Enrichment of IFN-\(\gamma\) producing cells in different murine adipose tissue depots upon infection with an apicomplexan parasite

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Here we report that lean mice infected with the intracellular parasite Neospora caninum show a fast but sustained increase in the frequency of IFN-\(\gamma\)-producing cells noticeable in distinct adipose tissue depots. Moreover, IFN-\(\gamma\)-mediated immune memory could be evoked in vitro in parasite antigen-stimulated adipose tissue stromal vascular fraction cells collected from mice infected one year before. Innate or innate-like cells such as NK, NK T and TCR\(\gamma\delta\)+ cells, but also CD4\(^+\) and CD8\(^+\) TCR\(\beta\)+ lymphocytes contributed to the IFN-\(\gamma\) production observed since day one of infection. This early cytokine production was largely abrogated in IL-12/IL23 p40-deficient mice. Moreover, production of IFN-\(\gamma\) by stromal vascular fraction cells isolated from these mice was markedly lower than that of wild-type counterparts upon stimulation with parasite antigen. In wild-type mice the increased IFN-\(\gamma\) production was concomitant with up-regulated expression of genes encoding interferon-inducible GTPases and nitric oxide synthase, which are important effector molecules in controlling intracellular parasite growth. This increased gene expression was markedly impaired in the p40-deficient mice. Overall, these results show that NK cells but also diverse T cell populations mediate a prompt and widespread production of IFN-\(\gamma\) in the adipose tissue of \(N.\) caninum infected mice.

The involvement of the adipose tissue in immune function has been increasingly recognized\(^1,2\). Indeed many cells of the immune system can be found in that tissue where some populations are enriched and/or display phenotypic characteristics distinct to those shown by cell counterparts present in lymphoid organs\(^3\). Since chronic low-grade adipose tissue inflammation has been associated with obesity-related diseases such as type 2 diabetes, many studies have addressed the immune components of adipose tissue in obese hosts\(^4,5\). However, the immune response to infection in the adipose tissue of lean hosts was studied in only a few reports that nevertheless showed that infections could have profound consequences in adipose tissue immune cell populations\(^6\). Increased numbers of macrophages and proinflammatory cytokines mRNA levels were observed in the adipose tissue of mice infected with diverse pathogenic agents such as adenovirus\(^3,7\), the bacterium \(Yersinia\) pseudotuberculosis\(^8\) and the protozoan parasites \(Trypanosoma cruzi\)^\(^7,8\).

\(N.\) caninum is an intracellular protozoan parasite, closely related to \(Toxoplasma gondii\), causative of clinical infections in diverse animal hosts occurring worldwide\(^9\). Neosporosis is particularly relevant in cattle where it is responsible for abortions causing heavy economic losses on dairy and beef industry\(^10\). Resistance against this infection has been associated with host production of pro-inflammatory cytokines IL-12 and IFN-\(\gamma\). Genetic deficiencies for these cytokines\(^10,11\) or their neutralization upon specific mAb administration\(^12,13\) confer lethal susceptibility to neosporosis in otherwise resistant murine strains. Accordingly, IL-12R\(\beta\) chain-deficient mice but not wild-type (WT) counterparts are also highly susceptible to \(N.\) caninum infection\(^14\). In non-infected hosts, several lymphocytic populations have been shown to produce IFN-\(\gamma\) in the adipose tissue such as CD8\(^+\) T cells\(^15\); CD4\(^+\) T cells\(^16\); CD11c\(^+\) dendritic cells\(^17\); CD11b\(^+\) macrophages\(^18\); and CD45\(^+\) monocytes\(^19\).
T cells24,25; invariant natural killer T (NKT) cells26, γδT cells27 and natural killer (NK) cells2. Increased IFN-γ mRNA levels, indicative of a Th1-type immune response, were previously observed in the gonadal adipose tissue of N. caninum infected hosts11. Therefore, we aimed here at determining whether production of IFN-γ could be promoted upon infection in distinct adipose tissue depots and which cell types could be the source of this cytokine. The obtained results show that distinct lymphoid cell populations in both visceral and subcutaneous adipose tissue contribute to IFN-γ production and that local early production of this cytokine is largely dependent on IL-12/IL-23 p40. Moreover, and interestingly, they also show that parasite-specific memory as revealed by IFN-γ production is maintained in the adipose tissue at least for one year upon the infectious challenge.

