Poorer NF-κB signaling by microfilariae in macrophages from BALB/c mice affects their ability to produce cytotoxic levels of nitric oxide to kill microfilariae

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Abstract **Upon activation with microfilariae (mf), macrophages from C57Bl/6 mice showed higher nuclear factor-κB (NF-κB) but lower activating protein 1 DNA-binding activity as compared to BALB/c macrophages. The C57Bl/6 macrophages produced cytotoxic levels of nitric oxide (NO) to kill *Setaria cervi* mf as compared to BALB/c macrophages. Inhibition of the NF-κB signal by pyrrolidine dithiocarbamate (PDTC) blocked NO production and microfilaricidal activity of C57Bl/6 macrophages and inclusion of the exogenous NO generator (SNP) in the PDTC treated C57Bl/6 macrophage cultures induced mf cytotoxicity. These results underscore that the NF-κB signal (induced in response to mf) is important for the NO-mediated microfilaricidal activity of macrophages.**

Keywords: Macrophage; Microfilariae; NF-κB; Nitric oxide

1. Introduction

The host factors play an important role in the outcome of infections [1–3]. This is well reflected in human filariasis, where some individuals are susceptible while the others are resistant residing in the intense transmission areas [4]. The molecular basis of such differential immune responses triggered by the host against the invaded parasites is not clearly understood. Identification of at least some of the host's determinants required for exhibiting natural immunity against pathogens will be of great importance in understanding the mechanism of regulation of filarial infection. Taking these into consideration, in this study we used two well established laboratory strains of mice [5,6], BALB/c (susceptible to microfilariae (mf)) and C57Bl/6 (resistant to mf) to explore possible roles played by the host factors in determining the outcome of filarial infection.

The natural immunity to microfilarial infection in human is mostly governed by the host's factor [7–9] although the molecular nature of such immunity is a contentious issue. The macrophages are shown to play important role in the initiation of innate immunity and the strategies for elimination of pathogens [10–12]. We have shown earlier that the macrophages are more crucial in inducing immunity to the microfilarial stage of filarial parasite than the T cell immune responses being involved [13]. Molecules induced during the effector activation of macrophage include free radicals such as nitric oxide (NO) [14–17] and various cytokines [18–22]. Induction of these effector molecules in activated macrophages is strongly controlled by various signal transcription factors, like nuclear factor-κB (NF-κB) and activating protein 1 (AP-1) [23–28]. Given the documented role of NF-κB/AP-1 factors in regulation of macrophage effector responses, we examined whether the microfilaricidal activity of macrophages is regulated at the level of signal transduction pathway and is responsible for host's natural immunity to mf stage of filarial parasite. Our data show that the host's immunity to mf stage is influenced by the macrophage effector functions through a regulation in the NF-κB/AP-1 signal cascades.

2. Materials and methods

2.1. Animal

The BALB/c mice were bred and maintained in the animal facility of Central Drug Research Institute (CDRI), Lucknow, India. C57Bl/6 mice were obtained as kind gift from National Institute of Immunology (NII), New Delhi, India. All the mice were 6–12 weeks old and experiments were performed as per the guidelines laid by the Institutional Animal Care and Usage Committee.

2.2. Preparation of mf antigens

Active and motile mf were collected in vitro from gravid female worms of *Setaria cervi*. About 1×10^{10} of *S. cervi* mf were sonicated using 12 cycles each of 4 min with 2 min cooling during intervals. Sonicate was centrifuged at $9000 \times g$ for 10 min. The supernatant was collected and kept frozen at −20 °C till further use.

2.3. Macrophage stimulation assay

The peritoneal exudate cells were harvested by injecting 4% thioglycolate broth as described elsewhere [29]. The cells were plated in 96-well tissue culture plate to a density of 5×10^5 cells/well in the presence of titrating doses of soluble extracts of *S. cervi* mf. Nitrite accumulation and the cytokine levels in the culture supernatants were determined after 48 h.

