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Th2 induction by *Nippostrongylus* secreted antigens in mice deficient in B cells, eosinophils or MHC Class I-related receptors

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Abstract

The populations of immune system cells necessary for induction of the Th2 response have yet to be fully defined. Among the most consistent natural stimuli for Th2 responses, are helminth parasites, such as the gastrointestinal nematode *Nippostrongylus brasiliensis*. Strong Th2 bias in immune responsiveness to live parasite infection can be reproduced by injection of a soluble *Nippostrongylus* excretory/secretory product (NES) collected from adult worm parasites in vitro. Injection of soluble NES induces a residual type-2 response (IL-13) even in IL-4-deficient mice, and drives a fully polar Th2 response in IL-5-deficient animals. A potent IL-10 response is observed irrespective of IL-4 or IL-5 gene status. While MHC Class II knockout animals fail to mount any IL-4 or IL-10 response, the Th2 bias is intact in Class I knockouts, indicating that CD8^{+} or NK-like T cells restricted to classical or non-classical Class I molecules do not play an essential role in Th2 induction. B cell-deficient μMT animals also show responses, which are strongly skewed to IL-4 production. Thus, Th2 induction by *Nippostrongylus* antigens is independent of B cells, or MHC class I presentation, and does not require a sufficiency of eosinophils in vivo.

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Keywords: T subset differentiation; Helminth parasites; Cytokines

1. Introduction

Nippostrongylus brasiliensis is a parasitic nematode of rodents, and is associated with the induction of highly polar Th2 type responses [1–4]. Studies on experimental infection of mice carrying defined cytokine gene deficiencies, or treated with specific cytokine reagents, have provided a remarkably clear understanding of the basis of protective immunity to this parasite. CD4^{+} T cells are stimulated to differentiate to Th2 cells, secreting IL-4 and IL-13, which evoke intestinal goblet cell proliferation and mucin hyper-secretion from non-lymphoid epithelial cells [5]. The actions of IL-4 and IL-13 on target cells in the intestine underlie expulsion of adult worms from the luminal environment. Thus, mice lacking the common receptor chain for IL-4 and IL-13 (IL-4Rα) are unable to expel *N. brasiliensis*, as are those deficient in the key cytoplasmic transducer linked to IL-4Rα, STAT-6 [6,7]. IL-13 is the critical contributor, as IL-13-deficient animals fail to expel adult worms [8], while mice lacking IL-4 retain a protective capacity for expulsion of parasites from the gut [2,7,8].

Other developments in *N. brasiliensis* infection, which are consonant with Th2 cell activation are an IL-5-dependent eosinophilia, mast cell hyperplasia and an IL-4-dependent polyclonal IgE response. This set of features is common to a broad range of helminth infections, and appear to play no role in the expulsion of *N. brasiliensis* itself in the context of a primary exposure. Eosinophils can, if sufficiently activated in IL-5 over-expressing animals, kill migratory larvae before they reach the intestinal tract [9,10]. It is likely that this protective barrier normally operates only in secondary infection

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30 [11]. Thus, infection generates an archetypal Th2 response, 31 in which the activity of adaptive and innate immune popula- 32 tions are closely intertwined through the production of key 33 cytokines.

34 In contrast to the clear analysis now available of Th2 ef- 35 fector mechanisms in gut nematode infections, we know little 36 of how the immune system becomes so firmly committed to 37 the Th2 pathway by the presence of the parasite. In particular, 38 the role of innate immune cells in Th2 initiation is of major 39 interest. We have shown, for example, that dendritic cells ex- 40 posed to *N. brasiliensis* antigen will initiate a Th2 response 41 in vivo [12], but other cell types may also be critical in this 42 pathway. For example, eosinophils are an important innate 43 cell population, which produces IL-4 early in *N. brasilien-* 44 *sis* infection [13]. In non-infection models, influential IL-4 45 production by basophils has long been known, perhaps de- 46 pendent on the cross-linking of B cell-derived IgE [14,15]. 47 Other investigators have argued that B cells themselves can 48 produce IL-4 or IFN-γ at critical junctures in the developing 49 response, thereby regulating Th subset differentiation [16]. 50 In addition, the NKT cell subset has the capacity to respond 51 rapidly to both polyclonal [17] and helminth parasite [18] 52 stimuli by producing IL-4, thereby creating an environment 53 favouring Th2 differentiation.