Results

IFN-γ is early produced in the adipose tissue of mice challenged with N. caninum tachyzoites. To determine which lymphoid populations could respond by producing IFN-γ in the adipose tissue of B6 mice infected intraperitoneally (i.p.) with N. caninum, we used flow cytometry and the gating strategy shown in Supplementary Fig. S1. The proportions of NK and NK T cells producing IFN-γ found were markedly increased as early as 24 h upon the parasitic challenge in all types of adipose tissue analysed (Fig. 1a,b). A slight increase in the frequency of IFN-γ− TCRγδ+ cells was also observed (Fig. 1c). Interestingly, CD8+ TCRγδ+ and CD4+ TCRγδ+ cells were also found to be early producers of IFN-γ in the infected mice, as detected in most adipose tissue depots analysed (Fig. 1d,e and Supplementary Fig. S2). Contrasting to this widespread cellular immune response detected in the gonadal, mesenteric and subcutaneous adipose tissue (GAT, MAT and SAT, respectively), only NK and CD4+ T cells produced IFN-γ in the mesenteric lymph nodes (MLN) of infected mice at frequencies higher than those detected in controls (Fig. 1a,e). CD4+ T cells simultaneously producing IL-10 and IFN-γ were also present at increased proportions in MAT, SAT and MLN of the N. caninum infected mice (Fig. 1e). Although early upon infection the proportions of IFN-γ−producing cells increased in all assessed populations, the numbers of NK cells producing IFN-γ per gram of adipose tissue were only found increased in SAT and those of TCRγδ+ cells in SAT, GAT and MAT (Supplementary Fig. S3). CD4+ T cells single producers of IL-10 were also detected at increased frequencies upon infection in GAT, MAT and MLN (Fig. 1e). Contrarily, CD4+ T cells single producers of IL-4 were slightly decreased in frequency and number in the GAT from infected mice (Supplementary Fig. S2). Altogether these results show that in the adipose tissue of N. caninum infected a prompt production of the host protective cytokine IFN-γ occurs, which is mediated by NK cells but also by different T cell populations.

Early production of IFN-γ in the adipose tissue of N. caninum infected mice is largely dependent on IL-12/IL-23 p40. In contrast to what was observed in WT mice the proportions of cells producing IFN-γ were not found above those of sham-infected controls in the MAT and SAT of infected IL-12/-IL-23 p40-deficient (p40−/−) mice except a detected increased frequency of IL-10 and IFN-γ− double-producing CD4+ T cells in the MAT (Fig. 2). Therein and interestingly CD4+ T cells single producers of IL-4 concomitantly decreased in both frequency and number (Supplementary Fig. S2). The effector function of IFN-γ includes the up-regulation of genes encoding proteins involved in inhibition of intracellular parasite growth such as interferon-inducible GTPases and inductible nitric oxide synthase28. In the infected WT mice a 14-, 26-, 17- and 2-fold increase was observed in all types of adipose tissue analysed (Fig. 1a,b). A slight increase in the frequency of IFN-γ− TCRγδ+ cells was also observed in the infected p40−/− mice (Fig. 1e). Although early upon infection the proportions of IFN-γ−producing cells increased in all assessed populations, the numbers of NK cells producing IFN-γ per gram of adipose tissue were only found increased in SAT and those of TCRγδ+ cells in SAT, GAT and MAT (Supplementary Fig. S3). CD4+ T cells single producers of IL-10 were also detected at increased frequencies upon infection in GAT, MAT and MLN (Fig. 1e). Contrarily, CD4+ T cells single producers of IL-4 were slightly decreased in frequency and number in the GAT from infected mice (Supplementary Fig. S2). Altogether these results show that in the adipose tissue of N. caninum infected mice a prompt production of the host protective cytokine IFN-γ occurs, which is mediated by NK cells but also by different T cell populations.

Production of IFN-γ in the adipose tissue of N. caninum infected mice is sustainably increased. Contrasting to the observation made 24 h upon infection when a marked increase in the frequency of IFN-γ−producing NK and NK T cells was observed in all adipose tissue depots analysed, by 7 and 21
Figure 1. Prompt increase in the frequency IFN-γ+ cells in the adipose tissue of N. caninum infected mice. Frequencies of (a) IFN-γ+ NK1.1+ TCRβ−TCRγδ− cells on total NK1.1+ cells, (b) IFN-γ+ NK1.1+ TCRβ+ TCRγδ− cells on total NK1.1+ TCRβ+ cells, (c) IFN-γ+ TCRγδ− NK1.1− cells on total TCRγδ− cells, (d) IFN-γ+ IL-10− CD8+ TCRγδ− NK1.1− cells on total CD8+ T cells and (e) IFN-γ+ IL-10+ CD4+ TCRγδ− NK1.1− and IL-10+ IFN-γ− CD4+ TCRγδ+ TCRγδ− NK1.1− cells on total CD4+ T cells, in the gonadal, mesenteric, omental and subcutaneous adipose tissue (GAT, MAT, OAT and SAT, respectively) and mesenteric lymph nodes (MLN) from wild-type C57BL/6 mice sacrificed 24 h after intraperitoneal challenge with 1 × 10⁷ N. caninum tachyzoites (NcT) or PBS, as indicated. Each symbol represents an individual mouse. Bars represent means of 9 mice per group pooled from 3 independent experiments. Statistically significant differences between different experimental groups are indicated (Mann-Whitney U, *P < 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001). Representative example of gating strategy used to define the respective cellular populations in the different depots of adipose tissue analysed. The example shown corresponds to MAT.
days this increase was slight and limited only to SAT in 7-day infected animals (Figs 5a,b and 6a,b). At this time point, the frequency of IFN-γ+ NKT cells actually decreased in the MAT (Fig. 5b). NK cells producing IFN-γ were also found increased in MLN by 7 days after the parasitic challenge while NKT cells did not respond in these lymphoid organs (Fig. 5a,b).

