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Co-existence of classical and alternative activation programs in macrophages responding to *Toxoplasma gondii*

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Abstract

Pro-inflammatory M1 macrophages are critical for defense against intracellular pathogens while alternatively-activated M2 macrophages mediate tissue homeostasis and repair. Whether these distinct activation programs are mutually exclusive or can co-exist within the same cell is unclear. Here, we report the co-existence of these programs in *Toxoplasma gondii*-elicited inflammatory macrophages. This is independent of parasite expression of the virulence factor ROP16 and host cell expression of signal transducer and activator of transcription 6 (STAT6). Furthermore, this observation was recapitulated by IFN- γ and IL-4 treated bone marrow-derived macrophages in vitro. These results highlight the multi-functionality of macrophages as they respond to diverse microbial and endogenous stimuli.

Keywords

Toxoplasma gondii; Macrophage; Virulence factor; Classical activation; Alternative activation

Following the Th1/Th2 paradigm for CD4⁺ T cells, two polarized phenotypes for macrophages, namely classically activated macrophages (M1) and alternatively activated macrophages (M2), have been described (Gordon, 2003). M1-type macrophages are critical for host defense against intracellular pathogens and have roles in antitumor immunity and autoimmune inflammation (Murray and Wynn, 2011). M2 macrophages are protective against helminth parasites and are important regulators of the wound healing response, tissue homeostasis and adiposity (Murray and Wynn, 2011). During the course of an inflammatory response, phenotypic switching of macrophages from an M1 to an M2 program has been observed, underscoring the dual role of macrophages in initiating and subsequently

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resolving inflammation. Whether this switch occurs by local conversion of M1 macrophages to anti-inflammatory M2 cells (Arnold et al., 2007) or by sequential recruitment from distinct precursor populations from the blood is currently being debated (Nahrendorf et al., 2007). The question of whether the M1 and M2 transcriptional programs are mutually antagonistic or can co-exist within the same macrophage cell remains unresolved (Jenkins et al., 2011). In this report, we demonstrate the co-existence of M1 and M2 activation programs within the inflammatory macrophages responding to *Toxoplasma gondii* at the single cell level. Furthermore, neither the parasite expression of the virulence factor ROP16 nor the host cell expression of signal transducer and activator of transcription 6 (STAT6) was required for the observed phenomenon. This phenomenon was faithfully reproduced in an in vitro experimental model system.

To address to what extent expression of M1 and M2 activation programs overlap in vivo, we investigated the expression of M1 and M2 markers in macrophages responding to *T. gondii* infection, where induction of the M2-associated arginase-1 (Arg1) has been reported (El Kasmi et al., 2008), against the backdrop of a classical Th1-dominated immune response (Yap et al., 2006). C57BL/6 wild type (WT) mice were procured from the Jackson Laboratory, USA. Mice were maintained and bred under specific pathogen-free conditions at the University of Medicine and Dentistry of New Jersey (UMDNJ, USA) animal care facility, in accordance with NIH guidelines. The use of animals for research was carried out under a protocol approved by the UMDNJ Institutional Animal Care and Use Committee. Female mice between 8–12 weeks of age were used for all experiments. The uracil auxotrophic CPS strain (Fox and Bzik, 2002) was maintained and propagated in confluent human foreskin fibroblasts (HFF) under Mycoplasma-free culture conditions. Upon the lysis of HFF cells, *T. gondii* tachyzoites were harvested, counted and gamma (γ) irradiated at 15,000 rads before use.

