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T-helper 1-like subset selection in *Mycobacterium bovis* bacillus Calmette–Guérin-infected resistant and susceptible mice

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SUMMARY

The *Bcg* gene has been shown to control natural resistance of mice to intravenous infection with low doses of *Mycobacterium bovis* (bacillus Calmette–Guérin; BCG). In the present study, we evaluated the impact of the *Bcg* gene on the development of T-cell reactivity during the early stages of infection. Congenic strains of mice, bearing 'r' and 's' alleles of the *Bcg* gene on B10.A and BALB/c backgrounds, were studied at different time-points for 2 weeks after infection. The *in vitro* proliferative response of spleen cells, induced by mycobacteria or concanavalin A, was depressed in the Bcg^s mice compared to the Bcg^r congenic mice 14 days after infection with 10^5 colony-forming units (CFU) of BCG. Polymerase chain reaction (PCR)-based methodology was used to compare the level of lymphokine gene expression in the spleens of infected congenic mice both *ex vivo* and after *in vitro* stimulation. In both cases, preferential expression of interferon-γ (IFN-γ), lymphotoxin, interleukin-2 (IL-2) and IL-2 receptor genes was observed. The lymphokine gene expression profiles indicated that T lymphocytes activated in the course of the BCG infection preferentially expressed the T-helper 1-specific pattern, irrespective of the allele of the *Bcg* gene. We showed that this bias in T-cell differentiation could not be attributed to either down-regulation of IL-4 gene expression or modulation of the macrophage co-stimulatory activity by live *M. bovis* BCG. We conclude that the mechanism of phenotypic expression of the *Bcg* gene resides in the differential ability of macrophages to be activated by lymphokines produced by protective T cells, rather than in the lack of these lymphokines in susceptible animals.

INTRODUCTION

Host interactions with infectious agents are characterized by the involvement of different immune effector mechanisms, which could be implicated both in the elimination of pathogens and in disease progression. T lymphocytes play a key regulatory role in the development and expression of these mechanisms through lymphokine production. The role of T cells in host resistance to infection could be correlated with the pattern of lymphokines, secreted by pathogen-specific T lymphocytes.

Since the T-helper 1 (Th1) and Th2 $CD4^+$ clones were described initially, many approaches have been used to demonstrate that the restricted pattern of lymphokine secretion by parasite-specific T cells is responsible for disease susceptibility/resistance.[1] In most cases, the development of the Th1-like subset, distinguished by the secretion of interleukin-2 (IL-2) and interferon-γ (IFN-γ), conferred resistance to intracellular pathogens (*Listeria major*, *Mycobacterium leprae*).[2,3] It is conceivable that the protective potential of certain sets of lymphokines would depend on their ability to augment or suppress effector reactions crucial for the survival and multiplication of particular pathogens.

The aim of the present study was to determine whether resistance/susceptibility to bacillus Calmette–Guérin (BCG) infection, conferred by the *Bcg* gene, correlates with the lymphokine spectrum expressed by the activated T cells and whether the quality of the macrophages, determined by the pleiotropic effects of the *Bcg* gene, contributes to the activation of the T cells, expressing a particular lymphokine profile. The *Bcg* gene is a single autosomal gene which controls the innate resistance of mice to several species of *Mycobacteria*.[4] This gene is identical or very closely linked to two other host resistance genes which control resistance to infection with *Salmonella typhymurium* (*Ity*) and *Leishmania donovani* (*Lsh*).[5] Recently, the most likely candidate for the *Bcg* gene has been cloned.[6] Interestingly, the gene (*Nramp1*) was found to be expressed exclusively in macrophages. The *Bcg* gene acts very early in the host response to infection, at the time of the initiation of the immune response, and is phenotypically expressed as an intrinsic ability of macrophages to control the growth of intracellular pathogens.[6,7] Recent reports have shown that Bcg^r macrophages are better antigen-presenting cells (APC) than their Bcg^s counterparts.[8,9] Considering the tentative role of APC

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Abbreviations: Con A, concanavalin A; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PPD, purified protein derivative; RT, reverse transcription.

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in T-lymphocyte subset activation, it seemed plausible that the contribution of innate resistance, determined by the *Bcg* gene, to the development of immune reactivity would be displayed at the lymphokine gene expression level, early in the course of the BCG infection.

MATERIALS AND METHODS

Mice

B10.A (*Bcg*^{s}) and A/J (*Bcg*^{r}) mice were obtained from the National Cancer Institute, Frederick, MD. Congenic B10.A. *Bcg*^{r} mice were constructed as described elsewhere[10] and maintained in our animal facility under specific pathogen-free (SPF) conditions. CD.2 (BALB/c.*Bcg*^{r}) mice were obtained as described elsewhere.[11] Both CD.2 and BALB/c mice were bred and maintained under SPF conditions in the animal facility of the Montreal General Hospital Research Institute.

Infection of mice

Mycobacterium bovis BCG (strain Montreal) was grown in Dubos liquid medium at 37° for 2 weeks before infection, filtered through a 5-μm filter (Millipore, Mississauga, Canada) to remove aggregates, and colony-forming units (CFU) of viable bacteria were determined as described in detail previously.[12] The BCG organisms were adjusted to the desired concentration in phosphate-buffered saline (PBS; Ca^{2+} and Mg^{2+} free) and injected in a volume of 0·2 ml into the lateral tail vein.

