Title:

A novel polymorphism in nitric oxide synthase interacting protein (NOSIP) modulates nitric oxide synthesis and influences mortality in human sepsis

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Abstract

Nitric oxide performs a wide variety of versatile functions in the immune system. But it's precise role in the pathogenesis of acute inflammation and sepsis is still controversial. In the present study, we demonstrate a novel mutation in nitric oxide synthase interacting protein (NOSIP) and its association with mortality in sepsis. We also show direct physical interaction of NOSIP with inducible nitric oxide synthase (iNOS), and demonstrate that differences in expression of NOSIP could influence differential induction of nitric oxide by monocytes/macrophages among species. Observations made in mice deficient in iNOS suggest that protective mechanism of nitric oxide in LPS induced inflammation is probably mediated by inhibiton of IL-1β synthesis. Differential nitric oxide production between mice and humans is also reflected upon IL-1β production between the species, where a clear inverse relationship emerges between nitric oxide and IL-1β. Thus, our study reveals NOSIP as an important regulator of inflammation by virtue of its ability to influence nitric oxide mediated inhibition of IL-1β synthesis and has opened up new avenues for therapeutic strategies against sepsis.

Introduction

Nitric oxide (NO) is a well-known modulator of a wide variety of immune functions[1]. After its discovery, the initial function of NO was ascertained as anti-microbial[2,3] and tumoricidal[4,5]. Later, NO was reported to regulate other facets of immune response like inhibition of T and B lymphocyte proliferation[6-10] and leukocyte trafficking[11]; modulation of cytokine, chemokine and growth factor production[12-16]; and T helper cell subtype differentiation[17-19].

Many reports in literature describe links between NO and sepsis. Demonstration of elevated levels of NO by-products in sepsis[20] and the fact that administration of NOS inhibitors were found to alleviate the hemodynamic manifestations of septic shock[21] led to the general belief that NO was detrimental to host during sepsis. This resulted in a large randomized controlled trial that involved treatment with a nonspecific NOS inhibitor[22]. However, the trial had to be terminated prematurely due to observed higher mortality in the treated group. On the other hand, a growing number of reports suggest a protective role for NO during inflammation and sepsis[23,24].

Nitric oxide synthase (NOS), the enzyme that makes NO in mammalian cells, has a multitude of interacting partners that regulate its function, localization and trafficking[25,26]. Of the three NOS isoforms, NOS1 (nNOS) and NOS3 (eNOS) binding proteins have been well characterized[25]. One such protein is nitric oxide synthase interacting protein (NOSIP), that was reported to interact with both nNOS[27] and eNOS[28] and mediate an inhibitory effect. On the contrary, interaction between NOS2 (iNOS) and NOSIP and it's role in regulating iNOS function has not been investigated.

A major source of controversy in literature has been differences in NO production among species, most notably between human and mouse[29,30]. It has been reported earlier that human monocytes and macrophages produce significantly lower amounts of NO than their mouse counterparts[31]. Whether such differences translate into differences in susceptibility to acute inflammation remains unknown

The present study was undertaken to resolve the controversies regarding function of nitric oxide in acute inflammation and sepsis. This study demonstrates for the first time a novel mutation in a regulatory region of human NOSIP gene which also was associated with increased mortality in sepsis. A combination of biochemical and imaging techniques were used to confirm interaction between iNOS with NOSIP. Using mice models of acute inflammation and human sepsis, this study demonstrates a protective role for nitric oxide during inflammation and sepsis. A significant negative association was observed between nitrite and inflammatory cytokines in both mice and humans. A search of literature revealed a hierarchy in nitrite production in species to LPS challenge/endotoxemia. A strong inverse association between plasma nitrite and toxicity/lethality due to LPS or sepsis was observed. Higher a species' nitrite production, higher is the absolute lethal dose (L.D.$_{100}$) of LPS, indicating that a host's susceptibility to LPS could, in part, be determined by its ability to produce nitric oxide. Mice deficient in *Nos2* gene were found to be more susceptible to LPS – mediated toxicity compared to wild type mice. Comparison of plasma cytokine levels between NOS2 deficient and wild type mice revealed increased IL-1β production in *Nos2* null mice, suggesting that protective mechanism of nitric oxide in acute inflammation could be via modulation of IL-1β activity. In the present study, it was also observed that species – specific differences in nitric oxide production could dictate differential susceptibility to inflammation and sepsis. Further, higher nitric oxide corresponded to lower induction of IL-1β which explains, in part, species specific sensitivity to acute inflammation. Our results also

indicate that differences in NO production between mice and humans could be a consequence differential expression of NOSIP between the two species.