54 One means of defining which cell types are critically re- 55 quired for Th2 development is to exploit available gene- 56 targetted transgenic mice, lacking particular components and 57 cell populations from the immune system. Surprisingly per- 58 haps, the IL-13-deficient mouse generates a strong IL-4 re- 59 sponse during *N. brasiliensis* infection [19], demonstrating 60 that IL-13 itself is not a prerequisite for Th2 development. 61 However, where gene-deficient animals show greatly en- 62 hanced susceptibility to infection [6–8], the dynamics of cy- 63 tokine responses over time are likely to be significantly al- 64 tered. We have therefore developed a model system in which 65 Th2 responses are induced by secreted antigens from cultured 66 *N. brasiliensis*. Th2 activation can be reproduced by injecting 67 isolated proteins from the parasite, without the introduction 68 of live organisms. Thus, induction of Th2 responses can be 69 studied in vivo in mice of different genetic backgrounds with- 70 out the confounding effect of variable levels of infection.

71 In this report, we investigate the requirements for differ- 72 entiation of the Th2 response to a helminth product in defined 73 gene-deficient animals. In particular, we studied whether key 74 accessory cell populations such as eosinophils or B cells are 75 necessary for Th2 induction, and whether $CD8^+$ or non- 76 conventional T cells (recognising MHC Class I-dependent 77 epitopes), may contribute to the type 2 outcome.

78 2. Materials and methods

79 2.1. Mice

80 The following strains and gene-targetted animals were 81 studied. IL-4-gene deficient (IL-$4^{o/o}$) [20] and IL-$5^{o/o}$ [21] 82 were a kind gift from Prof. Manfred Kopf, University of 83 Zürich. The β-2-microglobulin-deficient (β2$M^{o/o}$) mice [22] 84 were a gift from Matthias Merkenschlager, Imperial College, 85 London. MHC Class II knockout (deletion of I-A^b), and μMT 86 mice, both on the C57BL/6 background, were provided by 87 Prof. David Gray, University of Edinburgh.

88 2.2. Parasites and NES

89 The maintenance of *N. brasiliensis* in Sprague–Dawley 90 rats [2,23] and the collection of NES from adult worms 91 in vitro followed previously described protocols [24,25]. 92 Briefly, adult worms recovered from rats at d6 post-infection 93 were washed extensively and cultured in serum-free RPMI 94 1640 medium containing antibiotics and added glucose (to 95 1%). Supernatants collected from 1 to 7 days of culture 96 were pooled, concentrated over a 10,000 MW cut-off Amicon 97 membrane, and stored at ∼1 mg/ml in PBS. Heat-inactivation 98 of NES (hiNES), to abolish Th2-inducing activity was for 99 15 min at 95 °C [12,25].

100 2.3. Immunisations and culture

101 Mice were immunised in each hind footpad with either or 102 both 50 μg NES and 10 μg hen egg lysozyme (HEL) or oval- 103 bumin (OVA); in some experiments mice had been primed 104 by subcutaneous immunisation in the flank 21 or 28 days 105 earlier. Where stated antigens were emulsified in Complete 106 Freund's Adjuvant (CFA, Sigma) or Incomplete Freund's Ad- 107 juvant (IFA, Sigma). Popliteal lymph node cells (LNC) were 108 recovered 7 days following footpad immunisation. When sera 109 were required, animals were pre-bled on the day of primary 110 injection and again when the experiment was terminated after 111 secondary challenge. Four or five animals were used in each 112 experimental and control group and the data are presented as 113 mean ± S.E. of results from individual mice.

114 2.4. In vitro culture

115 LNC were cultured in DMEM containing 100 U/ml peni- 116 cillin, 100 μg/ml streptomycin, 4 mM glutamine and 10% 117 FCS. Cells were cultured at 2×10^6 ml in 0.2 ml cultures 118 containing various antigens (10 μg/ml) or controls (medium 119 only). Con A stimulation (2 μg/ml) was included in all exper- 120 iments. After 60 h, 100 μl of culture supernatant was removed 121 for cytokine assays.

122 2.5. Cytokine assays

123 Estimates of IL-4, IL-5, IL-10, IL-13 and IFN-γ contained 124 in supernatants were determined by capture ELISA, using 125 antibodies from Pharmingen unless otherwise stated. Where 126 multiple cytokines were assayed, supernatants from replicate 127 culture wells were pooled, and each cytokine assay performed 128 in duplicate. IL-4 ELISA included capture with antibody 129 11B.11 (gift of National Cancer Institute, Frederick, MD)

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at 4 μg/ml and detection with biotinylated anti-IL-4 (BVD6-24G2). IL-5 ELISA used TRFK5 at 1 μg/ml for capture and biotinylated TRFK4 for detection. IL-10 ELISA used JES5-2A5 at 4 μg/ml and biotinylated SXC-1 for detection. IL-13 ELISA used MAB413 (R&D Systems) at 2 μg/ml for capture and biotinylated BAF413 (R&D Systems) for detection. IFN-γ ELISA used R46A2 for capture and biotinylated XMG1.2 for detection. Levels of cytokine were determined from a standard curve constructed in DMEM-10 using recombinant murine cytokines (Sigma) for each assay. All cytokine capture assays used an Extravidin Alkaline Phosphatase (Sigma) amplification system and were developed using SigmaFast system substrate. Optical densities were measured at 405 nm and the data manipulated for curve fitting and interpolation using Deltasoft software.