2.4. Estimation of nitrite and cytokines

The NO produced from the activated macrophage reacts with oxygen and forms stable nitrite and nitrate. The nitrite accumulation in the

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Abbreviations: NF-κB, nuclear factor-kappa B; AP-1, activating protein 1; mf, microfilariae; NO, nitric oxide; iNOS, inducible nitric oxide synthase

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culture supernatants was measured for quantification of NO generation using Griess reagent [30]. Measurement of IL-10 and IL-12 cytokines was performed by two-site sandwich enzyme immunoassay as per the manufacturer's protocol (Pharmingen, San Diego, CA). Standard curves for the cytokines were obtained using recombinant standard proteins.

2.5. Parasite cytotoxicity assay

Actively motile *S. cervi* mf (500/well) were co-incubated in vitro with 5×10^5 peritoneal macrophages for 48 h in 96-well tissue culture plate. Wherever appropriate, macrophages were pretreated with various concentrations of aminoguanidine (AG, Sigma, St. Louis, USA) or 100 μM of pyrrolidine dithiocarbamate (PDTC; Sigma, USA) for 1 h. The mf cytotoxicity in the cultures was monitored by recording the percentage of live mf by microscopic observation and was confirmed by subsequent culturing of mf in the macrophage free medium for further 48 h.

2.6. Electrophoretic mobility shift assay

Macrophages from C57Bl/6 or BALB/c mice were stimulated for 1 h with 100 μg/ml of mf antigens. Nuclear extracts were prepared using NP-40 as described [31]. Protein concentrations were estimated using the bicinchoninic acid kit as per the supplier's instructions (BioRad, USA). About 7 μg of nuclear extracts was incubated for 30 min at room temperature with 1 ng of [γ^{32}P] end-labeled double stranded oligo-deoxyribonucleotides (NF-κB: 5′-TTGTTACAAGGGACTTT-CCGCTGGGGACTTTCCAGGGAGGCGTGG-3′ or AP-1: 5′-CGCTTGATGACTCAGCCGGAA-3′) in a buffer containing 2 μg of poly(dI–dC), 20 mM HEPES · KOH, pH 7.9, 0.5 mM DTT, 0.5 mM EDTA, and 5% glycerol. The DNA–protein complex was resolved on a 7% non-denaturing polyacrylamide gel in a running buffer containing 50 mM Tris–HCl of pH 7.5, 40 mM glycine and 10 mM EDTA. The specificity of the protein–DNA complex was verified by competing with 100-fold unlabeled oligonucleotide. The gel was dried and exposed to a phosphor imager (Fuji Bioimaging Analyzer, BAS 2500 with MacBAS, Fuji Medical Systems, Stanford CT, USA).

2.7. Infection with S. cervi *mf*

C57Bl/6 or BALB/c mice were infected intraperitoneally with 1×10^{10} *S. cervi* mf to stimulate macrophages in vivo and after 15 days mice were sacrificed to harvest peritoneal macrophages for activation assays.

2.8. Statistical analyses

All the data were analyzed using Student's *t* test wherever applicable.

3. Results

Macrophages are thought to constitute an important element in the innate defense properties against infections and have been implicated in governing resistance against infections [32–35]. Since the innate resistance responses of macrophages are largely controlled by the NF-κB transcription factors [36–38], we attempted to examine whether the anti-mf immunity is influenced by the NF-κB and other signal transcription factors in macrophages.

3.1. The macrophages from BALB/c mice are less efficient to kill the S. cervi *mf*

When actively motile *S. cervi* (a bovine filarial parasite) mf were co-incubated for 48 h with peritoneal macrophages from C57Bl/6 or BALB/c mice, the C57Bl/6 macrophages were more cytotoxic to the *S. cervi* mf as compared to the BALB/c macrophages (Fig. 1; $P < 0.001$). Similar to *S. cervi*, the *Brugia malayi* (a human filarial parasite) mf were more viable in the macrophage cultures from BALB/c as compared to C57Bl/6 mice (data not shown).

