pm 0.06	2.8 \pm 0.09	2.5 \pm 0.09	4.0 \pm 0.08*	3.1 \pm 0.07*	3.5 \pm 0.11*	2.7 \pm 0.08	18 \pm 2	43 \pm 3	43 \pm 4	26 \pm 4
	Drug	1.4 \pm 0.03	2.3 \pm 0.05	2.0 \pm 0.05	2.1 \pm 0.06	2.0 \pm 0.06	1.9 \pm 0.06	1.6 \pm 0.04	1.4 \pm 0.03	12 \pm 1	46 \pm 2	47 \pm 4	25 \pm 3
	Drug+DNA-hsp65	1.2 \pm 0.04	1.6 \pm 0.03	8.5 \pm 0.16*	0.3 \pm 0.01*	15.4 \pm 0.21*	0.3 \pm 0.01*	12.6 \pm 0.19*	0.1 \pm 0.01*	16 \pm 2	94 \pm 4*	55 \pm 5	10 \pm 1*
	Drug+DNAv	1.7 \pm 0.03	1.5 \pm 0.04	1.6 \pm 0.04	1.7 \pm 0.05	1.9 \pm 0.05	1.8 \pm 0.04	1.2 \pm 0.03	1.6 \pm 0.03	14 \pm 2	54 \pm 4	54 \pm 5	19 \pm 2
	Drug+BCG	1.7 \pm 0.06	1.6 \pm 0.05	3.6 \pm 0.09*	2.6 \pm 0.13	4.6 \pm 0.16*	2.6 \pm 0.09	5.7 \pm 0.02*	2.0 \pm 0.06	15 \pm 2	44 \pm 5	48 \pm 4	22 \pm 3
MDR-TB	Untreated	1.1 \pm 0.03	0.9 \pm 0.02	1.0 \pm 0.03	1.4 \pm 0.04	1.7 \pm 0.04	2.0 \pm 0.04	0.8 \pm 0.02	1.6 \pm 0.03	18 \pm 2	34 \pm 3	44 \pm 4	27 \pm 3
	DNA-hsp65	1.6 \pm 0.04	1.2 \pm 0.03	7.9 \pm 0.01*	0.6 \pm 0.02*	8.2 \pm 0.16*	0.4 \pm 0.02*	13.4 \pm 0.24*	0.1 \pm 0.01*	15 \pm 3	76 \pm 5*	49 \pm 4	13 \pm 1*
	DNAv	1.4 \pm 0.02	2.4 \pm 0.06	1.9 \pm 0.05	1.6 \pm 0.04	0.9 \pm 0.03	1.2 \pm 0.03	0.7 \pm 0.03	0.9 \pm 0.02	13 \pm 2	47 \pm 3	52 \pm 4	26 \pm 4
	BCG	1.5 \pm 0.04	1.5 \pm 0.04	1.7 \pm 0.06	1.7 \pm 0.05	2.3 \pm 0.07	1.4 \pm 0.05	3.1 \pm 0.10*	1.6 \pm 0.06	15 \pm 2	37 \pm 4	44 \pm 6	25 \pm 4
	Drug	1.2 \pm 0.02	1.8 \pm 0.05	0.9 \pm 0.01	1.9 \pm 0.06	1.3 \pm 0.03	1.6 \pm 0.05	0.7 \pm 0.02	1.3 \pm 0.02	12 \pm 2	31 \pm 1	60 \pm 4	31 \pm 3
	Drug+DNA-hsp65	1.3 \pm 0.02	1.4 \pm 0.03	3.6 \pm 0.09*	0.7 \pm 0.02*	6.3 \pm 0.21*	0.6 \pm 0.02*	4.6 \pm 0.13*	0.4 \pm 0.02*	16 \pm 2	71 \pm 3*	39 \pm 4	9 \pm 2*
	Drug+DNAv	1.3 \pm 0.04	2.0 \pm 0.08	1.1 \pm 0.03	1.6 \pm 0.04	1.1 \pm 0.03	1.5 \pm 0.04	1.0 \pm 0.03	1.5 \pm 0.04	17 \pm 3	34 \pm 2	48 \pm 3	19 \pm 2
	Drug+BCG	1.3 \pm 0.06	1.2 \pm 0.03	2.3 \pm 0.07	1.7 \pm 0.09	3.7 \pm 0.13*	1.0 \pm 0.08	4.0 \pm 0.21*	1.2 \pm 0.04	20 \pm 3	53 \pm 4	56 \pm 5	21 \pm 4

^{a,b}BALB/c mice were infected with *M. tuberculosis* and treated as described in Table 1.