Reagents and antibodies

Affinity-purified goat anti-mouse IgG (H + L) were purchased from Cedarlane (Hornby, Canada). Protein G-purified anti-CD3 monoclonal antibodies (clone 145-2C11) were a kind gift of Dr Owens (McGill University, Montreal, Quebec, Canada). Purified protein derivative (PPD) was obtained from Statens (Seruminstitute, Copenhagen, Denmark). Live *M. bovis* (BCG) strain Montreal was obtained from the Institute Armand-Frappier, Laval, Quebec, Canada. Ten micrograms of frozen stock of *M. bovis* BCG used for the *in vitro* stimulation contained $0{\cdot}8 \times 10^5$ CFU of the bacteria. Concanavalin A (Con A) was purchased from Pharmacia (Uppsala, Sweden) and used at a final concentration of 2·5 μg/ml.

Cells

Single-cell suspensions were prepared from the spleens of infected mice. Erythrocytes were lysed with a Tris-buffered solution of ammonium chloride (0·15 M), washed twice with Dulbecco's modified minimal essential medium (DMEM), containing 1% fetal calf serum (FCS), 20 mM HEPES and 100 μg/ml gentamycin, and resuspended in complete RPMI-1640 medium, containing 5% fetal bovine serum (FBS; HyClone, Logan, UT), 4 mM glutamine, 50 μg/ml gentamycin, 10 mM HEPES buffer, 1 mM sodium pyruvate and 1 × non-essential amino acid concentrate (all components Gibco-BRL, Grand Island, NY). Unseparated cell suspensions were stimulated at a concentration of $2{\cdot}5 \times 10^6$ cells per ml in 96-well flat-bottomed plates (Nunc, Roskilde, Denmark).

Cell separations

Spleen cells were cultivated on plastic Petri dishes at concentration of 5×10^6 cells/ml for 2 hr, at 37° in a CO_2 incubator. Plastic non-adherent cells were enriched for T lymphocytes by passage through a nylon-wool column (i.e. a 10-ml syringe containing 0·7 g of nylon wool; PolyScience, Niles, IL). After incubation of the column for 1 hr at 37°, nylon-wool non-adherent cells were eluted with 10 ml of prewarmed medium. These cells were treated with anti-Ia alloantiserum (Cedarlane), washed and the remaining Ia-positive and surface Ig-positive cells were removed by incubation on plastic Petri dishes coated with affinity-purified rabbit anti-mouse Ig antibodies (10 μg/ml). A panning procedure was repeated twice for 45 min at room temperature. Purified T lymphocytes were cultured at $2{\cdot}5 \times 10^5$ cells per well and did not respond to stimulation with anti-CD3 antibodies or mycobacterial antigens in the absence of APC. Plastic adherent cells obtained after a 2-hr incubation of spleen cell suspensions were washed extensively (six to seven times) with Ca^{2+}-free PBS and incubated for 15 min at room temperature in PBS containing EDTA (1 mg/ml). The cells were then detached by pipetting, washed twice and added to purified T lymphocytes at 10^4–10^5 cells/well.

Proliferation assay

Antigens or mitogens were added at the initiation of culture on the day of the cell preparation. After 40–48 hr, cultures were pulsed with [^{3}H]thymidine (specific activity 6·7 Ci/mM; ICN, Mississauga, Canada) at 0·5 μCi per well. Cells were harvested onto filter mats (Skatron, Sterling, VA) after 16 hr and the radioactivity was counted in a liquid scintillation β-counter (Beckman, Mississauga, Canada). Each culture was performed in triplicate. Spleen cells of two to three infected mice of each strain were processed separately and the statistical significance of differences in the proliferative response between mouse strains was calculated according to the Student's test.

Macrophage cell lines

C.D2Mϕ and BALBMϕ macrophage cell lines were established from the bone marrow of C.D2 (*Bcg*^{r}) and BALB/c (*Bcg*^{s}) mice, respectively, by transformation with J2 retrovirus, as previously described in detail.[12]

Lymphokine-containing supernatant

Spleen cells of intact BALB/c mice (5×10^6 per ml) were stimulated with Con A (2 μg/ml) and the supernatant was collected after 36 hr of incubation. After absorption of residual Con A with Sephadex G-25 (0·2 g/ml), the supernatant was sterilized by filtration and stored at −70° until analysis.

Analysis of lymphokine gene expression

A detailed description of the semi-quantitative analysis of lymphokine mRNA expression profiles based on reverse transcription-polymerase chain reaction (RT-PCR) is provided elsewhere.[13] In brief, the cells obtained from two wells in each group were pooled together and solubilized in 100 μl of guanidine isothiocyanate solution containing 0·04 pg of standard template (described below). Total cellular RNA was prepared by a single-step acidic phenol extraction, as previously described.[13] The total amount of RNA was transcribed into single-stranded complementary DNA using random priming and 200 U per sample of Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Gibco-BRL) in final volume of 20 μl (39°, 1 hr). Reverse transcriptase was then heat-inactivated (95°) and diluted with 30 μl of water. Four microlitres of the final solution was used for PCR amplification, using 0·5–2·0 μM

Table 1. Proliferative responses to mycobacterial antigens of cells

Mouse strain	*Bcg* allele	Antigen	Proliferation (c.p.m.)
B10.A-*Bcg*^{r}	*r*	BCG	14,300 (2500)*
		PPD	7300 (1100)
		—	1230 (210)
B10.A	*s*	BCG	450 (85)
		PPD	320 (80)
		—	960 (180)
CD.2	*r*	BCG	18,000 (2800)
		PPD	24,000 (4500)
		—	1780 (240)
BALB/c	*s*	BCG	6500 (2100)
		PPD	3200 (1600)
		—	1460 (210)

Bcg-congenic mice were infected intravenously with 10^5 CFU of live *M. bovis* BCG. Spleen cells were obtained 14–15 days after infection and stimulated with BCG (10 μg/ml, wet weight) or PPD (10 μg/ml) at a concentration of $2{\cdot}5 \times 10^6$ cells/ml. After 48 hr, the cultures were pulsed with [^{3}H]thymidine and harvested 16 hr later.