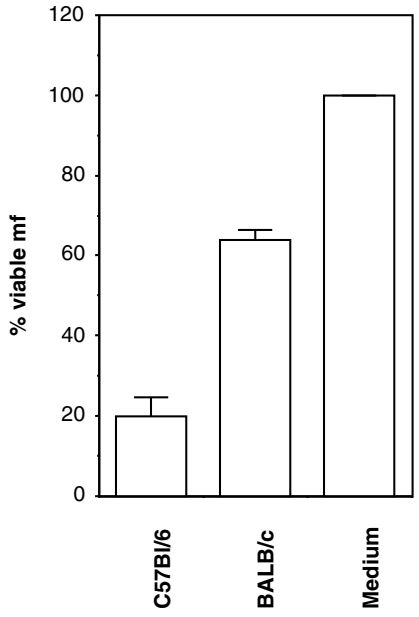

Fig. 1. Macrophage toxicity toward mf in vitro is poor in BALB/c macrophages. The active and viable mf of *S. cervi* (500/well) were co-cultured in vitro in 96-well tissue culture plate with peritoneal macrophages of C57Bl/6 or BALB/c mice (three/group) for 48 h. The motility and viability of mf were estimated and shown as means ± S.D. of three independent experiments. The motility and viability of mf in the wells that received no macrophage cells but only the culture medium were considered to be 100% viable and parasite cytotoxicity in the test samples was monitored based on percentage of normalized controls.

3.2. The higher microfilaricidal activity of C57Bl/6 macrophages is dependent on nitric oxide

As NO produced by the activated macrophages plays a key role in host defense [14–17,39], we examined whether NO is the basis for compromised microfilaricidal activity of BALB/c macrophages. We found that macrophages from C57Bl/6 mice produced significantly higher amounts of NO than that of BALB/c in response to soluble mf antigens (Fig. 2A, $P < 0.001$). Also, the NO levels were higher in the macrophage cultures from C57Bl/6 mice as compared to BALB/c during stimulation with live mf (data not shown). Since NO was a cytotoxic molecule [13,40], the enhanced production of NO by the C57Bl/6 macrophages may account for their increased microfilaricidal activity. To confirm this hypothesis, we used a specific NO/inducible nitric oxide synthase (iNOS) inhibitor, AG [41,42] in the C57Bl/6 macrophage cultures to confirm whether C57Bl/6 macrophages elicited microfilarial cytotoxicity via NO or not. The C57Bl/6 macrophages were pre-treated with titrating concentrations of the potential iNOS inhibitor AG before co-incubated with live mf. The C57Bl/6 macrophage cultures receiving no AG were taken as control. It was found that with higher doses of AG, the levels of NO induced fell steadily (Fig. 2B) in parallel with increased mf viability (Fig. 2C), suggesting that higher mf cytotoxicity showed by C57Bl/6 macrophage could be attributed to their ability to produce higher amounts of NO. Since IL-10 was also produced at a higher level by the C57Bl/6 macrophages, we further examined whether this was also reasoned for increased mf cytoxicity in C57Bl/6 macrophages. The addition of antibody to IL-10 in the C57Bl/6 macrophage cultures did not alter

