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Macrophages overexpressing tartrate-resistant acid phosphatase show altered profile of free radical production and enhanced capacity of bacterial killing

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Abstract

Activated macrophages and osteoclasts express high amounts of tartrate-resistant acid phosphatase (TRACP, acp5). TRACP has a binuclear iron center with a redox-active iron that has been shown to catalyze the formation of reactive oxygen species (ROS) by Fenton's reaction. Previous studies suggest that ROS generated by TRACP may participate in degradation of endocytosed bone matrix products in resorbing osteoclasts and degradation of foreign compounds during antigen presentation in activated macrophages. Here we have compared free radical production in macrophages of TRACP overexpressing (TRACP+) and wild-type (WT) mice. TRACP overexpression increased both ROS levels and superoxide production. Nitric oxide production was increased in activated macrophages of WT mice, but not in TRACP+ mice. Macrophages from TRACP+ mice showed increased capacity of bacterial killing. Recombinant TRACP enzyme was capable of bacterial killing in the presence of hydrogen peroxide. These results suggest that TRACP has an important biological function in immune defense system.

Keywords: Antigen presentation; Immune defense; Phagocytosis; NADPH oxidase; NOS

Certain types of leukocytes (e.g., activated macrophages, neutrophils) produce and release reactive oxygen species (ROS) and nitric oxide (NO) in response to phagocytosis or stimulation with various agents. The enzyme responsible for the production of superoxide is a multi-component NADPH oxidase that requires assembly at the plasma membrane to function as an oxidase. The generation of superoxide requires a large increase in the consumption of oxygen, and has therefore been termed as "respiratory burst" or "metabolic burst" (since the NADPH requirement is provided by a large increase in glucose metabolism through the hexose monophosphate shunt).

NADPH oxidase reduces molecular oxygen to superoxide anion [1,2] which in turn dismutates to hydrogen peroxide (H_2O_2) and this reaction is further accelerated by superoxide dismutase (SOD). Hydrogen peroxide is not a free radical but it plays a radical forming role as an intermediate in the production of more reactive ROS including hypochlorous acid (HOCl) by the action of myeloperoxidase [3,4] and most importantly, forming hydroxyl radical ($^{\bullet}OH$) via oxidation of transition metals. The two highly reactive ROS molecules thereby

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formed in phagosomes, HOCl and ·OH, are highly toxic to bacteria ingested by the phagocyte and mediate the direct antimicrobial effects of ROS. The NO radical (NO·) is produced in higher organisms by the oxidation of one of the terminal guanidonitrogen atoms of L-arginine [5]. This process is catalyzed by one of the three isoforms of the enzyme nitric oxide synthase (NOS). In macrophages, this isoform is inducible NOS (iNOS) which is stimulated by cytokines, lipopolysaccharides, and other immunologically relevant agents [6].

Type-5 acid phosphatase (acp5) or tartrate-resistant acid phosphatase (TRACP) is a member of the family of iron containing purple acid phosphatases. It is expressed in osteoclasts, activated macrophages, and dendritic cells [7–9]. TRACP phosphatase activity is required for normal bone resorption, since a knockout causes a mild osteopetrosis which is exacerbated if the lysosomal acid phosphatase is also mutated [10,11]. As activated macrophages are known to possess elevated levels of TRACP, it has been suggested that TRACP has an important role in immune defense system. This is supported by the findings that TRACP co-localizes with major histocompatibility complex II (MHC II) molecules and phagocytosed *Staphylococcus aureus* in alveolar macrophages [12]. Furthermore, TRACP-deficient mice showed reduced capacity to clear bacterial pathogens from the peritoneal cavity in vivo [13]. TRACP contains two iron atoms, one of which is redox active and able to generate ROS through the Fenton's reaction [14–16]. Continuous oxidation and reduction of the iron center of TRACP produce both hydroxyl (·OH) and superoxide ($O_2^{-\cdot}$) radicals, and these have been shown to degrade type I collagen in vitro [16]. In osteoclasts, TRACP has been suggested to participate in destruction of endocytosed bone matrix degradation products transported from the ruffled border to the cell surface [17,18]. Also, a macrophage-like cell line overexpressing TRACP showed increased ROS production, suggesting that the enzyme can generate ROS in vivo [12] and TRACP could have an analogous role in pathogen clearance in antigen presenting cells.