* SD in parentheses.

concentration of primers and 1 U of Taq polymerase (Biocan, Mississauga, Canada) per sample. Primer pairs specific for IL-2, IL-4, IFN-γ, tumour necrosis factor-β (TNF-β; lymphotoxin) and IL-2 receptor (IL-2R) mRNA were purchased from Clontech (Palo Alto, CA); CD3-γ chain-specific primers were prepared at Sheldon Biotechnology Centre (Montreal, Quebec, Canada). For the quantification of relative mRNA levels, PCR products were labelled with ^{32}P-dCTP (ICN; specific activity 10 mCi/ml) present in the PCR reaction mixture (1–2 μCi per sample). After 25 cycles of amplification (94°, 45 seconds; 65°, 30 seconds; 72°, 60 seconds), ^{32}P-labelled PCR products were separated by PAGE electrophoresis (4·2% of polyacrylamide). Fixed and dried gels were then exposed to X-ray films (Kodak, Rochester, NY). The amplification of cellular CD3 and IFN-γ mRNA was performed simultaneously with standard templates, which represent respective PCR products subcloned in pGEM 3 plasmid (Promega, Southampton, U.K.). The length of subcloned sequences was modified either by the insertion of a 123 bp DNA piece (CD3-M) or the deletion of 51 bp (IFN-M). Standard templates were prepared by transcription of subcloned and modified CD3- and IFN-γ-specific sequences using T7 RNA polymerase. The variability of the standard band density did not exceed 15% within one experiment. The integrated optical density of the bands was measured on SciScan 5000 (USB, Cleveland, OH) and the relative values of particular lymphokine gene expression were calculated as a percentage of the standard band. Each determination of the lymphokine gene expression profile was repeated two to three times, and the results were consistent and reproducible.

RESULTS

Proliferative responses of splenic T lymphocytes to mycobacterial antigens as a function of the *Bcg* gene

Spleen cells were obtained from mice of the *Bcg*-congenic pairs constructed on both B10.A and BALB/c genetic backgrounds,

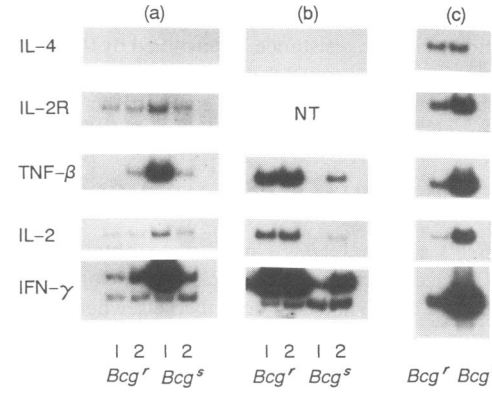

Figure 1. mRNA expression in bulk cultures of cells, obtained from B10.A and B10.A-*Bcg*^{r} mice infected with 10^5 CFU of BCG 2 weeks prior to *in vitro* stimulation. Cells ($2{\cdot}5 \times 10^6$/ml) were stimulated with BCG (lanes 1) and PPD (lanes 2) for 24 hr (a) and 72 hr (b) in a 96-well flat-bottomed plate. Cells from two wells per group were collected, pooled and lysed in guanidine isothiocyanate (GTC) solution, containing 0·04 pg of IFN-γ-specific control template (see the Materials and Methods) per sample. RNA was purified and amplified by RT-PCR, as described. (c) Control, the same cells stimulated with Con A (2·5 μg/ml) for 24 hr. NT, not tested.

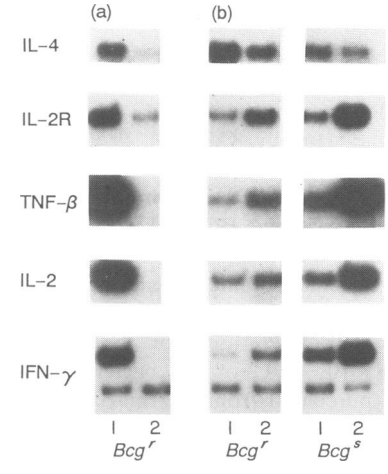

Figure 2. mRNA expression in cells, obtained from CD.2 and BALB/c mice infected with BCG 2 weeks before *in vitro* stimulation. (a) Cells were obtained from CD.2 (*Bcg*^{r}) mice infected with 10^6 CFU of BCG (Montreal). Lane 1, stimulation with PPD for 48 hr (10 μg/ml); lane 2, non-stimulated cells after 48 hr *in vitro*. (b) Cells were obtained from CD.2 (*Bcg*^{r}) and BALB/c (*Bcg*^{s}) infected with 10^5 CFU of BCG. Lane 1, stimulation with PPD for 48 hr; lanes 2, stimulation with *M. bovis* BCG (10 μg/ml) for 48 hr.

which were infected with 10^5 CFU of *M. bovis* BCG. The *Bcg*^{r} strains of both genotypes displayed normal reactivity, whereas the responses of *Bcg*^{s} mice were depressed (Table 1). The degree of T-cell hyporesponsiveness was consistently more pronounced in *Bcg*^{s} mice of the B10.A background and, in some experiments, reached the level of complete proliferative unresponsiveness. Complete unresponsiveness, either to Con A or to BCG and PPD, was never observed in infected *Bcg*^{s} mice of the BALB/c background.