Materials and methods

Reagents and kits

Gram-negative bacterial lipopolysaccharide (LPS, *E.coli* O55:B5), Ficoll Histopaque, Blood Genomic DNA extraction kit, Duolink Proximity Ligation Assay (PLA) kit, Nitrite/Nitrate estimation kit, Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), Bovine Serum Albumin (BSA), Phorbol 12-myristate 13-acetate (PMA), Paraformaldehyde (PFA) and Triton X-100 was obtained from Sigma chemicals. Dulbecco's Modified Eagle's Medium (DMEM), Foetal Bovine Serum (FBS), and Phosphate Buffered Saline (PBS) were from PAN Biotech GmbH. Acetic acid, citric acid, and dextrose was all from Fisher Scientific. Human 27-plex cytokine and Mouse 23-plex cytokine analysis kits were procured from Bio-Rad Laboratories. FACS lysing solution, Lyse/Fix buffer, Permeabilization buffer IV and FACSComp beads were bought from BD Biosciences. Brefeldin A was obtained from eBiosciences. *Taq* DNA polymerase for PCR was from Thermo Scientific. 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) and ProLong® Gold Antifade were purchased from Molecular Probes.

Antibodies

Anti-goat IgG Alexa Fluor 488 and anti-rabbit IgG Alexa Fluor 647 antibodies were purchased from Molecular Probes. Rabbit polyclonal antibody to iNOS was obtained from Abcam and goat polyclonal antibody to NOSIP was purchased from SantaCruz Biotechnolgies.

Human sepsis patients

The study was approved by ethics committees of Institute of Life Sciences and S.C.B. Medical College, and signed informed consent was obtained from all participants. 139 sepsis

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patients who were admitted to medical intensive care units at the Department of Medicine, S.C.B. Medical College and Hospital (Cuttack, India) were recruited for the study. For sepsis, patients were eligible for inclusion only if they had systemic inflammatory response syndrome and had a source of infection, proven or suspected. The acute physiological and chronic health evaluation II (APACHE II) scoring system was used to categorize the patients. Definitions of sepsis, severe sepsis, septic shock, and multi-organ dysfunction syndrome (MODS) were in accordance with published criteria[32,33]. The following categories of patients were excluded from the study: patients with diabetes mellitus, hypertension, nephrotic syndrome, chronic kidney disease (sonographic feature of CKD and/or GFR<30 ml/min), patients with cardiac failure and immunocompromised individuals. Blood was collected in vials containing 15% (v/v) Acetate Citrate Dextrose (ACD). This was followed by isolation of plasma by centrifugation at 2000 rpm for 10 min. Plasma was stored in single-use aliquots at -80°C.

Mouse model of endotoxemia

8-10 weeks old male C57Bl/6 mice were used for the study. All animal experiments were approved by the institutional animal ethics committee of Institute of Life sciences, Bhubaneswar. To simulate two groups based on lethality in human sepsis cases, a 5mg/kg non-lethal dose and 35mg/kg (lethal dose) of bacterial lipopolysaccharide (*E.coli* O55:B5, Sigma) was injected intraperitoneally. The animals were sacrificed at 2, 4, 8 and 16 hours post injection to mimic early and late stages of endotoxemia. Blood was collected in vials containing ACD as anticoagulant (15% v/v). collected blood was centrifuged at 2000 rpm for 10 minutes for isolation of plasma. Isolated plasma was stored in 100 μl single-use aliquots at -80°C.