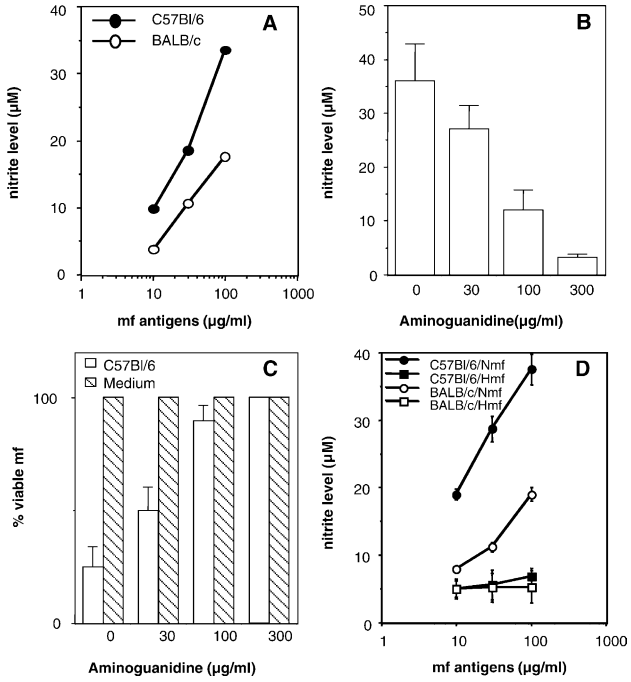

Fig. 2. The higher mf cytoxicity response of C57Bl/6 macrophages is dependent on NO. Peritoneal macrophages from C57Bl/6 or BALB/c mice were stimulated in triplicate with titrating doses of soluble extracts of *S. cervi* mf antigens for 48 h and the levels of NO induced were measured in the culture supernatants as means ± S.D. of three individual experiments (A). In another experiment, the *S. cervi* mf (500/well) were incubated in vitro with peritoneal macrophages from C57Bl/6 mice in the absence or presence of various concentrations of AG. After 48 h the nitrite accumulation in the culture supernatants (B) as well as the motility and viability of mf (C) are shown as means ± S.D. of three individual experiments. In another experiment, the peritoneal macrophages from C57Bl/6 or BALB/c mice were stimulated in triplicate with either native mf antigens (Nmf) or heat-denatured (95 °C for 5 min) mf antigens (Hmf). After 48 h, the levels of NO induced were measured in the culture supernatants as means ± S.D. of three different experiments (D).

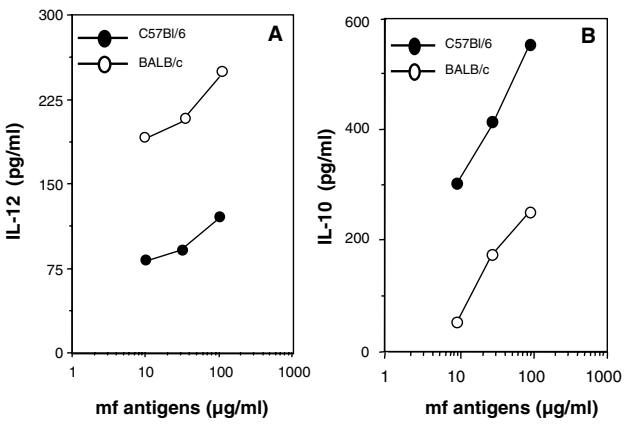

Fig. 3. Induction of IL-12 is poorer but IL-10 is higher in C57Bl/6 macrophages than in BALB/c macrophages. The culture supernatants used for estimating NO in Fig. 2A were used to measure the levels of IL-12 (A) and IL-10 (B) by two site sandwich EIA. Results shown are representatives of three independent experiments.

cytotoxicity pattern (data not shown), indicating that IL-10 is not directly cytotoxic to mf in the in vitro macrophage toxicity assay. This further strengthens the role of NO in induction of toxicity to mf.

Since the adult worms were collected from the peritoneal cavity of abattoir-slaughtered cattle, there is a chance of LPS contamination in the mf extracts. To rule out the possibility of LPS contamination, we used heat-denatured mf antigens (95 °C for 5 min) to stimulate macrophages harvested from both C57Bl/6 and BALB/c mice. As expected, the macrophages from the C57BL/6 mice produced higher levels of NO as compared to the BALB/c macrophages in response to the native mf proteins (Fig. 2D). But the NO production by macrophages from both C57Bl/6 and BALB/c mice was inhibited (see Fig. 2D) upon heat denaturation of the mf antigens. This therefore rules out the role of LPS contamination and supports the fact that the observed difference in the NO production level between the C57Bl/6 and the BALB/c mice was due to the stimulation generated by the mf proteins.