Figure 2. Impaired production of IFN-γ in the adipose tissue of infected IL-12/IL-23 p40−/− mice. Frequencies of (a) IFN-γ+ NK1.1+ TCRβ− TCRγδ− cells on total NK1.1+ cells, (b) IFN-γ+ NK1.1+ TCRβ+ TCRγδ− cells on total NK1.1+ TCRβ+ cells, (c) IFN-γ+ TCRγδ− NK1.1− cells on total TCRγδ− cells, (d) IFN-γ+ IL-10− CD8+ TCRβ+ TCRγδ− NK1.1− cells on total CD8+ T cells and (e) IFN-γ− IL-10− CD4+ TCRβ− TCRγδ− NK1.1−, IFN-γ− IL-10+ CD4+ TCRβ+ TCRγδ− NK1.1− and IL-10+ IFN-γ− CD4+ TCRβ− TCRγδ− NK1.1− cells on total CD4+ T cells in the mesenteric and subcutaneous adipose tissue (MAT and SAT, respectively) from IL-12/IL-23 p40−/− mice sacrificed 24 h after intraperitoneal challenge with 1 × 10^7 N. caninum tachyzoites (NcT) or PBS, as indicated. Each symbol represents an individual mouse. Bars represent means of 7–9 mice per group pooled from 2 independent experiments. (Mann-Whitney U, **P ≤ 0.01).

Representative example of gating strategy used to define the respective cellular populations in the different depots of adipose tissue analysed. The example shown corresponds to MAT.
Figure 3. Increased expression of interferon-inducible GTPases and nitric oxide synthase 2 in the adipose tissue of infected mice. Relative levels of immunity-related GTPase family M member 1 (Irgm1), interferon gamma induced GTPase (Igtp), guanylate binding protein 2, interferon-inducible (Gbp2), nitric oxide synthase 2, inducible (Nos2) and arginase (Arg1) mRNA, normalized to Non-POU-domain containing octamer binding protein (Nono) mRNA, detected by real-time PCR in the SVF of mesenteric adipose tissue of wild-type (WT) and IL-12/IL-23 p40−/− (p40−/−) mice 24 hours after intraperitoneal administration of 1 × 10⁷ N. caninum tachyzoites (NcT) or PBS. Each symbol represents an individual mouse. Bars represent means of 6 mice per group pooled from 2 independent experiments. (Mann-Whitney U, *P < 0.05; **P ≤ 0.01).
At day 7 upon infection a striking increase in the frequency of IFN-γ+ TCRγδ+ cells was observed in all adipose tissue depots analysed (Fig. 5c) that was still detected by 21 days after infection (Fig. 6c). T cells bearing αβ TCR also responded by producing IFN-γ in the infected animals with IFN-γ+ CD8+ and IFN-γ+ CD4+ T cells reaching proportions similar to those detected for γδ T cells (Figs 5c–e and 6c–e). Nevertheless, in the non-infected controls high proportions of αβ T cells either CD4+ or CD8+ producing IFN-γ were already detected (Figs 5d and 6d), that were higher than those found in the MLN of control mice (p < 0.0001 when comparing MLN vs GAT, MAT, OAT and SAT at any time point analysed, n = 9/group/time point). In agreement, others have reported a high frequency of IFN-γ-expressing T cells in visceral adipose tissue of lean hosts25. The frequency of IFN-γ+ CD4+ TCRβ+ cells increased upon infection in all depots analysed 7 days after infection, except OAT, and was still above controls by 21 days after infection (Figs 5e and 6e). Contrastingly, the proportions of IL-4-producing CD4+ T cells decreased in the GAT and MAT 7 and 21 days after infection and also in SAT in the later time-point (Supplementary Fig. S5). On the other hand, the frequency of IL-4 and IFN-γ double producers increased in MAT and OAT 7 and 21 days after infection and also in SAT 21 days after infection (Supplementary Fig. S5). The frequency of IL-10 and IFN-γ double producers within CD4+ T cells also increased 7 days upon infection in all tissues analysed, except OAT, and was still detected increased in GAT and MAT by 21 days (Figs 5e and 6e). IFN-γ+ CD8+ TCRβ+ cells were also found at increased proportions in MAT and SAT 7 days...
Figure 5. Sharp increase in the frequency of TCRγδ+ IFN-γ+ cells in the adipose tissue of *N. caninum* infected mice. Frequencies of (a) IFN-γ+ NK1.1+ TCRβ−TCRγδ− cells on total NK1.1+ cells, (b) IFN-γ+ NK1.1+ TCRβ−TCRγδ− cells on total NK1.1+ TCRβ+ cells, (c) IFN-γ+ TCRγδ− NK1.1+ cells on total TCRγδ− cells, (d) IFN-γ+ CD8+ TCRβ+ TCRγδ− NK1.1+ cells on total CD8+ T cells and (e) IFN-γ+ IL-10+ CD4+ TCRβ+ TCRγδ− NK1.1+ , IFN-γ+ IL-10+ CD4+ TCRβ+ TCRγδ− NK1.1+ and IL-10+ IFN-γ+ CD4+ TCRβ+ TCRγδ− NK1.1+ cells on total CD4+ T cells in the gonadal, mesenteric, omental and subcutaneous adipose tissue (GAT, MAT, OAT and SAT, respectively) and mesenteric lymph nodes (MLN) observed 7 days after intraperitoneal challenge with $1 \times 10^7$ *N. caninum* tachyzoites (NcT) or PBS, as indicated. Each symbol represents an individual mouse. Bars represent means of 9 mice per group pooled from 3 independent experiments. (Mann-Whitney U, *P* < 0.05; **P** ≤ 0.01; ***P** ≤ 0.001; ****P** ≤ 0.0001). Representative example of gating strategy used to define the respective cellular populations in the different depots of adipose tissue analysed. The example shown corresponds to MAT.
Figure 6. Sustained increase in the frequency of IFN-γ⁺ cells in the adipose tissue of *N. caninum* infected mice. Frequencies of (a) IFN-γ⁺ NK1.1⁺ TCRβ⁻ TCRγδ⁻ cells on total NK1.1⁺ cells, (b) IFN-γ⁺ NK1.1⁺ TCRβ⁺ TCRγδ⁻ cells on total NK1.1⁺ TCRβ⁺ cells, (c) IFN-γ⁺ TCRγδ⁺ NK1.1⁻ cells on total TCRγδ⁺ cells, (d) IFN-γ⁺ CD8⁺ TCRβ⁺ TCRγδ⁻ NK1.1⁻ cells on total CD8⁺ T cells, and (e) IFN-γ⁺ IL-10⁺ CD4⁺ TCRβ⁺ TCRγδ⁻ NK1.1⁻, IFN-γ⁺ IL-10⁺ CD4⁺ TCRβ⁺ TCRγδ⁻ NK1.1⁻ and IL-10⁺ IFN-γ⁺ CD4⁺ TCRβ⁺ TCRγδ⁻ NK1.1⁻ cells on total CD4⁺ T cells in the gonadal, mesenteric, omental and subcutaneous adipose tissue (GAT, MAT, OAT and SAT, respectively) and mesenteric lymph nodes (MLN) observed 21 days after intraperitoneal challenge with 1 × 10⁷ *N. caninum* tachyzoites (NcT) or PBS, as indicated. Each symbol represents an individual mouse. Bars represent means of 9 mice per group pooled from 3 independent experiments. (Mann-Whitney U, *P* < 0.05; **P** ≤ 0.01; ***P** ≤ 0.001; ****P** ≤ 0.0001). Representative example of gating strategy used to define the respective cellular populations in the different depots of adipose tissue analysed. The example shown corresponds to MAT.
Phenotype32,33, this likely explains the prompt production of IFN-\(\gamma\) in the infected mice. As the adipose tissue naturally presents a high frequency of T cells displaying a memory parasite inside macrophages than single IFN-\(\gamma\)-mediated long-term memory in adipose tissue of \(N.\ caninum\) infected mice. IFN-\(\gamma\) levels in the supernatants of mesenteric and subcutaneous adipose tissue (MAT and SAT, respectively) stromal vascular fraction cells cultured for 48 h alone (unstimulated) or in the presence of freeze-killed NcT (stimulated) recovered from control (PBS) or \(N.\ caninum\)-infected wild-type (NcT) mice, as indicated, one year after challenge. Each symbol represents an individual mouse. Bars represent means of 5–6 mice per group pooled from 2 independent experiments. (Mann-Whitney U, **\(P \leq 0.01\)).