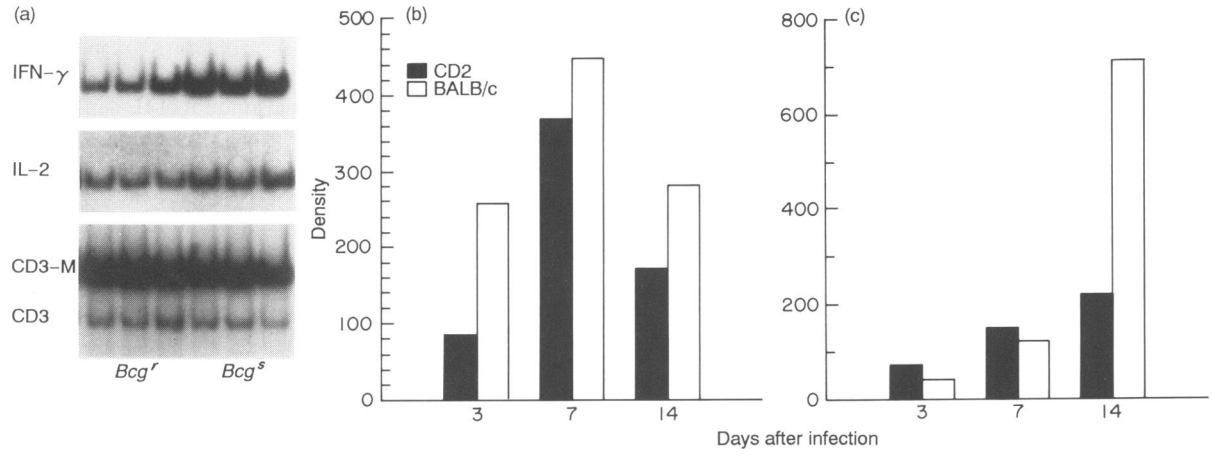

Figure 3. Expression of IL-2 and IFN-γ mRNA in the spleens of *Bcg* congenic mice *in vivo*. (a) Expression of IL-2 and IFN-γ mRNA 14 days after infection. CD.2 and BALB/c mice were infected intravenously with 10^5 CFU of *M. bovis* BCG. 0.5 μg of purified splenic RNA obtained from an individual mouse (three mice per group) was analysed by RT-PCR. (b) The dynamics of IL-2 mRNA expression. Total RNA was prepared from cells 3, 7 and 14 days after infection with *M. bovis* BCG (three mice per group) and analysed by RT-PCR with IL-2-specific primers. Optical density of the bands was measured on USB SciScan 5000. The values of optical density represent the average of three independent measurements in three individual mice. (c) The dynamics of IFN-γ mRNA expression. The RNA samples were prepared and analysed as in (b).

Table 2. Proliferative responses of BCG-reactive T lymphocytes after restimulation *in vitro* with BCG- or CD3-specific antibodies

Stimulation	CD-2	BALB/c
—	160	120
BCG alone	230	145
Anti-CD3 alone	85	80
Spleen cells alone	4650	1260
Spleen cells + BCG	18,200	3600
Spleen cells + anti-CD3	11,200	2850

Spleen cells obtained from *M. bovis* BCG-infected CD.2 and BALB/c mice were stimulated with BCG *in vitro* for 72 hr. Blast cells were purified by gradient centrifugation and after a 7-day rest period were restimulated with BCG (10 μg/ml) or plastic-bound CD3-specific antibodies (200 pg/well) at a concentration of 5×10^4 cells per well. Spleen cells obtained from naive syngeneic mice were depleted of T lymphocytes by Thy-1-specific antibody plus complement treatment and used as a source of accessory cells at a concentration of 5×10^5 cells/well. [^{3}H]thymidine (1 μCi/well) was added after 42 hr for an additional 8 hr. The numbers represent proliferative responses in counts per minute (c.p.m). Standard errors in triplicate did not exceed 15%.

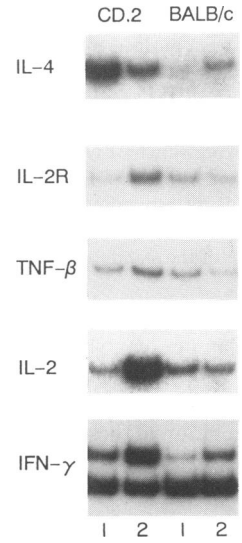

Figure 4. Analysis of lymphokine gene expression of BCG-reactive T cells after repeated stimulation *in vitro*. Cells of CD.2 (*Bcg*^{r}) and BALB/c (*Bcg*^{s}) mice infected with 10^5 CFU of BCG were obtained 2 weeks after infection and stimulated with BCG for 3 days. Viable cells were purified from the cultures by gradient centrifugation and cultured in the presence of mitomycin C-treated syngeneic spleen cells and 10% of lymphokine-containing supernatant for 6–8 days and viable cells (5×10^4 per well) were restimulated with BCG (10 μg/ml) or anti-CD3 antibody absorbed to plastic (100 ng/well) in the presence of syngeneic feeder cells. Lane 1, restimulation with anti-CD3 monoclonal antibodies; lane 2, restimulation with BCG. Total RNA was prepared after 48 hr and lymphokine mRNA expression was determined by RT-PCR technique.