We have previously generated a transgenic mouse line in which TRACP mRNA and protein are overexpressed, and in which bone calcium turnover is accelerated [19]. Here we have compared free radical production in macrophages of TRACP overexpressing (TRACP+) and wild-type (WT) mice, and studied bacterial killing activity of both recombinant TRACP enzyme and macrophages from TRACP+ and WT mice.

Materials and methods

Cell culture. For setting up bone marrow-derived macrophage culture femurs of 2-to 3-month-aged mice were dissected, bone marrow was flushed out with a 27-gauge needle, and cells were allowed to differentiate to macrophages for 5–7 days in RPMI medium supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10% fetal bovine serum (FBS), and 5 ng/ml M-CSF. Differentiation to macrophages was confirmed by F4/80 antibody staining, which is specific for monocyte/macrophage lineage [20].

Serum samples. The TRACP-overexpressing mice (TRACP+) were produced by pronuclear injection of a transgene containing the entire TRACP locus with an inserted SV40 enhancer [19]. In these mice, TRACP overexpression was similar in distribution to the expression of the endogenous enzyme. Transgene contains the three distinct promoters required for the expression in liver and kidney, macrophages, and osteoclasts [21]. Increased TRACP activity in transgenic mice was confirmed by measuring TRACP 5b activity from serum samples. Blood was collected from TRACP+ and WT FVB/N mice by heart puncture. Blood was allowed to clot for 30 min at room temperature and serum was separated by centrifugation. Serum samples were stored at −70 °C before analysis. Serum TRACP 5b activities were measured using immunoassay as described by Alatalo et al. [22].

Immunohistochemistry. Cells were fixed with 3% paraformaldehyde (15 min), permeabilized with 0.2% Triton X-100 (5 min), and blocked with 3% BSA/0.1% gelatine/PBS (30 min). For primary antibody incubations, cells were stained for 60 min with anti-TRACP rabbit polyclonal antiserum [23] diluted 1:600 with 0.5% BSA/0.2% gelatine/PBS or F4/80 rat monoclonal anti-mouse antibody (Serotec, Oxford, UK) diluted in 1:100. For TRACP visualization FITC-conjugated anti-rabbit antibody (Jackson Immuno Research Laboratories, West Grove, USA) in 1:50 dilution (30 min) was used as a secondary antibody. F4/80 antibody was followed by biotin-conjugated goat anti-rat antibody (DAKO, Glostrup, Denmark) diluted 1:400 (60 min) and samples were further incubated for 30 min with streptavidin-FITC (DAKO) in 1:150 dilution.

Measurement of intracellular oxidized state. ROS-levels of bone marrow-derived macrophages were measured using fluorescent probe, dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probes, Leiden, The Netherlands). This compound is capable of crossing the plasma membrane to enter into a cell, where after de-acetylation H_2DCF is susceptible to oxidation, generating a fluorescent product, DCF. Cells were grown almost confluent on 24-well plates. Medium was replaced with Hanks' solution containing 20 μM H_2DCFDA for 30 min, after which medium was changed to fresh Hanks' buffer containing 10μg/ml phorbol-12-myristate-13-acetate (PMA) as a stimulating agent. Fluorescence intensity was measured at 37 °C every 2 min using Victor^2 plate reader (Wallac, Turku, Finland) with an excitation filter of 485 nm and an emission filter of 535 nm.

Measurement of superoxide. Superoxide production was determined using a cell-impermeable, sulfonated tetrazolium salt WST-1, which is reduced by superoxide to a stable water-soluble formazan with high molar absorption coefficient [24]. Macrophages were grown on 96-well plates and the medium was replaced with 0.1 ml Hanks' solution containing 500 μM WST-1 with or without SOD (200 μg/ml). Samples were equilibrated at 37 °C and the reaction was initiated by adding 10 μg/ml PMA. Superoxide production was detected by measuring the reduction of WST-1 to a formazan at a wavelength of 450 nm using Victor^2 plate reader.

Measurement of nitric oxide. Nitric oxide was detected using DAF-FM diacetate (4-amino-5methylamino-2′,7′-difluorescein diacetate; Molecular Probes). This compound is essentially nonfluorescent until it reacts with NO to form a fluorescent benzotriazole. Cells were grown on 24-well plates with or without 10 μg/ml lipopolysaccharide (LPS) for 48 h before measurements. Cells were incubated in phenol red free Hanks' solution containing 5 μM DAF-FM diacetate for 45 min with or without PMA (10 μg/ml). Cells were washed to remove excess probe and incubated in fresh Hanks' buffer for an additional 20 min to allow complete de-esterification. Fluorescence intensity was measured using a Victor^2 plate reader with an excitation filter of 485 nm and an emission filter of 535 nm.