and 21 days after infection and also in OAT in the later time point (Figs 5d and 6d). Altogether these results show that \(N.\ caninum\) infection induced a marked response by T cells in the adipose tissue that is biased towards the production of IFN-\(\gamma\), a host protective cytokine in this infection.

Having observed that in the first weeks upon infection adipose tissue T cells predominantly produced IFN-\(\gamma\), we further determined whether this response could lead to antigen-specific memory in this tissue. Therefore, we isolated SVF cells from the MAT and SAT of infected mice one year after the i.p. parasitic challenge and stimulated them in vitro with freeze-killed NcT. As shown in Fig. 7, higher levels of IFN-\(\gamma\) were detected in culture supernatants of killed-NcT-stimulated SVF cells isolated from MAT and SAT of infected mice as compared to those of controls from non-infected mice. To determine if one year after the parasitic challenge mice were still infected and to evaluate the possibility of infection recrudescence, the parasite burden was determined in the lungs, a major target organ in acute neosporosis30, GAT, that was previously shown to be transiently colonized after a similar i.p. infection31, and brain, a target organ for chronic \(N.\ caninum\) persistence32. Parasitic DNA was detected in the brain of 2 out of 5 infected animals whereas no parasitic DNA was detected in GAT or lungs indicating that although a chronic infection was established, the parasite did not reactivate. Altogether, these results show that parasite-specific long-term immune memory was maintained in the adipose tissue even in the absence of detectable local parasite colonization.