To obtain *T. gondii*-elicited macrophages, we primed C57BL/6 mice with a non-replicative uracil auxotrophic CPS-null strain of *T. gondii* and harvested peritoneal exudate cells (PECs) on D5 as previously described (Ling et al., 2006). Indeed, western blot analysis of sort-purified F4/80⁺CD11b⁺ macrophages revealed the expression of both M1 (iNOS, IRF5, Irgm3) and M2 (Arg1, IRF4, FIZZ1) associated proteins (Fig. 1A). To determine whether the M1 and M2 markers are expressed by distinct macrophage subsets, we performed intracellular staining and flow cytometry to simultaneously monitor Irgm3 and IRF4 or FIZZ1, at the single cell level. The cells were blocked with 20 μ g/ml of anti-CD16/32 (2.4G2; BD Biosciences, USA) in FACS buffer (3% FBS, 0.1% azide in PBS pH 7.2). This was followed by surface staining of cells on ice with antibodies against F4/80 (BM8; eBioscience, USA), CD11b (M1/70; BD Biosciences), CD11c (HL3; BD Biosciences), CD4 (RM4-5; BD Biosciences) and CD8 α (53-6.7; eBioscience).

For intracellular detection, the surface stained cells were fixed with either 2% paraformaldehyde or BD Cytofix (BD Biosciences). Cells were then permeabilized with BD Perm/Wash buffer (BD Biosciences) and stained with rabbit anti-RELM α /FIZZ1 or normal rabbit IgG control followed by Zenon anti-rabbit reagent (Invitrogen, USA) staining. For detection of Irgm3, murine anti-mouse Irgm3 (BD Biosciences) conjugated to a fluorochrome was used. For detection of IRF4 and co-staining with Irgm3/FIZZ1, Irgm3 and

FIZZ1 co-stains, the cells were surface stained, fixed and permeabilized using a FoxP3 staining buffer set (eBioscience). The cells were stained with rat anti-human/mouse IRF4 (3E4; eBioscience) direct conjugate antibodies, rabbit anti-mouse FIZZ1 (PeproTech, USA), Zenon anti-rabbit reagent (Invitrogen) (Jenkins et al., 2011), and murine anti-mouse Irgm3 direct conjugated antibody. Samples were acquired using LSR II or FACSCalibur (Becton Dickinson, USA) and analysis was carried out using FlowJo v8.8.7 (Tree Star Inc., USA). In this study, macrophages were defined as being forward scatter^{high} and side scatter^{high}. These large granular cells were filtered for single cells and further gated exclusively on F4/80⁺CD11b⁺ cells. Expression of all intracellular markers was determined relative to corresponding isotype controls. Statistical analysis was carried out using GraphPad PRISM 4/Microsoft Excel. Data were analyzed by unpaired two-tailed *t* tests wherever applicable.

Surprisingly and unlike thioglycollate-elicited macrophages, nearly all of the *T. gondii*-elicited macrophages harvested 5 days after priming (D5) was positive for both Irgm3 and IRF4 (Fig. 1B, C). However, very few (5% double positive, DP) also expressed FIZZ1 (Fig. 1C). Despite the low frequency of FIZZ1⁺ macrophages on D5, essentially all of them expressed Irgm3 and IRF4, suggesting that the potential to express FIZZ1 is contained within the pre-existing dominant Irgm3/IRF4 DP population. Indeed upon rechallenge with *T. gondii*, FIZZ1 expression rapidly peaked at 8 h (Fig. 1B, C), declining within 24 h post-challenge (Fig. 1C). Importantly, at the peak of the FIZZ1 response, 94% of FIZZ1-expressing macrophages were also Irgm3-positive and 72% IRF4-positive (Fig. 1B). Taken together, our data provide a clear demonstration that the M1 and M2 programs can co-exist within the same macrophage cells responding to pathogen challenge.

The co-existence of the M2 program with the protective M1 response in *T. gondii*-elicited macrophages may result from an evasive maneuver of the parasite (Jensen et al., 2011). The virulence factor ROP16, a parasite encoded kinase, has been shown to phosphorylate host STAT3 and STAT6 (Saeij et al., 2007). We therefore determined whether expression of M2-associated markers required parasite expression of ROP16 or host expression of STAT6. The *STAT6*^{-/-} mice used for the experiments were obtained from Jackson Laboratory. The RH *ku80* (RH) and RH *ku80 rop16* (RH *rop16*) strains (Butcher et al., 2011) of *T. gondii* were developed in the laboratory of David Bzik (Dartmouth Medical School, Hanover, NH, USA). To our surprise, macrophages elicited in vivo by ROP16-deficient *T. gondii* parasites (Fig. 2A) or macrophages derived from STAT6-null mice (Fig. 2B) displayed the same induction and co-existence patterns for the M2 markers (IRF4, FIZZ1 and Arg1) assayed. These results suggest the existence of host factor(s) other than IL-4 or IL-13 that trigger induction of the M2 program and further indicate that this phenomenon is not a peculiarity of the specific *T. gondii* strains used to elicit the macrophages.