Bacterial killing assay. *Staphylococcus aureus* was grown to a mid log phase in Luria–Bertani medium, collected by centrifugation, suspended into 5% BSA/10% DMSO/PBS, and stored at −70 °C until used. The colony-forming units (c.f.u.) of the bacterial stock was determined by dilution and colony counting.

The involvement of TRACP in killing phagocytosed cells was tested using *S. aureus* and bone marrow-derived macrophages. Macrophage culture suspensions (0.4 ml) were mixed with bacterial suspensions (0.4 ml) and FBS (0.2 ml), and incubated with rotation at 37 °C for 45 min. One hundred microliters of the suspension was withdrawn, added to H_2O, and incubated for 15 min to lyse the macrophages. After vigorous vortexing, a dilution series was made in water and samples were plated on agar plates. Numbers of living bacteria (c.f.u.) were counted after overnight incubation.

When recombinant rat TRACP protein [25] was used, *S. aureus* were centrifuged down and resuspended in Hanks' solution. The bacterial solution (0.1 ml; approximately 75×10^6 bacteria) was incubated with recombinant TRACP (5 μg) with or without H_2O_2 (200 nM). As a control, *S. aureus* was also incubated without TRACP in the presence of 200 nM H_2O_2. Samples were incubated with rotation at 37 °C for 60 min. A dilution series of the samples were plated on agar plates, and c.f.u. was counted after overnight incubation.

Statistical analysis. Statistical analysis was performed with one-way analysis of variance, with $p < 0.05$ considered as statistically significant difference. One asterisk (*) indicates a p value between 0.01 and 0.05, two asterisks a p value between 0.01 and 0.001, and three asterisks (***) a p value of less than 0.001.

Results

Characterization of bone marrow-derived macrophages

In original description of the TRACP+ mice, the level of serum TRACP was not examined. To confirm the overexpression in mice that has been transferred between our two laboratories, we measured serum TRACP. The serum TRACP activity was approximately four times higher in TRACP+ mice than WT mice (data not shown). Overexpression in macrophages was examined in isolated bone marrow-derived macrophages. Previously such assays were based upon histochemical staining, which has a significant background due to other phosphatases. The macrophage phenotype of bone marrow-derived macrophages treated with M-CSF for 5–7 days was confirmed by staining with an antibody directed against the plasma-membrane differentiation marker, F4/80, which is a common marker to the mononuclear phagocyte system (Fig. 1A). This indicates that during cell culturing in the presence of M-CSF other cells died and final culture is a quite pure macrophage culture. Staining with anti-TRACP antibody showed clear TRACP protein expression in macrophages from TRACP+ mice whereas only weak staining was seen in WT macrophages (Figs. 1B and C). Hence, the transgene causes direct macrophage-specific overexpression of TRACP protein.

Effect of TRACP overexpression on intracellular ROS production

The intracellular oxidation status of bone marrow-derived macrophages was measured by following the oxidation of H_2DCF. ROS production by macrophages requires a trigger that can be mimicked by the protein kinase C agonist, phorbol myristate acetate (PMA). PMA-stimulated TRACP+ cells showed increased ROS production compared to WT macrophages (Fig. 2). This difference was seen within 10 min after addition of PMA ($p < 0.05$), and the difference was further increased during following period ($p < 0.01$).

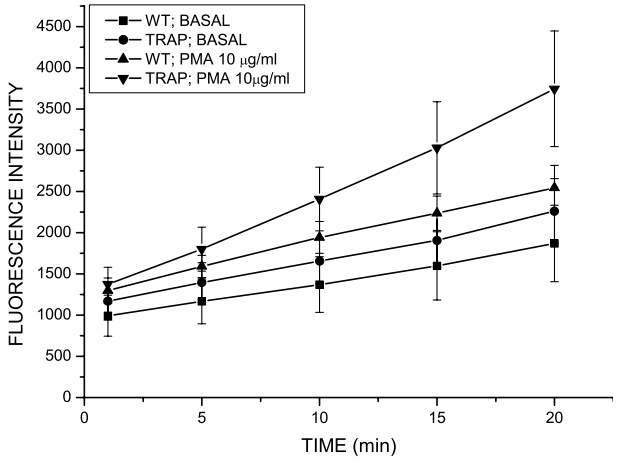

Fig. 2. Intracellular ROS production in WT and TRACP+ macrophages. Cells were stimulated with 10 μg/ml PMA and oxidation of H_2DCF was measured for 20 min using excitation and emission filters of 485 and 535 nm, respectively.