Discussion

In this work a prompt but sustained increase in IFN-\(\gamma\)-producing cells was observed in distinct adipose tissue depots of mice infected with \(N.\ caninum\). Bovine NK cells displayed increased IFN-\(\gamma\) production upon in vitro stimulation with \(N.\ caninum\)-infected bovine fibroblasts or with NcT33. Accordingly, in the infected mice higher proportions of IFN-\(\gamma\)-producing NK cells were observed in adipose tissue, concomitant with parasite detection. NK T cells were also early stimulated to produce IFN-\(\gamma\) in the adipose tissue of distinct anatomical locations. This effect was not observed in the MLN in accordance with the previous remarked particular characteristics of NK T cells present in adipose tissue3. Interestingly, CD4+ and CD8+ \(\alpha\beta\) T cells were also early producers of IFN-\(\gamma\) in the infected mice. As the adipose tissue naturally presents a high frequency of T cells displaying a memory phenotype32,33, this likely explains the prompt production of IFN-\(\gamma\) upon \(N.\ caninum\) infection. A previous report has shown that memory CD8+ T cells produce IFN-\(\gamma\) early upon infection in an antigen-independent manner, in response to IL-12 and IL-1834. Moreover, in vitro studies showed that CD8+ T cells isolated from the adipose tissue of lean mice produce IFN-\(\gamma\) in response to IL-12 and IL-18 alone35. As in p40−/− mice the increase in adipose tissue IFN-\(\gamma\)-producing CD8+ T cells proportions elicited by infection was abrogated it would be worth determining if it could depend on IL-12. The IL-12/IL-23 p40-dependent early production of IFN-\(\gamma\) in the adipose tissue was not confined to the CD8+ T cell population as it was also abrogated for NK T, TCR\(\beta\)-, and CD4+ T cells in infected p40−/− mice. In murine listeriosis, splenic NK1.1+ cells, CD8+ and CD4+ T cells were also shown to be early sources of IFN-\(\gamma\)35. Nevertheless, a slight increase in the proportions of CD4+ T cells simultaneously producing IFN-\(\gamma\) and IL-10 was still observed in \(N.\ caninum\) infected p40−/− mice. Interestingly in \(T.\ gondii\) infected hosts, a population of IFN-\(\gamma\) and IL-10 double-producing CD4+ T cells was shown to better control the replication of parasites inside macrophages than single IFN-\(\gamma\)-producers36. Therefore the increased frequency of CD4+ IFN-\(\gamma\)-producing IL-10+ T cells detected in the adipose tissue of infected p40−/− mice may nevertheless contribute to some control of local parasitic replication. In accordance with the increased proportions of these cells detected in the infected p40−/− mice, slightly increased levels of IFN-\(\gamma\) were detected in the culture supernatants of SVF cells isolated...
from MAT and SAT of infected p40−/− mice. This increase was however much lower than the one observed in similar cultures of cells obtained from infected WT mice, in accordance with the previously described impaired capacity of p40−/− mice to produce IFN-γ upon antigenic stimulation33. Moreover, freeze-killed NCT-induced in vitro the production of IFN-γ by MAT SVF cells of WT control mice while such effect was not induced in SVF cells of p40−/− mice further reinforcing the idea that the production of IFN-γ induced by N. caninum in the adipose tissue is mainly IL-12/IL-23 p40 dependent. Whether this effect could be mediated by IL-12, IL-23, p40 monomer or other putative heterodimer that can be formed with extracellular p40 monomer34 remains to be determined. As we detected these differences in IFN-γ production, we assessed whether this would translate into different expression levels of genes regulated by this cytokine, such as the ones encoding immunity-related GTPases (IRGs) and guanylate-binding proteins (GBP)s. These proteins are important for destruction of the parasitophorous vacuole in cells infected by the N. caninum related proteozoon T. gondii34. Increased expression levels of interferon-inducible GTPases mRNA were previously detected in the brain39,40 and spleen40 of N. caninum infected mice. In accordance, we observed here an up-regulated expression of Irgm1, Igt and Gbp2 in the adipose tissue early upon infection. Irgm1, IgtP and GBP2 have been shown to inhibit T. gondii replication in macrophages41,42. Therefore, a similar effect may also take place in the adipose tissue of N. caninum infected mice. Indeed, in p40−/− mice, where IRGs and GBP2 gene expression was only marginally up-regulated, a heavy parasitic colonization in the adipose tissue was previously observed 7 days after infection11. Nos2 expression, indicative of M1 type macrophage polarization, was found up-regulated in WT mice early upon infection whereas it was down-regulated in p40−/− mice. In vitro studies have shown that NO production by peritoneal macrophages induced by IFN-γ inhibits parasitic multiplication34. All these effector mechanisms that are down regulated or only slightly increased in the infected p40−/− mice can contribute to an impaired control of parasite replication locally in the adipose tissue. Moreover, Arg1 expression, that was found markedly up-regulated in the infected p40−/− mice, has been associated with increased host susceptibility in infections caused by other intracellular pathogens44.