Assessment of nitric oxide deficiency on LPS – mediated inflammation

8 – 10 weeks old male BALB/C mice were injected with 100 mg/kg L-NAME once every 24 hours for inhibition of nitric oxide synthesis. To examine effect of nitric oxide synthase inhibition on LPS mediated pathology, untreated or L-NAME treated mice were injected with 2 mg/kg LPS and mortality was monitored over a period of 4 days. For comparing LPS toxicity between wild type and *inos* null mice, 8 -10 weeks old male mice were injected with LPS at 15 mg/kg and either mortality was scored for 5 days or animals were sacrificed at 2, 6 and 12 hours to estimate cytokines in plasma.

Ex-vivo stimulation of human and mouse peripheral blood

Whole blood was withdrawn from healthy human volunteers by venepuncture or from normal healthy mice by cardiac puncture in acid citrate dextrose (ACD) anticoagulant at 15% v/v. Human donors were recruited from institute students. To test for cytokine expression, fifty microlitre whole blood was left untreated or stimulated with LPS at 1μg/ml for 2 hours in 37°C water bath along with brefeldin A (eBiosciences) at 1:1000 dilution. Post stimulation, cells were stained with fluorochrome conjugated specific antibodies and analysed by flow cytometry.

Measurement of cytokines in plasma

Human plasma was analysed using the human 27-plex cytokine panel (Bio-Rad) according to manufacturer's instructions and contained the following targets: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, Basic FGF, Eotaxin, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF, RANTES, TNF-α, and VEGF. Mouse plasma were analysed using the mouse 23-plex cytokine panel (Bio-Rad) as specified by the manufacturer and contained the following targets: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17, Eotaxin, G-CSF, GM-

CSF, IFN-γ, KC, MCP-1, MIP-1α, MIP-1β, RANTES, and TNF-α. All samples were read on a Bioplex 200 system (Bio-Rad). Concentrations of unknown samples were extrapolated from a 8-point standard curve fitted using a five-parameter logistic regression model.

Measurement of plasma nitrate and nitrite

Levels of nitrate and nitrite in human or mice plasma were estimated by a Griess colorimetric assay kit (Sigma chemicals) according to manufacturer's instructions. Briefly, nitrate in plasma was first converted into nitrite by treatment with nitrate reductase. Total nitrite was then estimated by a colorimetric reaction involving Griess reagent A (a solution of sulphanilamide in phosphoric acid) and Griess reagent B (Napthylethylenediamine in phosphoric acid). Absorbance was read at 540 nm. Unknown concentrations were estimated from a standard curve generated with known concentrations of sodium nitrite.

Assessment of intracellular localization of iNOS and NOSIP

Mouse and human whole blood was fixed, lysed and permeabilized followed by staining with anti-iNOS or anti-NOSIP antibodies. Followed by washing, the cells were analyzed on a AMNIS ImagestreamX for intracellular expression and colocalization. Alternately, the cells were fixed on a slide and co-localization was scored on a Leica Laser Scanning Confocal Microscope. For studying interaction between iNOS and NOSIP, *in situ* Proximity Ligation Assay was used using a commercially available kit (Sigma Duolink) according to manufacturer's instructions. In case of the human monocytic cell line THP-1, cells were grown in a coverslip - bottomed culture dish in complete IMDM media containing 10% fetal bovine serum. Cells were made to differentiate into macrophages by treatment with 10 nM Phorbol myristate acetate (PMA) for 48 hours. Following adherence, cells were washed with PBS and fixed with 2% paraformaldehyde (PFA). After rinsing with PBS, cells were permeabilized with 0.1% Triton X-100 following which they were incubated in a blocking

buffer containing 2% bovine serum albumin (BSA) and 2% fetal bovine serum (FBS) in PBS to minimize nonspecific binding of antibodies. After blocking, cells were first incubated with primary antibodies followed by fluorochrome conjugated secondary antibodies. Finally, cells were washed, counterstained with DAPI and image was acquired in a Leica SP5 confocal microscope.

Cloning, Expression and Purification of iNOS and NOSIP proteins

The human iNOS oxygenase domain containing 424 nucleotide sequence and NOSIP gene containing 301 nucleotide sequences were synthesized by GenScript and cloned in pET22b expression vector. The recombinant pET22b: iNOS and pET22b: NOSIP plasmid was used to transform competent E. coli BL21 (DE3) cells. The protein was over expressed using 0.2 mM IPTG at 250C. The harvested cells were resuspended and sonicated in 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.05% Triton X-100 and protease inhibitor (Sigma). The expressed proteins were purified by nickel nitrilotriacetic acid column using 250 mM imidazole.