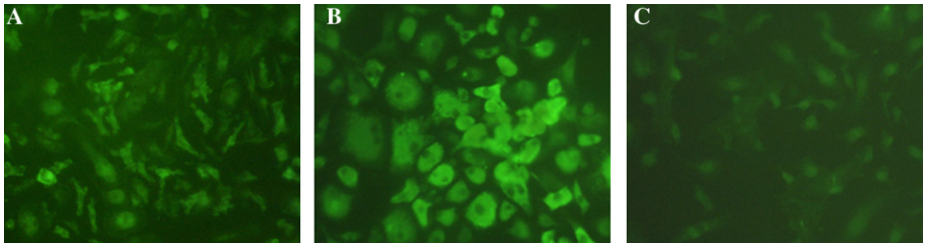

Fig. 1. Characterization of bone marrow-derived macrophages. (A) F4/80 positive macrophages. (B) TRACP protein expression in TRACP+ macrophages and (C) in WT macrophages. Cells were stained with F4/80 rat monoclonal anti-mouse antibody or rabbit anti-human TRACP antiserum followed by FITC-conjugated secondary antibody.

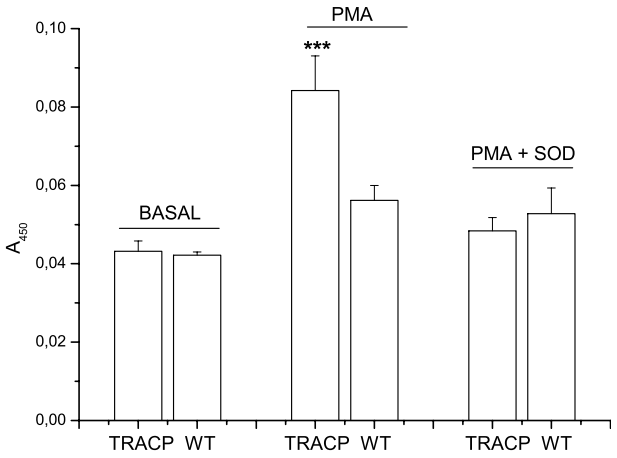

Fig. 3. Superoxide production in WT and TRACP+ macrophages. Amount of extracellular superoxide was measured by following a reduction of WST-1 to a formazan at a wavelength of 450 nm. Cells were stimulated with PMA (10 μg/ml) either in the presence or absence of SOD (200 μg/ml) to ensure the specificity of the measurement. Results are expressed as means ± SD, $n = 5$, $^{***}p < 0.001$.

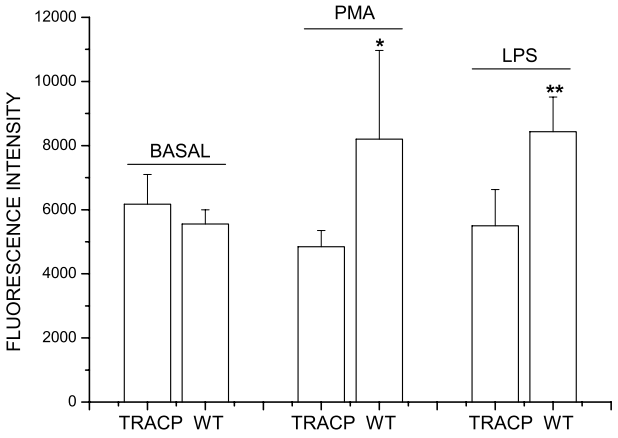

Fig. 4. Nitric oxide production in WT and TRACP+ macrophages. Cells were stimulated with PMA (10 μg/ml) or LPS (10 μg/ml). NO production was measured by following DAF-FM diacetate conversion to fluorescent compound using excitation filter of 485 nm and emission filter of 535 nm. Data are expressed as means ± SD, $n = 5$, $^{*}p < 0.05$, $^{**}p < 0.01$.

Without PMA stimulation, macrophages did not show significant difference in ROS production.