2.6. Flow cytometry and intracellular staining of $CD4^+$ T cells

Single cell suspensions were prepared from popliteal lymph nodes of mice that had been injected subcutaneously via the footpad 7 days previously with 50 μg/footpad of NES/complete Freund's adjuvant (CFA) or hiNES/CFA. In vitro, 2×10^6 cells/ml were stimulated with 50 ng/ml phorbol myristate acetate, 1 μg/ml Ionomycin and 20 μg/ml Brefeldin A for 6 h. After stimulation, cells were washed in FACS buffer (PBS with 0.5% bovine serum albumin) and resuspended at 2×10^6 cells/ml in FACS buffer with 10% normal rat serum for 15 min at 4 °C. At this point cells were stained with FITC anti-CD4 for 30 min at 4 °C, washed 3× in FACS buffer and fixed using a Cytofix/Cytoperm PlusTM kit (Pharmingen). After fixation cells were washed in CytoPerm/Wash solution and stained for 30 min at 4 °C with APC-conjugated anti-IL-4 mAb. After staining cells were analysed by flow cytometry using a FACSCalibur flow cytometer and BD CellQuest™ Pro software.

2.7. Statistical analyses

Data are described as mean and S.E. Differences in the log transformed mean values of data sets between experimental groups were compared using Student's unpaired *t*-test using Stata v8.0. *P*-values <0.05 were considered statistically significant and are noted in the figure legends. *P*-values were adjusted for multiple comparisons by applying Bonferoni correction.

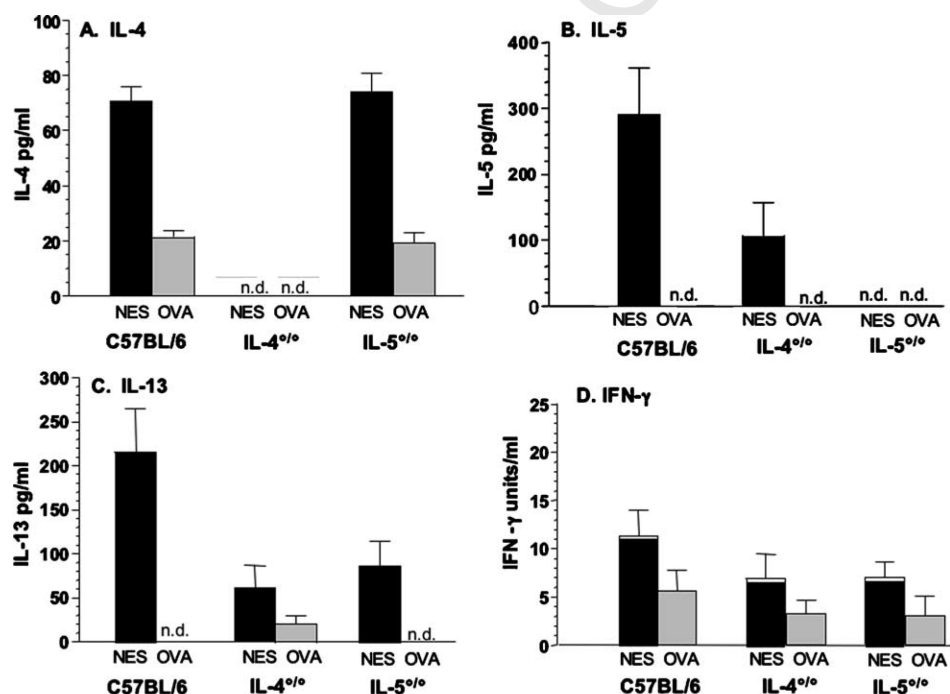

Fig. 1. In the absence of IL-4 or IL-5, responses to NES do not switch to Th1. Th1 and Th2-associated cytokine responses, as measured by IL-4 (A), IL-5 (B), IL-13 (C) and IFN-γ (D), are presented in wild-type C57BL/6 mice, and $IL\text{-}4^{o/o}$ and $IL5^{o/o}$ transgenic mice on the C57BL/6 background. Mice were immunised with NES/CFA 7 days before recovery of draining lymph node cells and in vitro challenge with NES (dark bars) or control protein (Ovalbumin, OVA, light bars). n.d. = not detectable, i.e. below assay cut-off values of 12 pg/ml for IL-4; 64 pg/ml for IL-5; and 20 pg/ml for IL-13. Data are mean and S.E. from 5 individual mice per group assayed individually. IL-4, IL-5 and IL-13 responses to NES are, in each case except the corresponding null mutants, significantly higher than the responses to OVA ($P < 0.05$). IFN-g responses to NES and OVA are not significantly different. IL-4 and IL-13 responses from C57BL/6 and $IL\text{-}5^{o/o}$ mice are not significantly different. The IL-13 response in $IL\text{-}4^{o/o}$ mice is significantly lower than wild type (<0.05), but the IL-5 response is not significantly different.