TCR-γδ+ cells have been shown to mediate host protection in other parasitic infections45,46. As the population of γδ T cells was the only one consistently showing elevated proportions of IFN-γ+ cells in all adipose tissue depots and time points analysed upon infection it would be worth exploring its role in the course of N. caninum infection. Others have already implicated γδ T cells in host defensive mechanisms against this parasite in the bovine host37. CD4+ T cells, also implicated in host resistance to neosporosis46, were found to be producing IFN-γ+ in the adipose tissue upon N. caninum infection. Using OVA-specific OT-II mice, others have shown that adipose tissue SVF macrophages can promote IFN-γ production by CD4+ T cells37. As macrophages were found at increased numbers in the adipose tissue of N. caninum infected mice41, it would be interesting to determine whether these leukocyte cells could be promoting local lymphocyte IFN-γ production observed therein. Indeed, a previous report showed that N. caninum-challenged bovine macrophages can promote IFN-γ production by CD4+ T cells49. T cells simultaneously producing IFN-γ- and IL-10 were found to increase in different adipose tissue depots of the infected mice. This IL-10 production by these cells can be important to prevent IFN-γ mediated-immunopathology as described in T. gondii infection49.

Helminth parasites have been shown to promote Th2-type responses in the adipose tissue12,13. In N. caninum infected mice increased splenic mRNA and serum levels of the Th2-type signature cytokine IL-4 were previously observed49,50. In the adipose tissue a decreased frequency of IL-4 single producer CD4+ T cells concomitant with increased proportions of CD4+ T cells producing both IFN-γ and IL-4 was found. Memory Th2 cells may acquire expression of IFN-γ when primed in conditions promoting Th1 development52. A similar phenomenon may occur in the N. caninum infected mice as the majority of resident T cells in the adipose tissue already present an effector-memory phenotype12,33. A distinct splenic T cell population producing both IL-4 and IFN-γ has been also described in mice infected with helminth parasites, which induce marked Th2-type responses53,54. Similar double producers were found here in the context of a parasitic infection that induced a marked Th1 bias. It would be worth determining in future studies whether the concomitant IL-4 and IFN-γ production could be a mechanism limiting an excessive Th1-type immune response.

A role in initiating and maintaining adipose tissue inflammation has been previously suggested for CD8+ T cells55. CD8+ T cells were shown to promptly respond by producing IFN-γ in the intestinal mucosa of N. caninum-infected mice49. Here we also showed a prompt but persistent increase in the frequency of CD8+ T cells producing IFN-γ in the adipose tissue of the infected mice that was more marked by 21 days of infection than at previous time points. As IFN-γ produced by CD8+ T cells was shown to play a significant host protective role in neosporosis46, this population may contribute to local protection against this parasite. A persistently increased frequency of CD8+ T cells producing IFN-γ was also recently reported in VAT of mice infected with Listeria monocytogenes13. Others have shown the presence of CD8+ memory T cells in fat pad up to 59 and 296 days after infection with L. monocytogenes and vesicular stomatitis virus, respectively73. Moreover, increased proportions of activated CD8+ T cells, as well as of CD4+ T cells, were observed 15 months upon infection in the adipose tissue of virally infected hosts13. We show here that MAT and SAT SVF cells isolated from mice one year after infection was established produce high levels of IFN-γ upon in vitro parasite antigen-recall indicating that memory cells can persist in these tissues in the long-term and are responsive to N. caninum.

The majority of the studies addressing the host immunity to N. caninum focused on the immune response occurring in lymphoid organs. Here, we have addressed the immune response to N. caninum infection occurring in a non-lymphoid tissue and have shown that upon the parasitic challenge an IFN-γ-mediated response is fast and concomitantly elicited in both visceral and subcutaneous adipose tissue. Moreover we have identified NK cells as well as TCR-γδ+ cells and distinct TCRβ3+ cell populations as cell sources of this host protective cytokine. Altogether, our results highlight the involvement of the adipose tissue in the host protective immune response to N. caninum.
Methods

Mice. Female WT B6 mice (7–8 week old) were purchased from Charles River and kept at the animal facilities of the Institute of Biomedical Sciences Abel Salazar (Porto, Portugal) during the experiments. IL-12/IL-23 p40-deficient B6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine, USA) and housed and bred also at IC-BAS in individual ventilated cages. Hiding and nesting materials were provided. Procedures involving mice were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and directive 2010/63/EU of the European parliament and of the council of 22 September 2010 on the protection of the animals used for scientific purposes, and Portuguese rules (DL 113/2013). Authorization to perform the experiments was issued by competent national board authority, Direcção-Geral de Alimentação e Veterinária (0420/000/000/2012 and 0421/000/000/2015).

Parasites. NCT (Nc-1, ATCC® [50843]) were obtained as previously described. As the virulence of N. caninum is attenuated if maintained for a long time in tissue culture, in all our experiments the parasites used underwent < 15 in vitro passages from the original ATCC vial. The viability of the used inocula was confirmed in highly susceptible p40−/− mice. Others have shown that 2 h freezing at −70 °C was enough to inactivate NcT. Therefore, for preparation of freeze-killed NCT, suspensions of live tachyzoites (prepared as described above) were centrifuged at 1500 × g for 15 min at 6 °C, the supernatant discarded and the NCT containing-pellet kept at least four days frozen at −80 °C. On the day of the experiment, the pellet was resuspended in RPMI-1640 medium supplemented with 10 mM Hepes, 85 IU/ml penicillin, 85 μg/ml streptomycin, 62.5 μg/ml of amphotericin B, 50 μM 2-mercaptoethylamine (all from all from Sigma-Aldrich, St Louis, USA) and 10% FBS (Gibco, MA, USA) (complete RPMI) by passing through a syringe with a 25G needle and applied to the cell cultures.