3.3. The C57Bl/6 and BALB/c macrophages reveal contrasting profiles of IL-12 and IL-10 cytokines

We have showed that NO negatively regulates IL-12 production and favors a Th2 type T cell response [29]. Having demonstrated that NO induction was less in BALB/c macrophages than in the C57Bl/6 macrophages (Fig. 2), we were interested to examine whether the induction profiles of IL-12 and other macrophage-induced cytokines were different in these two macrophages. The results showed that induction of the levels of IL-12 cytokine was less in C57Bl/6 macrophages as compared to the BALB/c macrophages in response to mf antigens (Fig. 3A). These observations further reiterate our earlier observation [29] that there is an inverse relation between IL-12 level and NO production, because NO probably serves as feedback inhibitor for IL-12 production. Therefore, the higher induction of IL-12 from BALB/c macrophages might be a consequence of poorer NO induction in them. Although the C57Bl/6 macrophages were not found to be potent inducer of proinflammatory cytokine IL-12, the anti-inflammatory cytokine IL-10 was induced at a level much higher than the level produced by the BALB/c macrophages (Fig. 3B).

3.4. Intraperitoneal inoculation of S. cervi mf in C57Bl/6 and BALB/c mice results in differential activation of peritoneal macrophages

The C57Bl/6 mice expel filarial parasites immediately after establishment of infection, whereas the BALB/c mice support survival of the parasites for a long time and are frequently used as surrogate model [5,6]. It was therefore important to examine whether the observed variations in macrophage responses in C57Bl/6 and BALB/c (Figs. 2 and 3) mice can still be emulated by the stimulation generated by confrontation to live filarial parasites in vivo. Therefore, we next infected C57Bl/6 and BALB/c mice with viable mf of *S. cervi* through intraperitoneal route and the spectrum of NO and cytokine production from the in vivo activated macrophages was compared. Macrophages from the mf infected C57Bl/6 mice showed enhanced NO production (Fig. 4A) as compared to the macrophages from mf infected BALB/c mice. The cytokine profiles revealed that induction of IL-10 was higher (Fig. 4B), whereas IL-12 cytokine was lower (Fig. 4C) in C57Bl/6 macrophages, supporting our in vitro data obtained from the thioglycolate elicited macrophages (see Figs. 2 and 3).

3.5. C57Bl/6 macrophages show higher NF-κB but poorer activating protein 1 DNA-binding activities as compared to the BALB/c macrophages

The expression of iNOS, responsible for higher output of NO in macrophages, is known to be predominantly regulated by the transcription factor, NF-κB [23,25,26]. The NF-κB transcription factor is known to be important for regulation of innate defense mechanisms of macrophages [43]. Therefore, we examined whether decreased NO production and poorer microfilaricidal activity of BALB/c macrophages were a consequence of deficient NF-κB activity in them in response to mf stimulation. Therefore, the DNA-binding activities of NF-κB transcription factor (an indicator of the activity status) were examined by electrophoretic mobility gel shift assay (EMSA) in the nuclear extracts prepared from mf antigen activated C57Bl/6 and BALB/c macrophages. The NF-κB activity was found to be more in the C57Bl/6 (Fig. 5A, lane 3) as compared to the BALB/c macrophages (Fig. 5A, lane 2). The basal activity of NF-κB was very poor in the unstimulated macrophages (data not shown). The specificity of the NF-κB-DNA complex was verified by a 100× cold competition using unlabeled NF-κB duplex oligonucleotides (Fig. 5A, lanes 4 and 5). The Western blot using the specific antibodies to p50 and p65 subtypes also revealed that the levels of the p50 and the p65 NF-κB transcription factors were more in the C57Bl/6 nuclear extracts as compared to the BALB/c (data not shown). Our studies thus indicated that both expression and activation status of NF-κB were higher in macrophages from C57Bl/6 as compared to the BALB/c mice.