3. Results

3.1. NES induction of type 2 differentiation does not require IL-4 or IL-5

We have previously shown in a range of mouse strains, that the injection of NES generates an antigen-specific Th2 response in draining lymph node $CD4^+$ T cells [25]. Th2 differentiation occurs whether NES is administered in soluble form, or in conjunction with Complete Freund's Adjuvant (CFA), but is abolished by heat-inactivation of the NES material [12,25]. To study the host cellular components, which may stimulate Th2 responsiveness, we first tested the ability of NES to generate type 2 cytokines in IL-$4^{o/o}$ and IL-$5^{o/o}$ mice on the C57BL/6 background. Popliteal lymph node cells (LNC) were recovered 7 days following primary immunisation. As shown in Fig. 1 , in the absence of IL-4 (Fig. 1A), detectable quantities of IL-5 and IL-13 were produced from LNC challenged in vitro with NES antigen (Fig. 1B and C). While IL-5 and IL-13 responses were lower in IL-$4^{o/o}$ than the levels observed in wild-type cells, the differences did not reach statistical significance. Notably, no shift towards Th1 (IFN-γ) was evident in mice deficient in IL-4 when immunised with NES (Fig. 1D).

The absence of IL-5 was found not to compromise the Th2 response in general. Not only were in vitro IL-4 responses unchanged in IL-$5^{o/o}$ cells (Fig. 1A), the amplification of polyclonal IgE following NES challenge in vivo remained intact in IL-5 gene-targetted animals (Fig. 2), consistent with earlier reports on *N. brasiliensis* infection of similar genotypes [26]. Thus, IL-5 is dispensible for a classic Th2 response to NES.

These results reinforce the conclusion that *Nippostrongylus* and NES provide a dominant Th2 influence in vivo that cannot be overturned by the lack of either IL-4 or IL-5. Such an outcome is consistent with the ability of IL-4 KO mice to expel *N. brasiliensis* via a Th2-type, IL-13-dependent pathway [6].

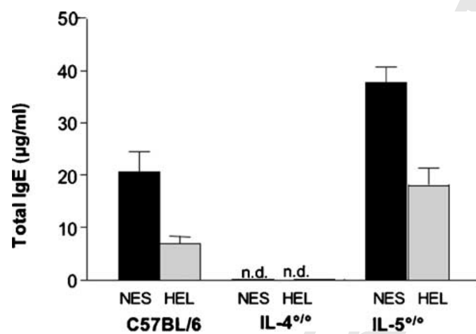

Fig. 2. Generation of polyclonal IgE responses by NES is intact in IL-5-deficient mice. Mice were immunised with NES/CFA or HEL/CFA, and boosted 28 days later with NES or HEL in Incomplete Freund's Adjuvant. Sera were sampled 7 days later for total IgE levels by ELISA. Data are mean and S.E. from 4 to 5 individual mice per group assayed individually. IL-5-deficient IgE responses do not differ significantly from those observed in wild type mice.

3.2. Loss of MHC Class I expression fails to alter Th2 responsiveness

To test whether Class I-dependent populations play any role in generating a Th2 response to NES, we studied mice in which MHC class I expression has been disrupted by deletion of the β-2-microglobulin gene. Such animals fail to express conventional Class I as well as non-conventional antigen-presenting molecules such as CD1. We found that these animals have vigorous, intact NES-specific IL-4 responses (Fig. 3A) and, like wild-type mice, only marginal IFN-γ production (Fig. 3B).