The effect of the *Bcg* gene on lymphokine mRNA expression

The observed depression of the proliferative response in *Bcg*^{s} mice could result from: (1) the lack of functionally active responding T cells; (2) the lack of T-cell activation due to insufficient expression of ligands for T-cell receptor (TcR) on the surface of APC; and (3) a block of the proliferation of activated T cells. To assess these possibilities, we used semi-quantitative RT-PCR to study the mRNA expression of the genes specifically associated with T-cell activation in the spleen cell populations, obtained from the resistant and susceptible congenic mice, 2 weeks after infection.[13]

B10.A genetic background. Spleen cells freshly obtained from infected B10.A mice expressed discernible amounts of

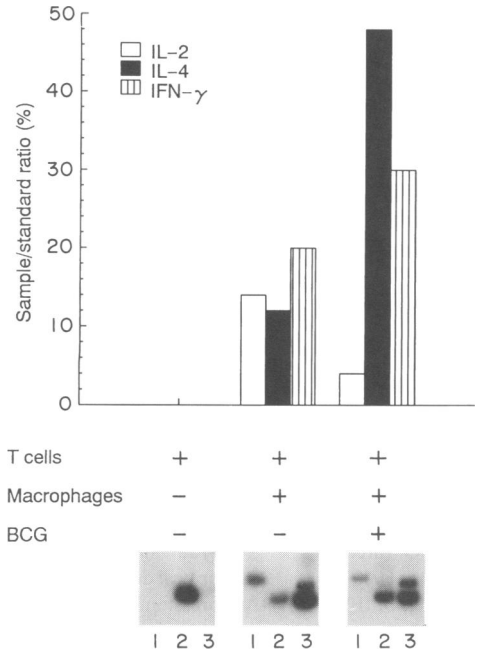

Figure 5. BCG infection of macrophage cell lines increases their co-stimulatory activity for T-lymphocyte proliferation and gene expression. T lymphocytes were purified from s of non-infected BALB/c mice as described in the Materials and Methods, and stimulated ($2{\cdot}5 \times 10^5$ per well) with anti-CD3 antibodies absorbed to plastic (100 ng/well). Cells of a macrophage cell line (CD.2Mϕ) were added at 5×10^3 per well. mRNA expression by T lymphocytes was determined by RT-PCR after stimulation with anti-CD3 antibodies for 48 hr. The optical density of the bands corresponding to IL-2 (lanes 1), IL-4 (lane 2) and IFN-γ (lane 3) mRNA was measured on USB SciScan 5000 and expressed as a percentage of the standard band.

IL-2, IL-2R, IFN-γ and TNF-β mRNA,which is characteristic of the activation of inflammatory-type T cells (data not shown). The expression of these lymphokines *in vitro* decreased by 48 hr in the absence of antigenic or mitogenic stimulation. As shown in Fig. 1a, *in vitro* stimulation of these cells with BCG, as well as with PPD, induced expression of IL-2, IL-2R, TNF-β and IFN-γ within 24 hr in the splenocytes of both the resistant and susceptible mice. No expression of IL-4 mRNA was detected in any case. It is noteworthy that, despite severe suppression of proliferative responses in the *Bcg*^{s} mice *in vitro*, their lymphocytes, stimulated with BCG, expressed higher levels of lymphokine mRNA, with IFN-γ and TNF-β being the most prominent. Figure 1b shows mRNA profiles of the same cells after 72 hr of stimulation. While the expression of IL-2, IFN-γ and TNF-β genes increased between days 1 and 3 of stimulation in cultures of spleen cell of resistant mice, it dropped significantly in BCG-stimulated *Bcg*^{s} splenocytes. Stimulation of *Bcg*^{s} lymphocytes with PPD (Fig. 1, lane 4) caused a moderate increase in IFN-γ and TNF-β gene expression in this time period, while proliferative responses of these cells were depressed (Table 1). IL-4 expression was not detected in any of the cultures.

To check whether the observed pattern of lymphokine gene expression was specific for stimulation with mycobacterial antigens, we studied mRNA expression of the same cells stimulated with Con A for 24 hr (Fig. 1c). Both *Bcg*^{r} and *Bcg*^{s} splenocytes stimulated with Con A expressed IL-2, IL-4, IFN-γ, TNF-β and IL-2R mRNA. As in the BCG-stimulated cultures, the expression of IL-2, IL-2R, IFN-γ and TNF-β was higher in susceptible mice, although their proliferative response to mitogen was dramatically depressed (data not shown). The emergence of an approximately equal amount of IL-4 mRNA in both 'r' and 's' splenocytes was detected after mitogen stimulation. Therefore, the lack of IL-4 expression by mycobacteria-reactive lymphocytes cannot be ascribed to non-specific negative regulation of this gene by micro-environmental influences in infected organs, but rather depends on the mode of T-lymphocyte activation.