Physical interaction by Surface Plasmon Resonance

Interaction of purified NOSIP with iNOS protein was monitored using Bio-Rad XPR 36 surface Plasmon resonance biosensor instrument. About 3 μg/ml of iNOS was immobilized on GLC chip by amine coupling method according to manufacturer's instructions. NOSIP was injected at concentration of 500nM with running buffer composed of PBS and 0.005 % Tween-20 at a flow rate of 50 μl/min. Molecular interaction was carried out at 20 °C. Further, kinetic parameter were determined, after fitting the association and dissociation curves to a 1:1 (Langmuir)-binding model. An activated channel without immobilized ligand was used to evaluate nonspecific binding. The response curves were also recorded on control surfaces. Results were calculated after subtraction of the control values using the ProteOn Manager software.

Identification of NOSIP gene polymorphism

Genomic DNA from whole human blood was isolated using a commercially available kit (Sigma) according to manufacturer's protocol. The *NOSIP* gene present on human chromosome number 19 spans over nine exons. A 503bp segment spanning exon 1 was amplified and checked for novel polymorphism in a cohort of 49 sepsis cases. Primer sequences for amplification are: Forward - 5' TCCCCATATTCCCACCAGTTTC 3'; and Reverse - 5' GCCGATGCTAGCTACCACTTGA 3'.. PCR was performed for 20 µl reactions containing 2 µl genomic DNA, 1X PCR buffer containing $MgCl_2$ (Sigma), 250 µM dNTP (Sigma), 10 pM of each of forward and reverse primers and 1U of Taq DNA polymerase (Thermo Scientific). PCR cycling conditions were as follows: an initial denaturation step at 94°C for 2 minutes; followed by 35 cycles of 94°C for 15 seconds, annealing at 56°C for 30 seconds and extension at 72°C for 30 seconds; and a final extension at 72°C for 5 minutes. Amplification was checked by running PCR products on a 2% agarose gel. The amplified PCR products were then sequenced on a Genetic Analyzer 3500 DNA sequencing platform (Applied Biosystems).

RNA extraction and qRT-PCR

Total RNA was extracted from mouse or human whole blood using Trizol LS reagent (Invitrogen). Isolated RNA was converted to complementary DNA (cDNA) using RT^2 first strand cDNA synthesis kit (Qiagen). The cDNA thus obtained was subjected to quantitative real-time PCR analysis with RT² SYBR® Green qPCR Mastermix (Qiagen) in 20 µl reaction volumes on a LightCycler 480 thermal cycler (Roche).

Statistical analysis

For comparison between two groups, either an unpaired t test or the nonparametric Man – Whitney U test was conducted. One way analysis of variance (ANOVA) followed by

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Bonferroni's multiple comparison test was carried out to compare means between three or more groups. Difference in survival among different groups was estimated by constructing Kaplan – Meier survival curves followed by assessment of significant difference using the log rank test. Fisher's exact test was used for comparison of genotype, allele frequencies and to test association of combined genotype distribution among various clinical categories. For all statistical comparisons, a p-value <0.05 was considered statistically significant.

Results

Direct binding of NOSIP with iNOS

Since it was already shown by others that NOSIP interacts with eNOS (endothelial NOS, NOS3) and nNOS (neuronal NOS, NOS1) and inhibits their function, the first objective was to investigate whether NOSIP also interacts with iNOS in a similar manner. The study was conducted by two different cell based assays and one cell free assay system using recombinant iNOS and NOSIP proteins. Co–localization of iNOS and NOSIP in primary human monocytes and the human monocytic cell line THP-1 indicated possible interaction between the two proteins (Figure 1A and B). To confirm this finding, a more definitive assay developed in recent times called proximity ligation assay (PLA) was employed to study interaction between iNOS and NOSIP in primary human monocytes. When PLA was performed with antibodies to iNOS and antibodies to NOSIP, bright fluorescent spots were observed that were otherwise absent if either of the two antibodies was used, indicating a direct physical interaction between iNOS and NOSIP in primary human monocytes (Figure 1C). Final demonstration of binding of NOSIP to iNOS came from Surface Plasmon Resonance studies with purified iNOS and NOSIP proteins, where a K_D value of 7.11×10^{-9} M confirmed a direct and intense interaction between the two proteins (Figure 1D).