3.3. B cells are not critical for Th2 induction by NES

A further important immune population is that of the B cell. In mice lacking B cells due to disruption of the membrane IgM receptor essential for B cell development (µMT mice, on the C57BL/6 background), we found that the Th2 responses induced by NES were comparable to those in the wild-type (Fig. 3A), and there was little evidence of any shift towards Th1 (IFN-γ) responsiveness (Fig. 3B). The presence of a Th2 response demonstrates that B cells are not essential for the induction of this phenotype, although these data do

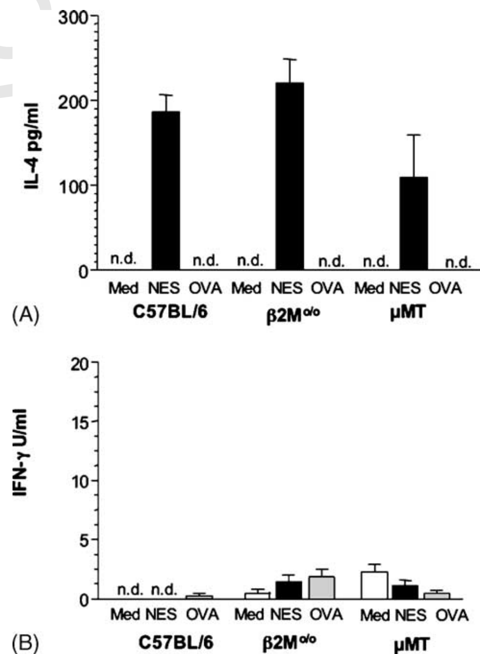

Fig. 3. (A) Th2 responses are intact in Class I-deficient and in µMT mice. IL-4 responses in wild-type C57BL/6 mice, and β2$M^{o/o}$ and µMT transgenic mice on the C57BL/6 background, immunised with soluble NES and OVA, 7 days before recovery of LNC and in vitro challenge with NES (dark bars) or OVA (light bars). n.d. = not detectable, i.e. below assay cut-off. Data are mean and S.E. from 4 (β2$M^{o/o}$) or 5 (C57BL/6, µMT) individual mice per group assayed individually and are representative of at least 2 experiments for each genotype. The IL-4 responses from the 3 strains of mice tested were not significantly different ($P > 0.05$). (B) Class I-deficient and in µMT mice do not generate Th1 responses to NES. IFN-γ cytokine production was measured in the same LNC cultures as presented in Panel A.

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226 not exclude the possibility that B cells participate in its am-
227 plification. This issue was explored further by enumerating
228 IL-4-responsive $CD4^+$ T cells in the draining lymph node
229 populations, by intracellular staining with antibody to IL-4.
230 As shown in Fig. 4, the frequency of IL-4-producing cells was
231 as high in NES-immunised μMT mice as in wild-type animals
232 (Fig. 4A and B), and sharply reduced by heat-inactivation of
233 the NES immunogen (Fig. 4C and D).

234 3.4. IL-4 Production is entirely MHC Class II-dependent

235 To test if some component of the IL-4 response to NES
236 is independent of MHC class II (for example, derived from
237 class I-restricted $CD8^+$ or NK T cells), we compared Th2 re-
238 sponses, in the form of IL-4 recall responses, in wild type and
239 MHC Class II-deficient animals homozygous for a null allele
240 at the I-A locus. As shown in Fig. 5A , the gene-targetted ani-
241 mals are incapable of mounting any detectable IL-4 response,
242 in contrast to wild-type strains or to the IL-$5^{o/o}$ animals. This
243 question was further explored by cytoplasmic staining of IL-
244 4 within lymph node cells from NES-immunised animals. As
245 shown in Fig. 5B, no intracellular IL-4 staining of cells from
246 the I-$A^{o/o}$ mice could be detected, in contrast to the wild-type
247 C57BL/6. While this does not exclude a contribution of Class
248 II-independent T cells at an earlier time point or in a distal
249 immune compartment, the lack of NES-specific serum anti-
bodies in I-$A^{o/o}$ animals (data not shown) indicates that the 250
absence of MHC Class II renders mice unresponsive to NES. 251

3.5. NES induces high IL-10 responses which are IL-4-independent 252 253

In the course of these experiments, we noted that NES 254
induces strong antigen-specific IL-10 responses among cells 255
from draining lymph nodes. IL-10 responses were found to be 256
unaltered in either IL-$4^{o/o}$ and IL-$5^{o/o}$ animals, which in fact 257
show a trend towards higher IL-10 release than cells from 258
wild-type animals exposed to a similar NES immunisation 259
(Fig. 6). No IL-10 was detected in Class II knockout mice, 260
indicating that B cells (at least within the lymph node) do not 261
contribute to this response, or can only do so once instructed 262
by a $CD4^+$ T cell. The production of IL-10 by IL-$4^{o/o}$ and 263
IL-$5^{o/o}$ is a robust property, and can be observed both in short- 264
term primary immunisations with soluble NES (Fig. 6A) and 265
on secondary challenge following administration in Freund's 266
adjuvant (Fig. 6B). 267

4. Discussion 268

Studies with mice genetically deficient in key immune cell 269
populations or cytokines have illuminated our understanding