Challenge infections. N. caninum infections were performed in 8–20 weeks-old female WT or p40−/− B6 mice by the i.p. route, by inoculation of 0.5 ml PBS containing 1 × 10⁶ tachyzoites. Mock-infected controls were similarly i.p. injected with 0.5 ml of PBS.

Collection of biological samples. Twenty-four hours, 7 and 12 months after infection, mice were isoflurane anesthetized for retro-orbital blood collection and euthanized by cervical dislocation. For flow cytometry analysis, GAT (VAT present in broad ligament of uterus and ovaries), MAT (VAT between the two peritoneal layers of the mesentery), OAT (VAT associated to the greater omentum; in the dissection, pancreas was carefully avoided), inguinal SAT (carefully avoiding inguinal lymph nodes) and MLN were removed and placed in Hank's balanced salt solution supplemented with 4% bovine serum albumin (BSA) and 10 mM Hepes Buffer (all from Sigma-Aldrich) for further analysis. In the one-year experiments, GAT, lungs and brain were collected from all mice and stored at −80 °C for DNA extraction.

Isolation of stromal vascular fraction cells. SVF cells were isolated as previously described in detail. Briefly, after collagenase II digestion, samples were homogenized to single-cell suspensions, passed through a 100 μm cell strainer and centrifuged at 280 × g for 10 min at 4 °C. Cells at the bottom, corresponding to the SVF were resuspended in complete RPMI medium for 48 h SVF cell cultures or in RPMI − 1640 supplemented with 10 mM Hepes, 60 IU/ml penicillin, 60 μg/ml streptomycin, 50 μM 2-mercaptoethylamine, and 10% FBS for SVF cell cultures to be used in flow cytometric analysis.

Flow cytometric analysis. For cytokine intracellular staining, SVF cells (1 × 10⁶ cells per well) isolated as described above were incubated for 4 h at 30 min at 37 °C with 500 ng/ml of ionomycin, 50 ng/ml PMA and 10 μg/mL of Brefeldin A (all from Sigma). Cells were pre-incubated with anti-mouse CD16/CD32 (clone 93) followed by surface staining with FITC anti-mouse TCR-β (clone GL3), APC anti-mouse NK1.1 (clone PK136), APC-eFluor® 780 anti-mouse CD8 (clone 53-6.7), eFluor® 450 anti-mouse TCR-γ (clone H57-597) (all from eBioscience, San Diego, CA) and Brilliant Violet 510™ anti-mouse CD4 (clone RM4-5) (BioLegend, San Diego, CA). Cells were then fixed with 2% formaldehyde, washed, permeabilized with 0.5% saponin (Sigma) and pre-incubated with anti-mouse CD16/Violet CD32 (clone 93) before intracellular staining with PE anti-mouse IL-10 (clone JES5-16E3), PerCP-Cyanine5.5 anti-mouse IFN-γ (clone XMG1.2) and PE-Cy7 anti-mouse IL-4 (clone 11B11) or respective isotype controls (PE Rat IgG2b, k (clone eB149/10H5); PerCP-Cyanine5.5 Rat IgG1 Isotype Control (clone eBRG1) and PE-Cyanine7 Rat IgG1 K (clone eBRG1). Data acquisition was performed on a FACSCanto II system (BD Biosciences, San Jose, CA) using the FACSDiva™ software (BD) and compensated and analysed in FlowJo version 9.7.5. (Tree Star, Inc., Ashland, OR). A biexponential transformation was applied to improve data visualization. Fluorescence minus one (FMO) gating was used to define the gates for IL-10−, IFN-γ− and IL-4− cells. Isotype controls were used to evaluate unspecific staining. Due to the high interference of PercpCy5.5 in the channel detecting PE-Cy7 and to assure that detection of IFN-γ− and IL-4 double production CD4+ T cells was not an artefact, in some experiments the same cells were also stained with FITC anti-mouse IFN-γ− (clone XMG1.2)(BD Biosciences) instead of PerCP-Cyanine5.5 anti-mouse IFN-γ− and no antibody was added in the PerCPCy5.5 channel (Supplementary Fig. 51c,d). By using this staining, similar frequencies of IFN-γ− and IL-4 double producer cells were obtained thus validating the presented results.