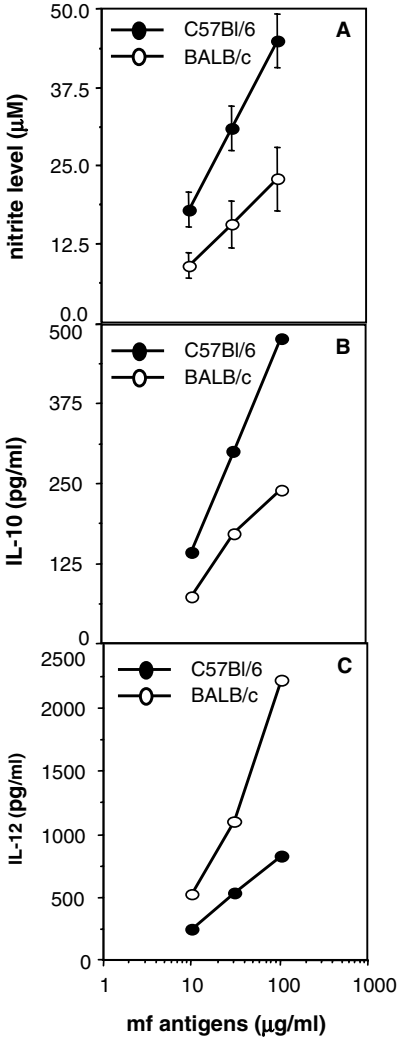

Fig. 4. Intraperitoneal implantation of *S. cervi* mf in C57Bl/6 mice results in differential activation of peritoneal macrophages. C57Bl/6 or BALB/c mice were infected intraperitoneally with 1×10^{10} *S. cervi* mf to stimulate macrophages in vivo. The peritoneal macrophages were harvested after 15 days and stimulated in vitro with varying doses of either PBS extracts of *S. cervi* mf for 48 h. The culture supernatants were harvested and levels of NO (A), IL-10 (B) and IL-12 (C) induced were measured in the culture supernatants. Results shown are representatives of three independent experiments.

Unlike NO, IL-12 was produced at higher levels in BLAB/c macrophages (Figs. 3 and 4). Since the AP-1 transcription factor strongly activates IL-12 transcription [28], we next examined whether higher IL-12 production in BALB/c macrophages occurred through increased AP-1 activity. Therefore, the DNA binding activity of AP-1 transcription factor was examined in the same nuclear extracts prepared from C57Bl/6 and BALB/c macrophages for measuring the active NF-κB levels. The data revealed that although the DNA binding activity of NF-κB was reduced (Fig. 5A), the AP-1 activity of BALB/c macrophages was increased (Fig. 5B, lane 2) as compared to C57Bl/6 macrophages (Fig. 5B, lane 3). Disappearance of the AP-1 band during cold competition with 100× unlabeled AP-1 duplex oligonucleotides further confirmed the specificity of this DNA-protein interaction in C57Bl/6 and BALB/c macrophages (Fig. 5B, lanes 4 and 5).

3.6. The NF-κB signal is critical for the NO-mediated microfilaricidal activity of C57Bl/6 macrophages

We reported earlier that the rate of mf clearance did not correlate with the levels of IL-12 induced in the milieu, rather the anti-microfilarial immunity was critically dependent on the iNOS/NO signal in macrophage [13]. Since iNOS/NO induction is mainly regulated by NF-κB [23,25,26], it is likely that