We next attempted to recapitulate our in vivo observations by simultaneously exposing bone marrow-derived macrophages (BMDMs) in vitro to IL-4 and IFN- γ , the canonical inducers of the M2 and M1 programs, respectively. This resulted in the additive or even synergistic upregulation of both M1 (iNOS, Irgm3) and M2 (Arg1 and Fizz1) associated markers, with no evidence for antagonism (Fig. 3A, B). Furthermore, single cell analysis of BMDMs treated with the IL-4/IFN- γ combination recapitulates the in vivo observation in that 96% of FIZZ1⁺ cells are also Irgm3⁺ (Fig. 3B).

Our data demonstrate that macrophages can respond to divergent environmental stimuli and simultaneously express M1 and M2 phenotypes. Induction of the M2 activation program within the same classically-activated macrophage would allow for efficient self-regulation of pro-inflammatory and cytotoxic responses during the progression of an immune response (El Kasmi et al., 2008). In addition to its potential role in the resolution of active inflammation (Stables et al., 2011), the expression of specific M2-associated enzymes may in itself be a functionally critical component of the antimicrobial armamentarium of these M1 macrophages. Recently, macrophages expressing an alternatively activated phenotype have been discovered to reside in the brain of *T. gondii*-infected mice. These cells mediate parasite cyst lysis via their production of chitinase (Nance et al., 2012). Our analysis of the phenotype of macrophages activated by *T. gondii* in the peritoneal cavity suggest that, instead of distinct M1 and M2 subpopulations, the chitinase-expressing macrophages could themselves be expressing the classical activated program activated by IFN- γ . Viewed more broadly, our observations are consistent with the potential for conversion of classically-activated into alternatively-activated macrophages through a dual M1⁺M2⁺ putative transitional stage, perhaps involving local tissue proliferation (Jenkins et al., 2011). Well-designed dual reporter mouse models will be required to track the dynamic evolution of macrophage phenotypes in vivo.

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References

- Arnold L, Henry A, Poron F, Baba-Amer Y, van Rooijen N, Plonquet A, Gherardi RK, Chazaud B. Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. *J Exp Med*. 2007; 204:1057–1069. [PubMed: 17485518]
- Butcher BA, Fox BA, Rommereim LM, Kim SG, Maurer KJ, Yarovinsky F, Herbert DR, Bzik DJ, Denkers EY. *Toxoplasma gondii* rhoptry kinase ROP16 activates STAT3 and STAT6 resulting in cytokine inhibition and arginase-1-dependent growth control. *PLoS Pathog*. 2011; 7:e1002236. [PubMed: 21931552]
- El Kasmi KC, Qualls JE, Pesce JT, Smith AM, Thompson RW, Henao-Tamayo M, Basaraba RJ, Konig T, Schleicher U, Koo MS, Kaplan G, Fitzgerald KA, Tuomanen EI, Orme IM, Kanneganti TD, Bogdan C, Wynn TA, Murray PJ. Toll-like receptor-induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens. *Nat Immunol*. 2008; 9:1399–1406. [PubMed: 18978793]
- Fox BA, Bzik DJ. De novo pyrimidine biosynthesis is required for virulence of *Toxoplasma gondii*. *Nature*. 2002; 415:926–929. [PubMed: 11859373]
- Gordon S. Alternative activation of macrophages. *Nat Rev Immunol*. 2003; 3:23–35. [PubMed: 12511873]
- Jenkins SJ, Ruckerl D, Cook PC, Jones LH, Finkelman FD, van Rooijen N, MacDonald AS, Allen JE. Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation. *Science*. 2011; 332:1284–1288. [PubMed: 21566158]
- Jensen KD, Wang Y, Wojno ED, Shastri AJ, Hu K, Cornel L, Boedec E, Ong YC, Chien YH, Hunter CA, Boothroyd JC, Saeij JP. *Toxoplasma* polymorphic effectors determine macrophage polarization and intestinal inflammation. *Cell. Host Microbe*. 2011; 9:472–483.