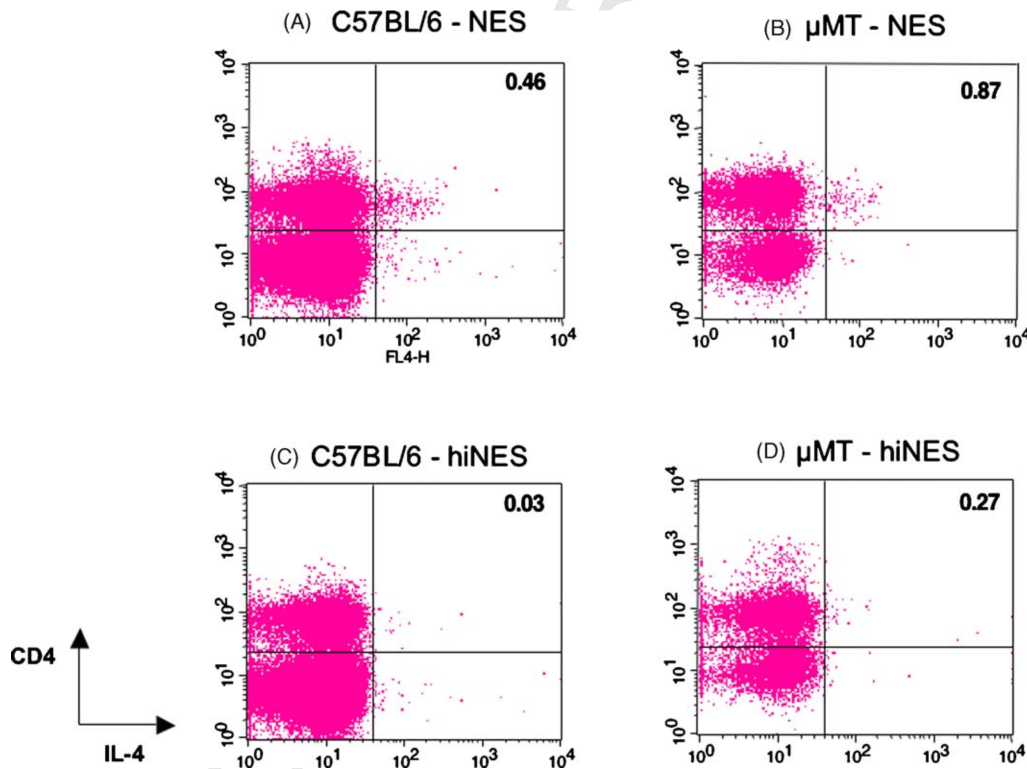

Fig. 4. Frequency of IL-4-producing $CD4^+$ T cells is equivalent in wildtype and μMT mice. Cytoplasmic staining of $CD4^+$ lymph node T cells for IL-4, harvested 7 days following immunisation with NES (A,B) or with heat-inactivated NES (hiNES, C,D), in wild-type (A,C) or μMT (B,D) mice. Data are from representative individual animals; no significant difference was found between wildtype and μMT groups.

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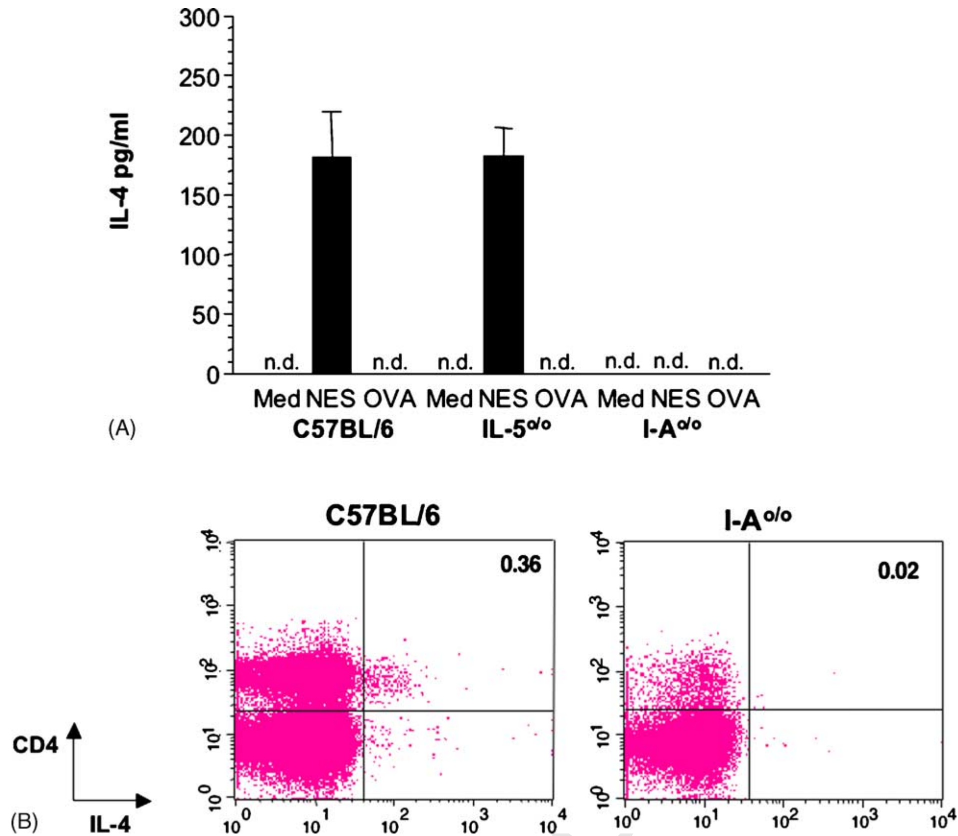