A mutation in NOSIP gene leads to increased protein expression and and is associated with higher mortality in patients with sepsis

Multiple sequence alignment of NOSIP protein sequence between different species ranging from zebrafish to humans revealed remarkable conservation (Figure 2A). Therefore, it became important to search for novel polymorphisms in the NOSIP gene that may have significant functional consequences. A 503bp fragment spanning the first exon of NOSIP was amplified and sequenced. Sequence matching of 49 DNA samples isolated from sepsis patients with that of the database revealed presence of a four nucleotide deletion (TCTC) downstream of exon one (Figure 2B) in about 55% (27/49) individuals. Comparison of protein expression of NOSIP in healthy volunteers showed increased intracellular expression of NOSIP in individuals with deletion allele (Figure 2C). qRT-PCR analysis of comparison of gene expression in normal healthy laboratory controls clasified according to NOSIP gene mutation revealed higher expression of IL-1β, TNF-α, iNOS and NOSIP in individuals with deletion allele (Figure 2D), suggesting increased inflammation in these individuals. Not surprisingly analysis of mutant genotype distribution in patients with sepsis revealed significantly higher mortality among individuals displaying homozygous deletion mutation with a significantly high odds ratio (5/8 deaths, P-value = 0.0159, OR = 10.56, 95% C.I. = 1.611 to 69.15) (Table 1).

Taken together, above data suggests that higher intracellular expression of NOSIP could lead to increased risk of sepsis associated mortality by inducing increased inflammation.

Inverse association of plasma nitrite with inflammatory cytokines

The observation that increased NOSIP may be harmful to host during acute inflammation by lowering NO synthesis suggests a role played by NO during acute inflammatory disorders. In

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order to gain insight into the role of nitric oxide in acute inflammation, plasma nitrite levels were estimated by the Griess colorimetric reaction and a multivariate correlation analysis of plasma cytokines and nitrite was conducted. A significant negative association was observed between plasma nitrite and inflammatory cytokines, as shown in Table 2. This observation indicated a possible protective role of nitrite in human sepsis. To test this hypothesis experimentally, male C57Bl/6 mice were injected with two different doses of LPS and plasma cytokines and nitrite were estimated at 2, 4, 8 and 16 hours post injection. As shown in Table 2, plasma nitrite negatively correlated with inflammatory mediators in a mouse model of experimental endotoxemia. These observations further suggested a protective role for nitric oxide in pathogenesis of endotoxemia.

Deficiency in nitric oxide synthesis increases susceptibility to acute inflammation by increased IL-1β production

To investigate the effect of nitric oxide deficiency on LPS – induced inflammation, male BALB/c mice were injected by IP route with 100 mg/kg of Nω-Nitro-L-arginine methyl ester (L-NAME) hydrochloride once every 24 hours to block nitric oxide synthase activity. L-NAME is a structural analogue of L-Arginine that inhibits all three isoforms of nitric oxide synthase. Untreated and L-NAME treated mice were then injected with 2 mg/kg of LPS (sub-lethal dose that does not induce mortality in normal mice) and mortality was assessed over a period of 96 hours. Figure 3A reveals comparison of LPS induced mortality between untreated and L-NAME treated animals – the results clearly demonstrate that inhibition of nitric oxide synthesis is detrimental to host in a mouse model of endotoxemia. This was further tested by studying LPS induced mortality in mice deficient in Nos2 gene. Comparison of a sub-lethal dose of LPS between wild type C57Bl/6 mice and Nos2 null revealed that 15mg/kg of LPS failed to induce mortality in wild type mice, while about 84% of the animals in Nos2-/- group died at the same dose of LPS (Figure 3B). This further confirmed the