^cLymphocytes were obtained from the pooled inguinal and mesenteric lymph nodes. Plastic- and nylon-adherent cells were removed and the frequencies of cells producing IFN- γ (type-1 phenotype) or producing IL-4 (type-2 phenotype) in the presence of PMA (10 ng/ml) and monoclonal antibody against CD3 (YCD3-1, 50 ng/ml; Gibco-BRL) were determined by limiting dilution ELISPOT analysis.¹⁹

^dLymph node T cells were stained with a fluorescent isothiocyanate (FITC)-labelled mAb specific for CD44 or CD62L (Pharmingen).¹⁹ Cells were fixed in 2% paraformaldehyde and analysed in FACScan (Becton Dickinson, San José, CA, USA) by using gate analysis for lymphocytes comparing the intensity of fluorescence of cells from treated groups with that of control cells incubated with an unrelated and isotype-matched antibody. Asterisks indicate statistically significant differences for IFN- γ and IL-4 producing T-cell frequency from treated mice in relation to untreated mice (Student's *t*-tests, $P < 0.001$). These data are from a typical experiment ($n = 5$) repeated 3 times with similar results.

Traditionally, the protection against TB has been regarded as due to phagocytosis and killing of *M. tuberculosis* by immunologically activated macrophages or cytotoxic T lymphocytes.^{10,15,16} This is a result of a type-1 cellular response in which IFN- γ is produced by antigen-specific T lymphocytes, as distinct from a type-2 response, in which T cells produce IL-4.¹⁶ IFN- γ is the main macrophage-activating factor, and it has been shown to be essential for protection.^{9,10,22} To test if the therapeutic treatment had modified T-cell priming, we estimated the frequencies of T cells that typified type-1 or type-2 immune responses in the lymph nodes of treated and untreated mice. DNA-hsp65 combined with drug therapy eliminates *M. tuberculosis* bacilli by establishing a cellular immune response that is dominated by antigen-specific T lymphocytes where both produce IFN- γ (Table 2) and are cytotoxic towards infected cells (data not shown). The same pattern of response was observed regarding the antimycobacterial effect of cytotoxicity induced by bulk lymph node T cells from these animals (data not shown). Both functions are probably required to maximize effective antimycobacterial immunity in mice and in humans.² In contrast, antigen-specific T cells that produce IL-4 (Table 2) and are not cytotoxic are abundant in control groups and during groups treated

with drugs only. Such type-2 responses do not contribute to complete the elimination of bacilli and so a shift in the balance towards type-1 responses by DNA-hsp65 might be beneficial. Moreover, the memory/activated T cells (expression of CD44 and CD62L, respectively), which mediate the long-lived host response against TB, were characterized in mice infected with *M. tuberculosis* which were treated further with a different schedule of treatment. The therapeutic activity of DNA-hsp65 alone or in combination with drug treatment was associated with the presence of lymph node T-cell populations in which a high expression of CD44 and a low expression of CD62L were prominent even after 6 months from the beginning of treatment; whereas in control groups or after treatment with drugs alone the majority of T cells presented a low expression of CD44 and a high expression of CD62L (Table 2).

Upon and during infection, the first cell type generally encountered by the tubercle bacillus is the macrophage. The macrophage response against *M. tuberculosis* is of primary importance in host defense. However, *M. tuberculosis* is extremely well adapted to the hostile environment within the macrophage and can survive and replicate where other pathogens perish.¹⁵ Moreover, macrophages actively participate as antigen-presenting

Table 3 Modulation of adhesion, activation and signaling markers of pulmonary macrophages during development of TB as well as under treatment with DNA-hsp65 and/or drugs^a

Macrophage mediators	Time after treatment	Not infected	Infected with H37Rv and treated with:				
			Not treated	DNA-hsp65	DNAv	Drug	Drug+DNA-hsp65
IL-10 (pg/ml)	Time 0	612 ± 29	1480 ± 125	935 ± 88	1130 ± 141	1280 ± 91	1090 ± 115
	3 months	540 ± 32	1765 ± 142	2359 ± 142	1271 ± 76	977 ± 69	1895 ± 25
	6 months	390 ± 19	1912 ± 164	2418 ± 139	1248 ± 89	918 ± 77	2328 ± 31
IL-12 (pg/ml)	Time 0	123 ± 15	870 ± 45	914 ± 56	776 ± 67	748 ± 63	825 ± 55
	3 months	242 ± 22	238 ± 23	3350 ± 214*	824 ± 58	943 ± 70	3571 ± 228*
	6 months	178 ± 17	188 ± 34	3890 ± 202*	692 ± 64	868 ± 68	3214 ± 214*
MHC I (MFI)	Time 0	40 ± 2	38 ± 2	34 ± 1	34 ± 3	51 ± 3	48 ± 3
	3 months	44 ± 3	20 ± 1	66 ± 4*	43 ± 2	62 ± 4	173 ± 6*
	6 months	43 ± 5	17 ± 1	181 ± 6*	64 ± 5	75 ± 3	197 ± 8*
MHC II (MFI)	Time 0	38 ± 4	40 ± 4	43 ± 2	44 ± 3	50 ± 2	51 ± 5
	3 months	44 ± 2	22 ± 2	123 ± 7*	58 ± 4	68 ± 6	212 ± 10*
	6 months	46 ± 5	25 ± 2	175 ± 8*	61 ± 2	74 ± 5	285 ± 11*
CD18 (MFI)	Time 0	55 ± 3	89 ± 3	94 ± 5	77 ± 5	68 ± 3	76 ± 4
	3 months	50 ± 4	68 ± 3	128 ± 8*	65 ± 3	60 ± 2	165 ± 9*
	6 months	51 ± 4	62 ± 2	186 ± 4*	54 ± 4	74 ± 5	219 ± 13*
CD86 (MFI)	Time 0	62 ± 5	53 ± 4	51 ± 2	48 ± 2	84 ± 5	60 ± 3
	3 months	68 ± 4	28 ± 3	188 ± 7*	51 ± 3	93 ± 3	190 ± 12*
	6 months	57 ± 3	24 ± 1	194 ± 8*	54 ± 5	111 ± 7*	293 ± 9*
NO $\mu\text{M}/10^5$ cells	Time 0	24 ± 3	28 ± 5	32 ± 4	28 ± 3	25 ± 3	23 ± 2
	3 months	28 ± 4	31 ± 4	87 ± 5*	42 ± 4	65 ± 4	114 ± 10*
	6 months	17 ± 3	21 ± 4	76 ± 6*	32 ± 3	58 ± 3	157 ± 12*