Effect of TRACP overexpression on superoxide production

WST-1 reduction was used to detect superoxide production of macrophages. PMA increased superoxide production both in WT and TRACP+ macrophages but the increase was significantly higher in TRACP+ cells (Fig. 3). PMA-stimulated WST-1 reduction was extensively inhibited (~90%) by addition of SOD indicating specificity of the measurement for the superoxide anion.

Effect of TRACP overexpression on intracellular NO production

To extend our study to the production of other reactive intermediates in the inflammatory responses, NO synthesis was determined using DAF-FM diacetate [26]. LPS and PMA activated NO production in WT macrophages, whereas no significant change was observed in TRACP+ macrophages (Fig. 4).

Bacterial killing assay

To study the effect of free radicals produced by TRACP on bacteria, bone marrow-derived macrophages were incubated with *S. aureus*. In these experiments, we were able to demonstrate that TRACP overexpression increased the capacity of bacterial killing (Fig. 5A). However, it should be noted that owing to the high standard deviation not all experiments demonstrated clear difference between WT and TRACP+ cells. For this reason, and to eliminate the possibility that TRACP overexpression has unrelated pleiotropic effects on the macrophages, we decided to introduce recombinant TRACP protein directly to bacteria. Recombinant TRACP protein was incubated with *S. aureus* and H_2O_2. TRACP protein was significantly more effective in killing bacteria than H_2O_2 alone (Fig. 5B). In the absent of H_2O_2, TRACP alone had no microbial activity, suggesting that the mechanism is, indeed, dependent upon the ability of TRACP to catalyze ROS generation (Fig. 5C).

Discussion

Oxidative killing is an important mechanism of host defense, as evidenced from the phenotype of individuals that lack ROS production, for example in chronic granulomatous disease. In the innate immune system, phagocytes are activated, and they produce ROS and reactive nitrogen species (RNS) in amounts sufficient to kill invading bacteria. Antigenic peptides generated within macrophages by the break down of pathogens are presented by MHC II molecules to the antigen receptors of T lymphocytes. TRACP expression in antigen presenting cells may indicate a role for it in antigen processing, especially in the light of its potential as a Fenton catalyst in ROS production. Macrophages from TRACP-deficient mice showed enhanced PMA- and interferon-γ-induced superoxide production, and also LPS-induced NO production was increased compared to WT mice [13].

We found altered free radical production in macrophages derived from TRACP+ mice. General ROS levels measured using H_2DCFDA revealed that TRACP overexpression increases ROS production when