- Ling YM, Shaw MH, Ayala C, Coppens I, Taylor GA, Ferguson DJ, Yap GS. Vacuolar and plasma membrane stripping and autophagic elimination of *Toxoplasma gondii* in primed effector macrophages. *J Exp Med*. 2006; 203:2063–2071. [PubMed: 16940170]
- Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol*. 2011; 11:723–737. [PubMed: 21997792]
- Nahrendorf M, Swirski FK, Aikawa E, Stangenberg L, Wurdinger T, Figueiredo JL, Libby P, Weissleder R, Pittet MJ. The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. *J Exp Med*. 2007; 204:3037–3047. [PubMed: 18025128]
- Nance JP, Vannella KM, Worth D, David C, Carter D, Noor S, Hubeau C, Fitz L, Lane TE, Wynn TA, Wilson EH. Chitinase dependent control of protozoan cyst burden in the brain. *PLoS Pathog*. 2012; 8:e1002990. [PubMed: 23209401]
- Saeij JP, Collier S, Boyle JP, Jerome ME, White MW, Boothroyd JC. *Toxoplasma* co-opts host gene expression by injection of a polymorphic kinase homologue. *Nature*. 2007; 445:324–327. [PubMed: 17183270]
- Stables MJ, Shah S, Camon EB, Lovering RC, Newson J, Bystrom J, Farrow S, Gilroy DW. Transcriptomic analyses of murine resolution-phase macrophages. *Blood*. 2011; 118:e192–208. [PubMed: 22012065]
- Yap GS, Shaw MH, Ling Y, Sher A. Genetic analysis of host resistance to intracellular pathogens: lessons from studies of *Toxoplasma gondii* infection. *Microbes Infect*. 2006; 8:1174–1178. [PubMed: 16513380]

Highlights

- We report the co-existence of M1 and M2 activation programs in *Toxoplasma*-elicited macrophages at the single cell level
- The superimposition of the M1 and M2 activation is independent of parasite expression of the virulence factor ROP-16.
- The expression of M2-associated genes in *Toxoplasma*-activated macrophages does not require host cell expression of STAT6

Fig. 1.

Co-existence of M1 and M2 macrophage (MΦ) activation programs in *Toxoplasma gondii* elicited macrophages at the single cell level. Mice were primed with an i.p. injection of irradiated 2×10^6 carbamoyl phosphate synthase-deficient (CPS) tachyzoites as previously described (Ling et. al., 2006). At day 5 after initial priming (D5), another dose (1×10^6 i.p.) of parasites was administered (rechallenge). The peritoneal exudate cells (PECs) were harvested by peritoneal lavage with RPMI media (Invitrogen) supplemented with 1% FBS on designated day 5 (D5+0 h; no rechallenge), 8 h (D5+8 h), 24 h (D5+24 h) and 48 h (D5+48 h) after rechallenge for the kinetics experiment. For all other experiments, only