BALB/c genetic background. Splenocytes, obtained from infected, susceptible (*Bcg*^{s}) BALB/c mice expressed higher levels of lymphokine mRNA than their *Bcg*^{r} congenic counterparts, infected with the same dose of BCG (Fig. 2b). At this point, the bacterial burden in the spleens of *Bcg*^{s} mice was at least 10 times or more higher than that observed in the spleens of *Bcg*^{r} mice (data not shown). To test whether a higher antigenic load in susceptible mice could account for the observed differences, spleen cells from CD.2 mice infected with a 10 times higher BCG dose (10^6 CFU) were tested in parallel (Fig. 2a). In fact, the expression of IL-2, IL-2R, TNF-β and IFN-γ mRNA in these mice increased and reached the levels observed in susceptible mice. It is noteworthy that the increase of the BCG load in this case resulted in predominant activation of Th1-specific lymphokines. Thus, the comparison of lymphokine expression patterns in *Bcg* congenic mice on BALB/c background revealed similar profiles of lymphokine gene expression in both resistant and susceptible mice.

Analysis of lymphokine gene expression *in vivo*

Total RNA was prepared from the spleens of CD.2 (*Bcg*^{r}) and BALB/c (*Bcg*^{s}) mice at 3, 7 and 14 days after infection with 10^5 CFU of *M. bovis* BCG. The levels of IFN-γ and IL-2 mRNA expression were then tested by RT-PCR (Fig. 3). The expression of IL-2 increased by day 7 after infection and then decreased by day 14 in infected organs of both resistant and susceptible mice (Fig. 3b). The expression of IFN-γ mRNA steadily increased during the period of observation (Fig. 3c) in both strains of mice. Nevertheless, the absolute amount of IFN-γ mRNA was significantly higher in the spleens of BALB/c mice 14 days after infection (Fig. 3a). In these mice, the level of IFN-γ mRNA expression increased about 12-fold from day 3 to day 14, whereas in CD.2 mice the increase was only threefold. Interestingly, increased levels of IFN-γ expression *in vivo* correlated with the depression of proliferative responses in *Bcg*^{s} mice after stimulation *in vitro*.

Suppression of lymphocyte proliferative responses in *Bcg*^{s} mice results in inactivation of BCG-reactive T cells

Our data indicated that inflammatory-type immune responses developed early after the BCG infection in both the innately resistant and susceptible mice. The significant difference between susceptible and resistant congenic mice of both genetic backgrounds was observed at the level of proliferative responses, which were supressed in susceptible mice. One could expect that in T-cell populations which have undergone suppression, the Th2 phenotype is preferentially expressed. To test this hypothesis, spleen cells obtained from infected mice were stimulated with BCG *in vitro* for 48 hr. Viable cells were then

recovered using gradient centrifugation. The amount of viable cells obtained from non-suppressed (*Bcg*^{r}) cultures was consistently two- to fivefold higher than from the cultures of splenocytes obtained from susceptible mice. Viable cells were kept in culture in the presence of syngeneic feeder cells and 10% of lymphokine-containing supernatant for 6–7 days. These cells were then recovered by centrifugation on a Lympholyte–M gradient. Equal amounts of cells were stimulated with plastic-bound anti-CD3 antibodies or BCG in the presence of mitomycin C-treated syngeneic splenocytes depleted of T cells. Both stimuli effectively induced the proliferation of CD.2 (*Bcg*^{r}) T lymphocytes, while T cells of BALB/c (*Bcg*^{s}) mice were almost unresponsive (Table 2). Lymphokine mRNA expression was also studied in the same cultures. As shown in Fig. 4, the expression of mRNA of all lymphokines studied was higher in *Bcg*^{r} cells. Thus it appeared that the suppression of antigen-induced proliferation of lymphocytes from BCG-infected, susceptible mice was followed by the development of T-cell unresponsiveness, revealed at the level of both gene expression and proliferation. These results showed that the suppression of the proliferative activity of *Bcg*^{s} T lymphocytes did not result in activation of Th2-specific gene expression, but rather in the inactivation of T lymphocytes.

In contrast to lymphocytes of BALB/c mice, T cells of *Bcg*^{r} congenic mice responded well both to BCG and to anti-CD3 stimulations. The higher proliferative response of these cells to BCG than to anti-CD3 antibodies may reflect an enrichment of the responding cells with mycobacteria-reactive T lymphocytes after the first cycle of *in vitro* stimulation with BCG. In agreement with previous data, these cells, having been restimulated with BCG, preferentially expressed the Th1-specific pattern (Fig. 4, lane 2). Interestingly, the same population stimulated with plastic-bound anti-CD3 antibodies expressed relatively increased IL-4 and decreased IL-2 and IFN-γ levels (Fig. 4, lane 1). Therefore, it appeared that, although the population of BCG-reacting T lymphocytes exhibited preferential induction of IL-2, TNF-β and IFN-γ gene expression in response to BCG stimulation, it retained the functional flexibility and was capable of modulation of the lymphokine profile.