Fig. 5. NES does not elicit IL-4 responses in MHC Class II-deficient animals. (A) LNC from C57BL/6, MHC Class II deficient (I-$A^{o/o}$), and IL-5-deficient mice on the C57BL/6 background immunised with soluble NES and OVA. Animals were challenged in vitro with NES, OVA, or medium alone (Med). Secreted IL-4 responses were measured by ELISA. n.d. = not detectable, i.e. below assay cut-off. Data are mean and S.E. from 5 individual mice per group assayed individually. (B) Intracellular cytokine staining for IL-4 in lymph node $CD4^+$ T cells from C57BL/6 and MHC Class II deficient (I-$A^{o/o}$) mice immunised 7 days previously with NES/CFA. Data are from representative individual animals.

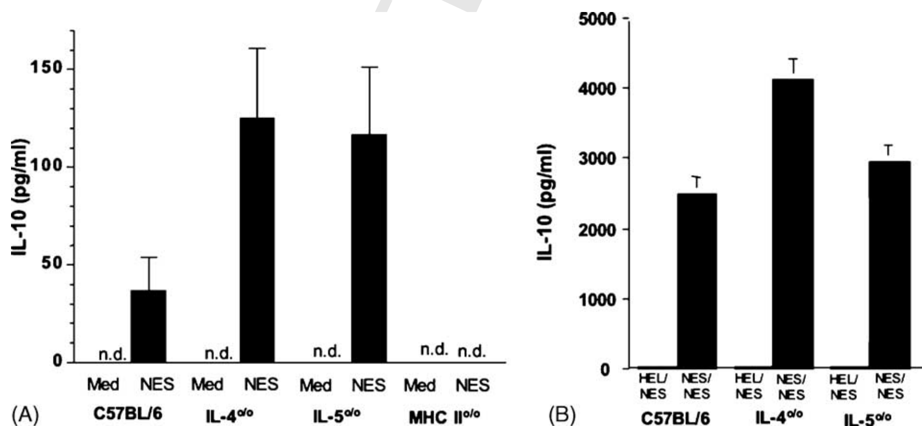

Fig. 6. Strong IL-10 responses in vitro to NES are intact in IL-$4^{o/o}$ and IL-$5^{o/o}$ mice. (A) Primary IL-10 responses of lymph node cells, 7 days following immunisation with soluble NES, in wild-type C57BL/6, and IL-$4^{o/o}$ and IL$5^{o/o}$ on the C57BL/6 background. Cultures were challenged in vitro with NES or medium alone (Med). n.d. = not detectable, i.e. below assay cut-off. Data are mean and S.E. from 5 individual mice per group assayed individually. Differences in IL-4 production between the 3 strains of mice do not reach statistical significance. (B) Secondary IL-10 responses of lymph node cells to NES. Mice were primed with either NES-CFA or HEL-CFA at day 0 and boosted with the same antigen in IFA at day 21. At day 28 lymph node cells were recovered and challenged in vitro with NES. Data are mean and S.E. from 5 individual mice per group assayed individually. The IL-10 response from IL-$4^{o/o}$ mice was significantly higher than either the C57BL/6 wild type or the IL$5^{o/o}$ genotype ($P < 0.05$).

of resistance to gastrointestinal helminths [27]. The absence of IL-13, IL-4Rα or STAT-6 all disable protective immunity and permit *N. brasiliensis* survival [6,7]. This widely-used infection model has also been exploited to analyse the genesis of Th2 differentiation: to date, eosinophils [13,28] and dendritic cells [12] have been implicated in the specific case of *N. brasiliensis*, while in other helminth parasites the involvement of B cells [29] and NKT cells [18] has been suggested. In our investigations, we chose to use the secreted protein fraction from adult *N. brasiliensis* (NES), as responses in mice with differing specific immune deletions would not be influenced by differential susceptibilities to chronic parasite infection.