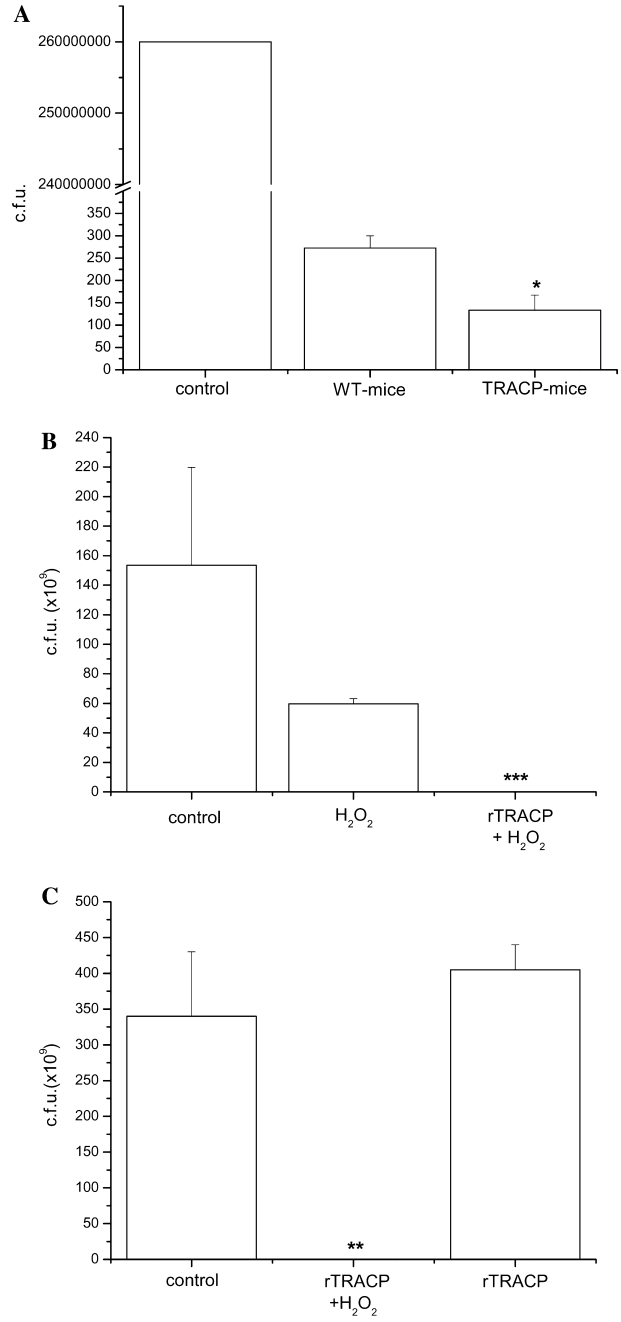

Fig. 5. The effect of TRACP on the viability of bacteria. (A) Macrophages from WT and TRACP+ mice were incubated with *S. aureus* for 45 min, after which the aliquots of solutions were plated on agar plates and c.f.u. was counted after overnight incubation. (B,C) Recombinant TRACP (rTRACP; 5 μg) and *S. aureus* were incubated for 60 min with or without H_2O_2 (200 nM), and c.f.u. were determined as above. In the control only bacteria were grown. Data are expressed as means ± SE, $n = 3$, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

macrophages are activated. This is consistent with our earlier findings where TRACP-transfected cells showed elevated levels of hydroxyl radicals compared to parental cells as detected using the deoxyribose method [12]. H_2DCFDA has been shown to detect at least H_2O_2 [27], $^{\bullet}OH$ [28], and peroxyl radicals ($HOO^{\bullet}$) [29]. Overall ROS production started to increase within 10 min of addition of stimulus, so we can assume that TRACP can respond quickly in a phagocytosis event. When ROS species produced by TRACP were studied more specifically, superoxide anion production was determined to be elevated. When redox-active ferrous ion of TRACP reacts with H_2O_2 by the Fenton's reaction, it produces a ferric ion and a hydroxyl radical. Ferric ion is still able to react with H_2O_2 to form a superoxide anion and regenerate a ferrous ion, so TRACP can also produce superoxide if enough H_2O_2 is available. Increased superoxide levels in TRACP-deficient macrophages were speculated to be a compensatory mechanism balancing radical levels by NADPH oxidase produced superoxide [13]. Surprisingly, both LPS- and PMA-stimulated NO production in WT macrophages but no significant change in TRACP+ macrophages was observed. We suggest that although ROS and RNS pathways are independent, they may compete for a common substrate (O_2), or their products may inactivate each other (e.g., (O_2^- and NO) [30]. An alternative is that ROS interfere in some way with the transcriptional induction of iNOS, which requires the redox-sensitive NF-κB pathway [31–34]. Further experiments should be done concerning cytokine profile of TRACP+ macrophages since Bune et al. [13] showed that TRACP-deficient macrophages had enhanced secretion of proinflammatory cytokines, tumor necrosis factor-α, and interleukin-1β and -12.

To determine if enhanced free radical production in TRACP+ macrophages has any effect on their capacity to destroy phagocytosed material, bacterial killing experiments were performed. Macrophages from TRACP+ animals showed a tendency to higher capacity for bacterial killing but we were not able to get repeatedly statistically significant difference compared to WT macrophages. When we used recombinant TRACP protein in similar assays, it killed bacteria significantly more effectively than H_2O_2 alone, which was added as a substrate for TRACP. When hydrogen peroxide was left out, TRACP protein lost its bacterial killing capacity, suggesting that this killing was due to function of radicals produced by TRACP. Further evidence that bacterial killing is linked to radical formation, and not, e.g., to phosphatase activity of TRACP, rises from the fact that experiments were done at neutral pH. The optimal pH for phosphoester hydrolysis of TRACP is close to 4.5 and for ROS generating activity to 6.5 [35]. One possible reason for the difference between results from TRACP+ macrophages and recombinant protein assays is that difference in ROS production between TRACP+ and WT macrophages was not high enough to lead into clear difference in bacterial killing experiments.

In summary, TRACP is able to produce ROS after appropriate stimulus and in macrophages these radicals most likely have a direct destructive effect on phagocy-

tosed material. These findings strongly support earlier ideas of the role for TRACP in immune defense system of macrophages.

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