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hypothesis that absence of nitric oxide inducing enzyme and consequently nitric oxide leads to increased susceptibility to LPS. To test possible mechanisms of the observed phenomenon, wild type and Nos2-/- mice were injected with LPS at a dose of 15mg/kg and the animals were sacrificed at 2, 6 and 12 hours post injection for estimation of cytokines in plasma. The results shown in Figure 3C reveals significantly higher production of interleukin-1β (IL-1β) in mice lacking the Nos2 gene. However, plasma TNF-α level were comparable (Figure 3C), suggesting that protection against endotoxemia/sepsis in wild type could have been mediated by inhibition of NLRP3 inflammasome pathway by nitric oxide. Indeed, an independent study conducted by another group also suggests such a possibility[34].

To test whether nitric oxide − mediated inhibition of IL-1β activity can be used to predict differences in LPS toxicity among species, human and mouse whole blood were stimulated ex-vivo with LPS and intracellular IL-1β and TNF-α were measured by flow cytometry. The assumption for this study was that human monocytes which are known to sysnthesize insignificant nitric oxide in comparison to mouse monocytes would systhesize relatively more IL-1β. Figure 3D shows that this indeed is the scenario- human peripheral blood monocytes produced significantly higher IL-1β as compared to mice upon stimulation with LPS. However, TNF-α production between the two species was comparable (Figure 3D). A comparison of intracellular iNOS and NOSIP expression between circulating monocytes of human and mouse revealed comparable levels of iNOS but significantly higher levels of NOSIP in human cells, suggesting that low NO production by human monocytes could be a consequence of higher inhibition of iNOS activity by high expression of NOSIP (Figure 3E).

Next, a search of existing literature was performed to assess association between plasma nitrite and susceptibility to acute inflammation or sepsis. Data on susceptibility to LPS were either obtained from the present study as in the case of C57Bl/6 mice, or derived

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from a previously published report[35]. Data on plasma nitrite of rabbit, sheep and guinea pig were from previously published reports while plasma nitrite values of human and mouse were from the present study. A strong direct association between plasma nitrite levels and higher lethal dose to LPS or sepsis was observed (Figure 3F), suggesting that higher a species' ability for nitrite production, higher is it's ability to resist pathology asscociated with inflammation.

The above findings demonstrate that nitric oxide protects against experimental endotoxemia by inhibiting IL-1β activity. Further, differential synthesis of nitric oxide and consequently IL-1β may be used to predict species – specific differences in sensitivity to acute inflammatory response syndromes.

Discussion

Nitric oxide (NO) is synthesised by many cell types in immunity and inflammation[36]. The principal enzyme responsible for nitric oxide production in immune cells is the inducible type – 2 isoform of nitric oxide synthase (iNOS or NOS2), which produces high – level sustained NO synthesis during acute inflammation. There is considerable confusion regarding the role of nitric oxide in sepsis[37]. Use of iNOS inhibitors for treatment in the past yielded disappointing results in preclinical models. Given the results of the current study, this is not surprising since iNOS-derived NO displays potent anti-inflammatory effects. A recent study by Fletcher *et al.* now documents an unexpected therapeutic potency of the transfer of *ex vivo* expanded fibroblastic reticular cells (FRCs) into mice with endotoxemia or cecal-ligation-and-puncture induced sepsis which was dependent on iNOS activity expressed by the FRCs[38]. In a similar sepsis model, iNOS-dependent upregulation of cGMP and subsequent activation of TACE (expansion of TACE) was found to protect against organ injury[39]. Other studies in animal models have also documented possible protective role of NO in

endotoxemia[40]. The present study was undertaken to investigate the role of NO in acute inflammation and sepsis.

The single most important finding of the present study was identification of a novel modulator of iNOS activity. Nitric oxide synthase interacting protein (NOSIP) was initially reported to interact with eNOS and inhibit its function by sequestering it in Golgi complex[28]. Subsequently, interaction of NOSIP with nNOS was also reported[27]. However, till date interaction between NOSIP with iNOS and its role in immune response has not been reported and the current study fills this lacuna in literature. The study demonstrates physical interaction of NOSIP with iNOS (both in intact cells and in cell free systems) and suggests that differences in intracellular levels of NOSIP among species could be responsible for differential nitric oxide metabolism. More significantly, a four nucleotide deletion upstream of first exon of *NOSIP* gene in humans was found to be associated with increased intracellular expression of NOSIP protein and increased risk of mortality in patients with sepsis. This observation has opened up an entirely new area of therapeutic strategy against sepsis and possibly for management of acute inflammation in general.