^aBulk bronchoalveolar lavage (BAL) macrophages were stimulated with PMA (10^{-7} M, Sigma) and lipopolysaccharide (LPS from *Escherichia coli*, 50 ng/ml, Sigma) in RPMI 1640 medium containing 2 mM L-glutamine, 1% non-essential amino acids, 1% sodium pyruvate, gentamicin and penicillin-streptomycin as antibiotics (complete medium; Gibco BRL) and 5% FCS (Sigma). Results for IL-10 and IL-12 were determined by ELISA (pg/ml).²⁰ Expression of surface markers such as MHC I, MHC II, CD18 and CD86 were given as median fluorescence intensity (MFI) by FACScan analysis. The cells were stained by the same procedure described in Table 2. No production was evaluated by the method of Greiss. Asterisks indicate statistically significant differences for adhesion, activation and signaling markers of pulmonary macrophages from treated mice in relation to untreated mice (Student's *t*-tests, $P < 0.001$). These data are from a typical experiment ($n = 5$) repeated 3 times with similar results.

cells and are also potent effectors of inflammation because of their capacity to secrete a wide array of chemical mediators.²³ The characterization of these mediators are extremely important to better understand the role of macrophages in the initiation and modulation of the immune response in TB under treatment with DNA-hsp65 and drugs. Macrophages from the bronchoalveolar lavage fluid (BAL) of animals which were previously infected and after infection treated with DNA-hsp65 or with the combination of DNA plus drugs synthesize high levels of IL-12 and MHC class I and class II molecules that could enhance both innate (natural killer cells) and acquired (B and T cells) immunity (Table 3). These BAL macrophages also produce nitric oxide (NO) in high quantity and express CD18, CD16/32 and CD86 (Table 3) which are accessory molecules that interact with receptors on T cells to enhance adhesion, activation and signaling (costimulation).^{24–26} All these properties (MHC expression, secretion of IL-12 and the expression of costimulatory and activation molecules) are regulated after exposure to DNA-hsp65, before or after infection with *M. tuberculosis*. Thus, we suggest that BAL macrophages under DNA-hsp65 or DNA-hsp65 plus drugs can capture and process antigens, display larger amounts of MHC-peptide complexes at their surface and regulate their costimulatory molecules, and migrate from inflammatory tissue to lymph nodes for the induction of immunity. In the presence of BAL macrophages and of the IL-12 they produce, these T cells turn into IFN- γ -producing Th1 cells,²⁶ permitting a better elimination of pathogen and avoiding secondary reinfection. On the contrary, we demonstrated that BAL macrophages from infected animals or those from infected and treated only with drugs are more suppressive, correlated with a persistent inflammatory reaction without resolution of infection, and expressed lower levels of CD16/32, CD86 and MHC class I and class II molecules. The macrophages from these animals lack the IL-12, costimulatory and MHC class I and class II molecules that are essential to drive clonal expansion of the T cell, the production of cytokines, and development of killer cells.²⁶

In recent years, the elucidation of cellular and molecular components and the use of new experimental approaches have started to reveal the underlying working principles of the immune system.^{27,28} Consequently, it is now possible to identify new areas and new strategies for intervention.²⁹ Likewise, we demonstrated in this study that the new combination approach using immunotherapy and chemotherapy should offer several improvements over the existing regimens to treat TB. It was possible to shorten the total duration of effective treatment and/or significantly reduce the total number of doses needed to be taken under DOTS supervision. Moreover, it improves the treatment of MDR-TB, which cannot be treated with isoniazid and rifampicin, and/or provide a more effective treatment of latent TB infection, which is essential to eliminate TB. Thus, the effect of chemotherapy and immunotherapy alone or simultaneously could be assessed in humans.^{30,31}

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