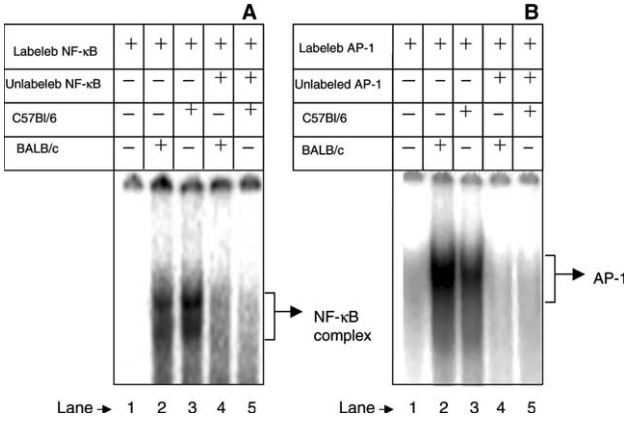

Fig. 5. C57Bl/6 macrophages show higher NF-κB but lower AP-1 activity as compared to BALB/c macrophages. EMSA was carried out to detect DNA binding activity of NF-κB (A) or AP-1 (B) with 7 μg of nuclear extracts prepared from C57Bl/6 or BALB/macrophages stimulated with 100 μg/ml of *S. cervi* mf extracts. Lane 1 represents free probes (A and B). In lane 2 the probes were incubated with nuclear extracts from BALB/c macrophages and in lane 3 the C57Bl/6 nuclear extracts were used. Cold competitions were performed using 100-fold excess unlabeled NF-κB or AP-1 oligonucleotides as indicated (lanes 4 and 5). Results shown are representatives of three independent experiments.

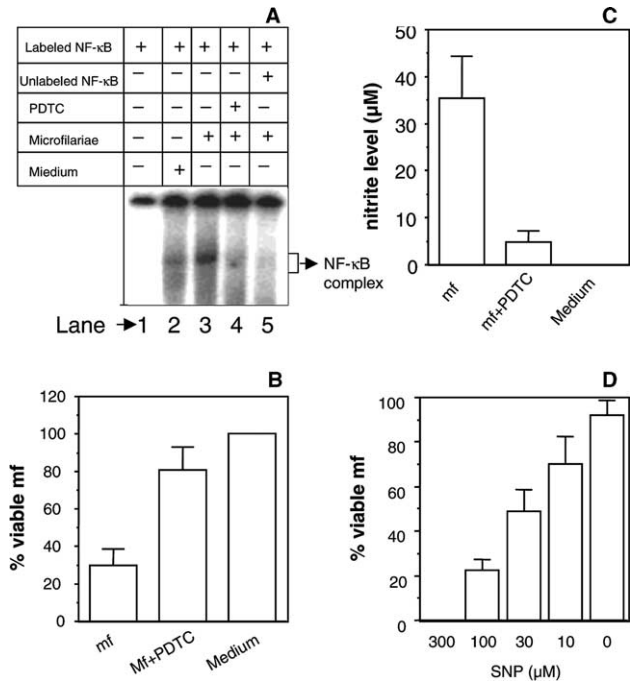

Fig. 6. The NF-κB signal is important for the NO-mediated cytotoxicity of C57Bl/6 macrophages against *S. cervi* mf. Around 5×10^5C57Bl/6 macrophages were co-cultured with active and viable *S. cervi* mf (500/well) in the absence or presence of the NF-κB inhibitor PDTC (100 μM). The NF-κB activity was examined after 1 h by EMSA (A). Microfilarial viability (mean ± S.D.; B) and nitrite accumulation in the culture supernatants (mean ± S.D.; C) were measured after 48 h. In another experiment, the active and viable mf of *S. cervi* (500/well) were co-incubated with the PDTC treated C57Bl/6 macrophages receiving titrating concentrations of the exogenous NO generator SNP and mf viability in these cultures was monitored after 48 h (D). The motility and viability of mf in the wells that received no macrophages were normalized to be 100% viable and parasite cytotoxicity in the test samples was monitored based on the percentage viability of normalized controls. Results shown are representatives of three independent experiments.