Modulation of macrophage co-stimulatory activity by live *M. bovis* BCG is not responsible for preferential activation of Th1-specific gene expression

To directly address the impact of the BCG infection of macrophages on their co-stimulatory activity, we studied the activation of purified T cells by surface-bound anti-CD3 antibodies. This treatment was reported to induce T cells from unprimed animals to secrete both IL-2 and IL-4.[14,15] In this model the TcR cross-linking (signal 1) was uniformly provided by immobilized ligand-mimicking antibodies, so the T-cell activation was proportional to the accessory signal delivery, provided by added macrophages or macrophage cell lines (see the Materials and Methods). Rigorously purified mouse T lymphocytes did not respond to anti-CD3 stimulation and most of the cells died within 2 days of culture. The addition of $2–10 \times 10^4$ splenic adherent cells or $2–10 \times 10^3$ cells of macrophage cell lines reconstituted the T-lymphocyte proliferative responsiveness (data not shown), thus indicating that macrophages were capable of providing appropriate accessory activity for anti-CD3-stimulated naive T cells. As shown in Fig. 5, after 48 hr of stimulation with anti-CD3 antibodies in the presence of macrophage cell lines, T cells expressed IL-2 and IFN-γ, as well as IL-4 mRNA. Overnight pretreatment of macrophage cell lines with live BCG before adding them to T lymphocytes led to increased expression of both IFN-γ and IL-4 genes by anti-CD3-stimulated T-cells. Essentially similar lymphokine expression patterns were observed upon co-stimulation with both CD.2 and BALB/c macrophage cell lines.

Thus the co-stimulatory activity delivered by macrophages to purified intact T lymphocytes stimulated with plastic-bound anti-CD3 antibodies was adequate to induce expression of both Th1- and Th2-specific lymphokines. Hence, the modulation of co-stimulatory activity of macrophages by their infection with live *M. bovis* BCG was insufficient to account for the selective activation of Th1-like lymphocytes observed in most cases of immunization with BCG.

Furthermore, we found that pre-infection of spleen cells *in vitro* with live BCG, 24 hr before Con A stimulation, did not down-regulate the IL-4 mRNA expression induced by this mitogen (data not shown).

DISCUSSION

The analysis of IL-4 and IL-10 mRNA expression in lepromatosis leprosy lesions indicated that Th2- or Th0-like cells were activated *in vivo*.[16] On the other hand, clones of human PPD-reactive CD4$^+$ T cells selectively produced Th1-specific lymphokines.[17] The present study was aimed at establishing the influence of macrophages on lymphokine gene expression by mycobacteria-reactive T lymphocytes in mice. The results demonstrated preferential activation of cells expressing IFN-γ, lymphotoxin and IL-2 early after infection with live *M. bovis* BCG. This Th1-like pattern developed both in *Bcg*^{r} and *Bcg*^{s} congenic mouse strains. Comparison of *Bcg*-congenic strains of B10.A and BALB/c background revealed substantial differences in lymphokine profiles of mycobacteria-reactive T lymphocytes. Namely, IL-4 mRNA was expressed in BALB/c but not B10.A mice, although IFN-γ, lymphotoxin and IL-2 mRNA expression was predominant in both cases. Obviously this pattern was not controlled by the *Bcg* gene, since the lymphokine gene expression was qualitatively similar in *Bcg*^{r} and *Bcg*^{s} mice either on a B10.A or a BALB/c background.

Both H-2 and non-H-2 genes were shown to influence lymphokine production in mice infected with *Mycobacteria*. The H-2^d haplotype of BALB/c mice was shown to determine the poor IFN-γ response of BALB/c mice to several antigens, including BCG.[18,19] Several peculiarities of mycobacteria-related immune reactivity distinguishing non-H-2 genes of BALB/c and B10 background have also been reported.[20–22] Thus BALB/c mice produced antibodies to many different mycobacterial antigens, whereas B10 mice responded only to a few, irrespective of H-2 haplotype. This phenomenon was reported after BCG vaccination[20] as well as in chronic tuberculosis infection induced by intraperitoneal injection of *M. tuberculosis*.[21] In the latter case, levels of IL-4 mRNA were higher in the spleens of BALB/K (H-2^k)-than in B10.BR (H-2^k)-infected mice, whereas IFN-γ mRNA levels were the same. BALB.K mice, chronically infected with *M. tuberculosis*, also had significantly higher CFU counts in the spleens and lungs compared to B10.BR mice.[22] Since both strains bear the same

alleles of H-2, *Bcg*[11] and *Tbc-1* genes,[23] other, yet unidentified, genes seem to contribute to the differential activation of lymphokine genes in mycobacterial infections and to the control of bacterial multiplication.

The successful attempt to correlate the lymphokine profile with resistance was reported in leprosy patients.[16] IL-2 and IFN-γ mRNA expression was revealed by RT-PCR in tuberculoid leprosy lesions. In contrast, IL-4, IL-5 and IL-10 mRNA predominated in the multibacillary form.[16] CD8$^+$ T-suppressor clones, derived from immunologically unresponsive leprosy patients, produced IL-4 after antigen stimulation.[2] We observed the expression of IL-4 mRNA by mycobacteria-activated cells. Remarkably, this expression was much higher after intravenous than after subcutaneous infection (authors' unpublished observations). The ability of BCG-reactive T lymphocytes to express IL-4 mRNA developed within the first 2 weeks after infection, since almost no IL-4 mRNA was detected when the cells were stimulated with BCG or PPD at 3, 7 and 9 days after infection (data not shown). Recent observations by Huygen *et al.*[19] also demonstrated the production of IL-4 by cells of BCG-infected BALB/c, but not C57BL/6, mice when stimulated with mycobacterial antigens.