An inverse association of plasma nitrite with inflammatory cytokines was observed in human sepsis and mouse model of endotoxemia, suggesting a reciprocal regulation of nitric oxide and inflammatory mediators. Species-specific differences in nitric oxide synthesis have been well documented in literature[29,30,41]. Whether such differences translate to observed differences in toxicity to LPS was one of the key questions that has been addressed in the present study. The results reveal a clear positive association between nitrite oxide production and lethal dose of LPS, suggesting that a given species' ability to tolerate inflammatory insult is, at least in part, dictated by its ability to produce nitric oxide. This conclusion may have important bearings to a study that demonstrated proteins in serum rather than intrinsic cellular differences may play a role in regulating variations in resilience to microbe-associated

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molecular patterns between species[35]. It becomes apparent that identification of such proteins may open up new areas of targeted therapy. However, given the sheer number of different proteins in serum, this is an arduous task. The observations made in the current study could assist in narrowing down the search field by initially probing enzymes involved in nitric oxide biosynthesis.

Another important finding of the present study was increased IL-1β production in mice lacking a functional *iNOS* gene, suggesting that protective effect of nitric oxide in endotoxemia is mediated through inhibition of IL-1β synthesis. However, TNF-α levels remained comparable between wild type and iNOS deficient mice. This is particularly interesting especially in the light of an earlier study that demonstrated no difference in LPS toxicity between wild type and TNF-α knockout mouse[42]. On the other hand, mice deficient in IL-1β converting enzyme fared much better in comparison to wild type mice when injected with 800 μg LPS intraperitoneally[43]. These observations suggest a central role for IL-1β in mediating pathogenesis due to endotoxemia and sepsis. Similar to nitric oxide, species specific dichotomy in terms of IL-1β production was observed between human and mouse circulating immune cells that negatively correlated with nitric oxide production, further demonstrating IL-1β could be a central pathogenic hub in endotoxemia.

Despite advances in both understanding of basic biology as well as better critical care support, mortality due to sepsis remains unacceptably high[44]. Currently, no effective therapy exists to combat human sepsis. Given the protective role of nitric oxide in host response to sepsis, it is tempting to suggest use of nitric oxide donors for treatment of sepsis. Indeed, several studies have attempted NO supplementation in sepsis, and a systematic review and meta-analysis of such studies revealed that this line of therapy could be promising[45]. However, due to extremely short half-life of nitric oxide, NO donors need to be administered at very frequent intervels, thus potentially raising the cost of treatment. In this regard, the

identification of NOSIP as a key regulator of NO synthesis immediately opens up exciting possibilities for targeted therapy. Designing small molecule inhibitors of iNOS-NOSIP interaction could result in increased NO production which, in turn, should control inflammation. However, considering the conserved nature of NOSIP with ability to regulate NO production by neuronal, endothelial and immune cells extreme caution should be exercised before designing such therapies as the overall function of NOSIP in other cellular processes need to be evaluated extensively. While in the curent study we have reported interaction between iNOS and NOSIP, discovery of a novel mutation in human NOSIP gene and its association with mortality in human sepsis and role in induction of IL-1β by monocytes on stimulation with LPS we are yet to unambigouly demonstrate that knockdown of NOSIP gene in human monocytes would result in increased synthesis and release of Nitric Oxide – investigations in progress are addressing this critical issue.