the NF-κB signals are critical for the NO-mediated microfilaricidal action of C57Bl/6 macrophages. Therefore, we blocked the NF-κB signal in C57Bl/6 macrophages using 100 μM of PDTC (a pharmacological inhibitor of NF-κB, [44,45]), and looked for the mf viability in these macrophage cultures after 48 h of co-cultivation. The results showed that inhibition of NF-κB signals in C57Bl/6 macrophages by PDTC (Fig. 6A) significantly increased mf viability (Fig. 6B, $P < 0.001$) in parallel with reduction in NO induction (Fig. 6C, $P < 0.001$). It is now interesting to confirm whether NF-κB influences macrophage cytotoxicity responses through a regulation on NO induction. Therefore, we next used sodium nitroprusside (SNP, an exogenous generator of NO) in the C57Bl/6 macrophage cultures treated with PDTC (to block the NF-κB signal and thus the production of endogenous NO) and then looked for mf cytoxicity in these cultures. Inclusion of SNP in these cultures resulted in dose dependent killing of mf (Fig. 6D).

4. Discussion

Presence of granuloma covering the surface of filariae supports the importance of macrophages in anti-filarial immunity [46,47]. We show here that the NF-κB/AP-1 signal in macrophages in response to mf stimulation varies between C57Bl/6 and BALB/c mice and the NF-κB signal is critical for the NO-mediated microfilaricidal activity of C57Bl/6 macrophages. The poorer toxicity responses against the mf stage in BALB/c mice may be because of their inability to produce cytotoxic levels of NO due to defective NF-κB signals. This may be a single piece of the puzzle, since the signaling network leading to macrophage activation in C57BL/6 and BALB/c mice is a complex phenomenon and needs to be elucidated further.

Although the NO signal was poorer the IL-12 induction was higher in BALB/c, which may be because of increased AP-1 activity in them. It is not clear as to why in BALB/c, the AP-1 activity was more and NF-κB was down. It is possible that the NF-κB and the AP-1 transcription factors cross regulate each other, or it may reflect the compensatory role of other activation pathways (AP-1) when one pathway is down (NF-κB). It is also possible that many complex interactions between various transcription factors are actually involved in control of effector functions of C57Bl/6 and BALB/c macrophages. It is pertinent to note that *Leishmania* sp. infected C57Bl/6 mice show a Th1 bias (unlike filarial infection) and govern protection against the infection [48]. It was likely that the inflammatory events observed in the infected C57Bl/6 mouse were particularly influenced by the *Leishmania* sp. parasite proteins.

Although the protective role of IL-10 in anti-mf immunity can never be ignored in vivo, the NO pathway appeared to be of crucial importance leading to direct cytotoxicity to the mf in C57Bl/6 mice, since the NO/iNOS inhibitor AG blocked the microfilaricidal activity of C57Bl/6 macrophages. Macrophages from BALB/c mice, in contrast, exhibited poorer induction of NO and cytotoxicity to mf. We have shown earlier that delayed clearance of filarial parasites in bruton's tyrosine kinase (Btk) deficient mice is associated with compromised NO induction [29] and macrophage effector responses but not on IL-12 or Th1 responses [13,49]. There is evidence that NO is directly cytotoxic to other nematode [40]. Furthermore, it has been shown that mice that are resistant to filarial parasites can become susceptible when administered with NO inhibitor [50]. The fact that NO pathway is not well demonstrated in human macrophage [51], the concept of NO mediated killing of filarial parasites as defined in the murine model may be confusing in human filariasis. But since filarial parasites reside in the lymphatics of mammalian host in close proximity to endothelial cells that can produce NO [52], the importance of NO in anti-filarial immunity in human may not be ignored. In conclusion, for the first time we report here a role for the NF-κB/AP-1 signal transduction cascades to regulate the levels of NO production and cytokine balances and thereby influence the host's ability to elicit resistance to filarial infection.

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