D5+0 h and D5+8 h time points were used. (A) Western blot analysis of M1 (iNOS, IRF5 and Irgm3) and M2 (IRF4 and FIZZ1) marker expression in lysates of naïve and FACS sorted F4/80⁺CD11b⁺ *T. gondii* elicited macrophages (harvested on D5). Peritoneal macrophages (F4/80⁺CD11b⁺) were sorted from CPS primed D5+0 h PECs. Alternatively, naïve or adherent macrophages were harvested from CPS primed D5+0 h PECs by plating for 4 h and extensive washing with cold PBS to remove non-adherent cells. Macrophages were lysed in radioimmunoprecipitation assay (RIPA) buffer (Sigma, USA) supplemented with protease inhibitors (Thermo Scientific, USA). Total protein was resolved on 12% SDS-PAGE gel and transferred onto polyvinylidene fluoride (PVDF) membrane. The membranes were blocked and probed with primary antibodies; rabbit anti-mouse FIZZ1 (PeproTech), rat anti-mouse IRF4 (eBioscience), murine anti-mouse arginase1 (BD, Biosciences), murine anti-mouse Irgm3 (BD Biosciences), rabbit anti-mouse IRF5 (Cell Signaling Technology, USA), rabbit anti-mouse iNOS (BD Biosciences) and subsequently developed using appropriate secondary reagents and standard methods. (B) Representative FACS plots of primed (D5+0 h) and rechallenged (D5+8 h) *T. gondii*-elicited macrophages gated on F4/80⁺CD11b⁺ cells ($n = 5$ mice/group). (C) This depicts the kinetics of induction of M1 and M2 activation programs in *T. gondii*-elicited macrophages at the single cell level. Time course of induction of M1 and M2 markers at the single cell level, ($n = 5$ mice per time point) is indicated. PECs were harvested at the indicated time points and used for monitoring M1 and M2 markers by intracellular staining (FACS). The Pie charts depict the fractional distribution of the frequencies of cells among F4/80⁺CD11b⁺ macrophages across aforementioned time points. Single positive, double positive (D.P) and double negative (D.N) macrophage frequencies are indicated according to the legend for each corresponding row.

Fig. 2.

Co-existence of M1 and M2 macrophage activation programs in *Toxoplasma gondii*-elicited macrophages is not dependent upon parasite ROP16 kinase or host signal transducer and activator of transcription 6 (STAT6). Fractional distribution of percentage positive cells among F4/80⁺CD11b⁺ macrophages in (A) RH/ROP16 deficient *T. gondii*-elicited and (B) CPS-elicited wild type (WT) and STAT6^{-/-} macrophages. The data are the averages of $n = 3-4$ mice per group. Single positive, double positive (D.P) and double negative (D.N) frequencies are depicted according to the legend for each corresponding row. All comparisons were not statistically significant except in IRF-positive cells (S.P. + D.P. populations) in STAT6^{-/-} mice during the Day 5 (D5)+0 h time point, ($P = 0.04$) and in Irgm3 and IRF4 D.P. populations, ($P = 0.04$). PECs (D5+0h) were harvested from (C) RH or ROP16-deficient *T. gondii* primed and (D) CPS-elicited WT or STAT6^{-/-} mice. Enrichment for adherent macrophages was achieved by plating and extensive washing of non-adherent cells. Lysates were prepared and used for western blotting for Irgm3 and Arg1 expression.

Fig. 3.

The co-existence of M1 and M2 macrophage activation programs can be demonstrated in bone marrow-derived macrophages (BMDMs) in vitro. Bone marrow cells were harvested by the lavage of marrow cavities of femurs and tibia/fibula of C57BL/6 wild type female mice. The cells were cultured in DMEM supplemented with 10% FBS in the presence of 40 ng/ml of recombinant murine macrophage-colony stimulating factor (mouse M-CSF) for 7 days. Cells were harvested with Cell Stripper (Cellgro/Mediatech, USA) and cultured either with plain medium or with IFN- γ (10 ng/ml), IL-4 (20 ng/ml) individually or in combination for 60 h at 37°C. All of the cytokines were procured from PeproTech. (A) Western blot analysis of M1s (iNOS, Irgm3) and M2s (Arg1, FIZZ1) in lysates of untreated or cytokine treated BMDMs. (B) Representative FACS plots of untreated or cytokine treated BMDMs gated on F4/80⁺CD11b⁺ cells.