Despite the fact that IL-4-expressing cells appear in BALB/c but not in B10.A and B10.A-*Bcg*^{r} mice, the BCG growth is controlled as effectively in CD.2 (*Bcg*^{r}) as in IL-4 non-producing B10.A-*Bcg*^{r} mice. Moreover, BALB/c (*Bcg*^{s}) mice are capable of developing effective mechanisms of bacterial clearance 2–3 weeks after infection. Thus, the expression of IL-4 does not confer disadvantages on BALB/c mice in comparison with B10.A mice over the course of a primary BCG infection. Compared with early detrimental effects of endogenously produced IL-4 in mouse cutaneous leishmaniasis,[1] the balance between Th1 and Th2 subset development was maintained in BCG infection so that the activation of Th2-like cells did not inhibit the development of protective immunity. Therefore, the development of a Th2-like subset may play an essential role in the pathogenesis of only some infections, caused by intracellular parasites such as cutaneous leishmaniasis. In fact, in experimental mouse visceral leishmaniasis caused by *L. donovani*, the susceptibility to infection correlated inversely with the level of Th1 cytokine production (specifically IFN-γ), rather than with Th2 subset activation.[24]

Co-stimulatory activity of specialized APC is considered to provide an important signalling mechanism which contributes substantially to the selection of Th subsets. In fact, cross-linking of TcR in the absence of co-stimulary activity induced anergy[25] or apoptosis[26] in Th1 but not Th2 clones. Optimal proliferation of Th1 clones required accessory activity provided by macrophages, whereas Th2 clone proliferation was supported by B cells.[27] Modulation of co-stimulatory activity of APC with lipopolysaccharide LPS or various lymphokines was shown to be necessary to support the proliferation of Th1 or Th2 clones. However, the role of distinct APC characteristics in shaping restricted lymphokine profiles of unselected naive lymphocytes is poorly understood.

We presumed that changes in co-stimulatory activity of macrophages after infection with *M. bovis* BCG could account for selective Th1 subset activation in this infection. To assess this possibility, we studied the influence of macrophages on the activation of purified naive T lymphocytes by plastic-absorbed anti-CD3 antibodies. In this case signal 1 (TcR triggering) was consistently provided by antibodies, so that activation of T cells would be proportional to accessory activity provided by the added APC population. Purified T cells activated with anti-CD3 antibodies did not express lymphokine genes after 48 hr of stimulation and died in the absence of APC. Expression of IL-2, IL-4 and IFN-γ mRNA was detected after 48 hr of stimulation, provided that splenic adherent cells or macrophage cell lines were added. Infection of macrophage cell lines, exhibiting either *Bcg*^{r} or *Bcg*^{s} phenotype,[12] resulted in the increased expression of both IL-4 and IFN-γ mRNA by T lymphocytes. These results demonstrate for the first time that co-stimulatory activity provided by cells of macrophage origin is sufficient for the activation of T cells expressing lymphokines which are characteristic of both subsets of T cells, in the case of anti-CD3 stimulation. Infection of macrophages with live BCG did not change this pattern, but did induce an increase in their co-stimulatory activity. Therefore, the precise mechanisms of Th1 subset selection are yet to be elucidated.

The data presented in this report also addressed the relationship between the parameters of T-cell activation *in vivo* and *in vitro*. We demonstrated that higher activation of cells in *Bcg*^{s} mice *in vivo* was accompanied by the depression of their proliferative responsiveness *in vitro* compared to the *Bcg*^{r} congenic mice. Therefore, the *in vitro* T-lymphocyte hyporesponsiveness in this case could not be regarded as an indication of the deficiency of T-cell activation. In another report,[28] macrophages obtained from the lung interstitial tissue of mice infected with *M. tuberculosis* H37Rv exhibited extremely potent suppressor activity. Nevertheless, the T lymphocytes purified from the same compartment were able to respond to stimulation with mycobacterial antigens *in vitro*. This observation is in agreement with previous reports of Orme & Collins,[29] which demonstrated the development of protective *Mycobacteria*-reactive T lymphocytes in mice infected with atypical *Mycobacteria*, despite the presence of suppressor cells capable of inhibiting their proliferative responses *in vitro*. Thus, it appears that the cells capable of down-regulating of T-lymphocyte proliferative responses *in vitro* do not necessarily impair the development of protective cells *in vivo*. Moreover, the inhibition of proliferation of the responding T cells could promote their maturation towards an effector phenotype and increase the expression of effector lymphokines. It still remains to be elucidated whether it uncouples mRNA expression and lymphokine production or blocks T-lymphocyte responses to secreted lymphokines.

Overall, the presented data have led us to the following conclusions. Firstly, the suppression of proliferative responses of T lymphocytes *in vitro* is dependent on the innate susceptibility to the BCG infection and is controlled by the *Bcg* gene. Despite suppression of their proliferative responses *in vitro*, splenic T lymphocytes of susceptible mice are functionally activated and able to express Th1-specific lymphokine mRNA both *in vivo* and upon restimulation with mycobacterial antigens *in vitro*. Secondly, the development of BCG infection in both *Bcg*^{r} and *Bcg*^{s} mouse strains results in preferential activation of T lymphocytes expressing a Th1-like functional phenotype. This bias appears to result from the selective activation of Th1-specific genes early after an encounter with live *M. bovis*, rather than from preferential expansion of the Th1 clones. Finally, the lack of balance between the Th1 and Th2 subset activation is not involved in the *Bcg*-gene controlled susceptibility to *M. bovis* BCG infection.

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