Our first experiments show that a vestigial Th2 response to NES remains in the absence of IL-4, consistent with original observations that IL-$4^{o/o}$ mice can expel *N. brasilensis* infections [2,7,8]. Resistance of IL-4-deficient mice is due to the induction of a related type 2 cytokine, IL-13 [6–8] acting through the IL-4Rα receptor on non-lymphoid epithelial cells in the intestinal tract.

The ablation of the IL-5 gene results in greatly diminished, but not altogether abolished, eosinophil levels [21]; residual eosinophils may be stimulated by eotaxin, and other cell types (particularly B1 cells) are also depleted in the absence of IL-5 [11]. In in vivo infection with *N. brasiliensis*, eosinophils are a major contributor of IL-4 from an early time-point [13], and numerically dominate the cellular response in terms of IL-4 production by day 9 [28]. Eosinophil IL-4 synthesis has also been shown to be independent of the Th2-controlling transcription factor STAT-6 [28], suggesting that these cells could initiate a type 2 response prior to the onset of T cell subset differentiation. However, our data showing intact Th2 responses in IL-$5^{o/o}$ animals casts doubt on the relative importance of eosinophils in this regard, although we cannot formally exclude such a role. Future studies with eosinophil-specific lineage-ablated mice will be necessary to establish whether these cells are prerequisites of the Th2 response to infection, or a prominent but secondary participant in that response.

The relative roles of MHC Class I and II presentation were also studied by using appropriate gene-targetted mice. Surprisingly clear-cut results were obtained: in the absence of Class II, no trace of a type 2 response could be observed, even among the innate cell populations. This finding is consistent with published work showing that NES-specific IL-4 production is essentially confined to the $CD4^+$ population of T cells [25], and that without CD4 ligation (to MHC Class II) the Th2 response to *N. brasiliensis* is absent [30].

In contrast, Class I-deficient animals were fully responsive to NES in terms of their Th2 phenotype. The primary hypothesis we set out to test was that non-conventional T cells (e.g. NKT), recognising CD1 or other non-classical but β2-microglobulin (β2M)-dependent ligands, were initiating skewed Th responses to this parasite. Earlier studies with other helminths, such as *Brugia malayi* [18] had suggested this might be the case, although other investigators have tested β2$M^{o/o}$ animals in *N. brasiliensis* infection and reported an intact Th2 response [31]. We likewise find that β2$M^{o/o}$ mice are fully competent to mount a Th2 reaction to NES, and we therefore conclude that NKT cells do not play an essential role in the generation of this response.

Relatively few studies have examined the role of B cells in the Th2 response to nematode antigens, despite the fact that B cells have the ability to induce Th2 differentiation, for example through OX40L expression [32,33]. Hall et al. reported, in a model of *Onchocerca*-induced keratitis, that Th2 stimulation was equivalent in wild type and μMT mice, but that the latter showed significantly less pathology [34]. We have now demonstrated that Th2 responses to NES are intact in μMT mice, and that no shift to Th1 occurs, suggesting that while B cells can drive and amplify Th2 development there is no requirement for them to do so in the context of NES stimulation.

Of particular interest is our finding that high levels of IL-10 are released in response to NES challenge, even in IL-$4^{o/o}$ animals. This implies that IL-10 release may not simply be a consequence of Th2 expansion, but may be required to initiate Th2 responses to helminths. This is exactly the conclusion proposed from studies on *Trichuris muris* and *Trichinella spiralis*, in which IL-10 deficiency abolishes the capacity of mice to mount a protective Th2 response [35,36]. Future studies with IL-10 gene-targetted animals could explore not only the relationship between nematode infection and Th2 generation, but also the possible involvement of IL-10 in down-regulatory interactions in chronic helminth infection [37].

Our studies have focussed on the initial source of IL-4 as a central ingredient of the Th2 environment. Interestingly, other investigators have indicated that the nature of TCR ligation and signalling is also important: for example, the Th2 response to *Nippostrongylus* infection is absolutely dependent upon CD4 expression by the naïve T cell population [30], arguing that *both* an appropriate cytokine milieu *and* a suitable strength-of-signal are necessary for Th2 development. Similarly, the absence of CD28 abolishes the Th2 response to *N. brasiliensis* infection [38]. However, in both cases there is no evidence of a consequent switch to the Th1 phenotype. Hence, the qualitative selection towards the Th2 pathway is more likely to be through non-TCR signals, while adequate TCR ligation remains the *sine qua non* for responsiveness.

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