IFN-γ detection in culture supernatants of SVF cells. WT and p40−/− MAT and SAT SVF cells were added to 96 well plates (3.5 × 10⁵ SVF cells/well) and cultured for 48 h in complete RPMI alone or with 1.75 × 10⁶ freeze-killed NCT at a ratio cell:NCT of 1:5; prepared as described above. IFN-γ levels in culture supernatants were quantified with Ready-Set-Go™ ELISA (eBioscience) according to manufacturer's instructions.
Cytospin immunohistochemistry. Cytospins of SVF cells isolated from MAT and SAT of mice sacrificed 24 h after infection with *N. caninum* were prepared as follows. The slides were methanol fixed and specifically stained for *N. caninum* by a previously described protocol12. Briefly, peroxidase activity was blocked by treatment with 0.3% hydrogen peroxide in methanol (Merck, Darmstadt, Germany) for 20 min. Sections were then incubated in a moist chamber for 20 min with normal rabbit serum (Dako, Glostrup, Denmark) diluted 1:5 in 10% BSA (Sigma), to eliminate non-specific staining. Excess serum was removed and the sections were incubated at room temperature, 1h45 min with goat anti- *N. caninum* polyclonal serum (VMRD, Pullman, WA) diluted 1:1500. Sections incubated with anti- *N. caninum* antibody were washed and incubated for 30 min at room temperature with the peroxidase-labeled rabbit anti-goat secondary antibody (Millipore, Billerica, MA, USA) diluted 1:500. The colour in all sections was developed by incubation with 3,3′-diaminobenzidine (Dako). After counterstaining tissue sections with Mayer’s Haematoxylin (Merck), slides were mounted in Entellan (Merck). A positive reaction was indicated by the presence of brown cytoplasmic staining.

**PCR for the detection of NcT.** DNA from the brain, lungs, and GAT of infected and PBS-treated mice, or from NcT to use as positive standards, was extracted and *N. caninum* DNA was detected as previously described in detail13. DNA samples corresponding to 103 to 100 NcT were included as external standards.

**RNA isolation and real time PCR analysis.** Total RNA extraction (from 10⁴ MAT SVF cells of WT and p40⁻/⁻ mice) and cDNA synthesis were performed as previously described in detail11. Real-time PCR was then used for the semi-quantification of *Irgm1*, Igtp, Gbp2, Nos2 and Arg1 mRNA expression levels with the Kapa SYBR Fast qPCR Kit (Kapa Biosystems Inc, Wilmington, MA) in a Rotor-Gene 6000 (Corbett life science, Sydney, Australia). As reference genes we used *Hprt* and *Nono*. For the quantification of mRNA expression levels, the reaction was performed in a final volume of 10 μL containing 0.2 μM of each specific primer11,40:

| Forward Primer | Reverse Primer |
|----------------|----------------|
| CTCTCTGGGACCCTCATTG | AGGAGCTGTCATTAGGGACATC |
| Nos2 forward: ACATTGTGGCCCTCTGTGTG | Nos2 reverse: AGTCGCGGCTCATTAAAGC |
| *Nos2* forward: TGAGTACCTGGAACATTCACTGAC | *Nos2* reverse: GTCTATGTCTGTGGGCCTGA |
| Gbp2 forward: GTGAGGGCATGCGATAG | Gbp2 reverse: CTCTTGAGGCGGTCAACTGAC |
| Hprt forward: GATTTCTCTCTCTGTGTG | Hprt reverse: GCATTTTTCTCGGGACGG |
| Irgm1 forward: CTCTCTGGATCAGGGTTTGAGGAGTA | Irgm1 reverse: GCATTTTTCTCGGGACGG |

Individual relative gene expression values were calculated using the following formula: 2⁻ⁿ(sample/positive control) x 100.

**Statistical analysis.** Statistical significance of results was determined by non-parametric Mann-Whitney U test calculated with GraphPad Prism 6.0 software. (⁎P ≤ 0.05; ⁎⁎P ≤ 0.01; ⁎⁎⁎P ≤ 0.001; ⁎⁎⁎⁎P ≤ 0.0001). The data presented is from 2 to 3 pooled independent experiments with n = 6–9 mice/group as indicated in respective figure legends. Each individual mouse is represented in figures by a symbol and bars represent means of each experimental group.

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Acknowledgements
The authors are most indebted to Professor Artur Águas for fruitful discussions. The authors are also thankful to undergraduate students Daniela Costa e Sousa and Sofia Jesus for the assistance in some experiments. This work was supported by FEDER through COMPETE and by national funds through FCT-FCOMP-01-0124-FEDER-020158 (FCT reference: PTDC/CTV/122777/2010). Luzia Teixeira was supported by Fundo Social Europeu and Programa Operacional Potencial Humano through FCT Investigator Grant IF/01241/2014. Pedro Ferreirinha and Alexandra Correia were respectively supported by FCT fellowships SFRH/BD/76900/2011 and SFRH/BPD/91623/2012.

Author Contributions
L.T. conceived, designed, and conducted the experiments, analyzed the data and wrote the manuscript. R.M.M. and P.F. participated in the experiments and contributed to data analysis. J.M., F.B., J.M. and A.P. participated in experiments and contributed to data acquisition. P.G.F. and A.C. participated and assisted in the design of the experiments and contributed to data analysis. M.V. assisted in the design of experiments and contributed to the interpretation of data and manuscript writing. All authors read and approved the final manuscript.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Teixeira, L. et al. Enrichment of IFN-γ producing cells in different murine adipose tissue depots upon infection with an apicomplexan parasite. Sci. Rep. 6, 23475; doi: 10.1038/srep23475 (2016).

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