Acknowledgements

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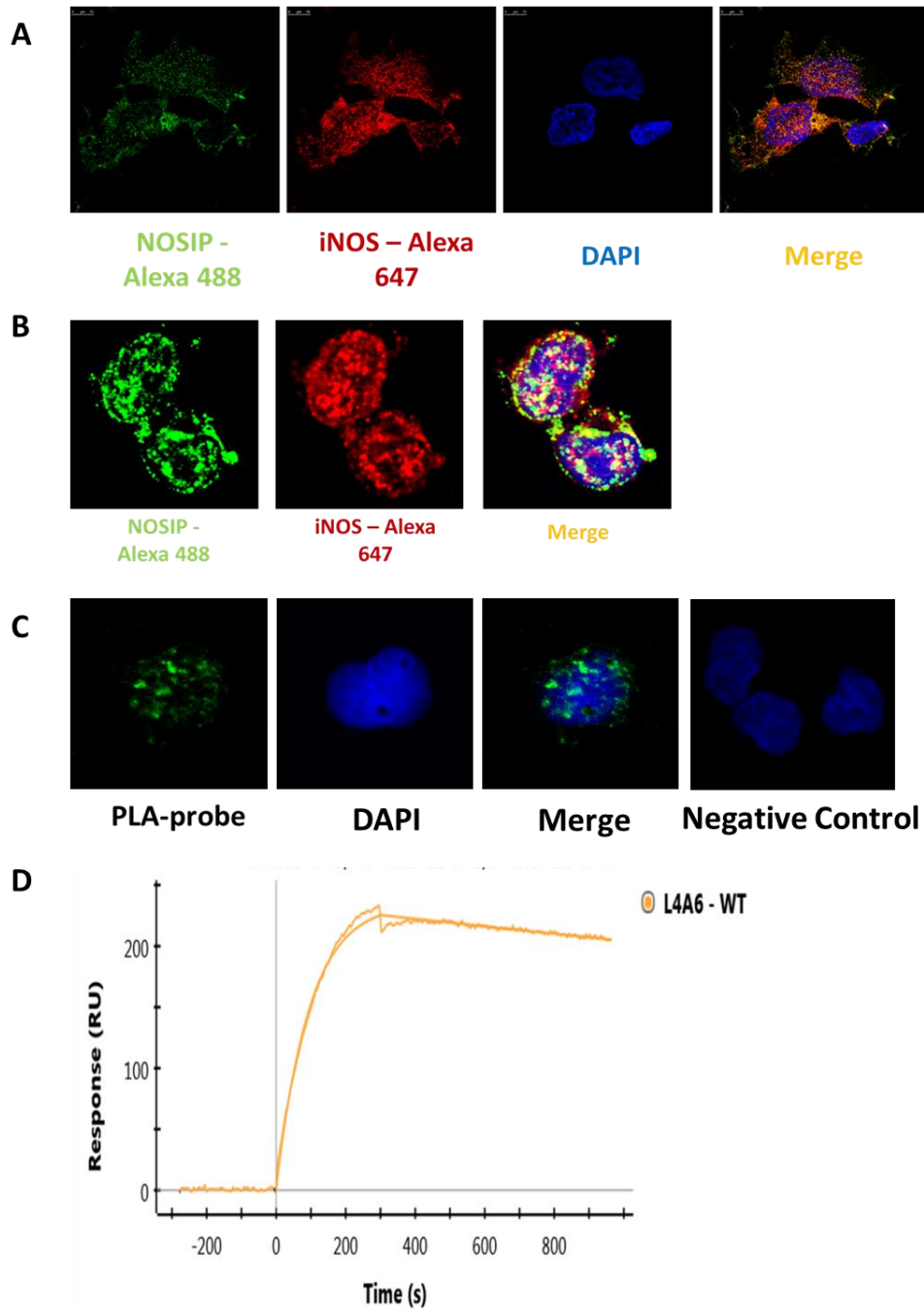
Figure 1. Interaction of NOSIP with iNOS. Cellular localization of iNOS and NOSIP in monocytic cell – line THP – 1 (A) and primary human monocytes (B) showing co - localization of the two proteins. (C) In situ proximity ligation assay demonstrating direct physical interaction between iNOS and NOSIP in primary human monocytes. Each speckle corresponds to one interacting pair. Negative control cells with only one primary antibody shows no background staining. (D) Interaction between immobilised solid phase iNOS and

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NOSIP as an analyte as measured by surface plasmon resonance. iNOS 3µg/ml and NOSIP 500nM were used for the study. K_D=7.11E-09M, Chi2=25.31 was obtained in Kinetic − Langmuir analysis.
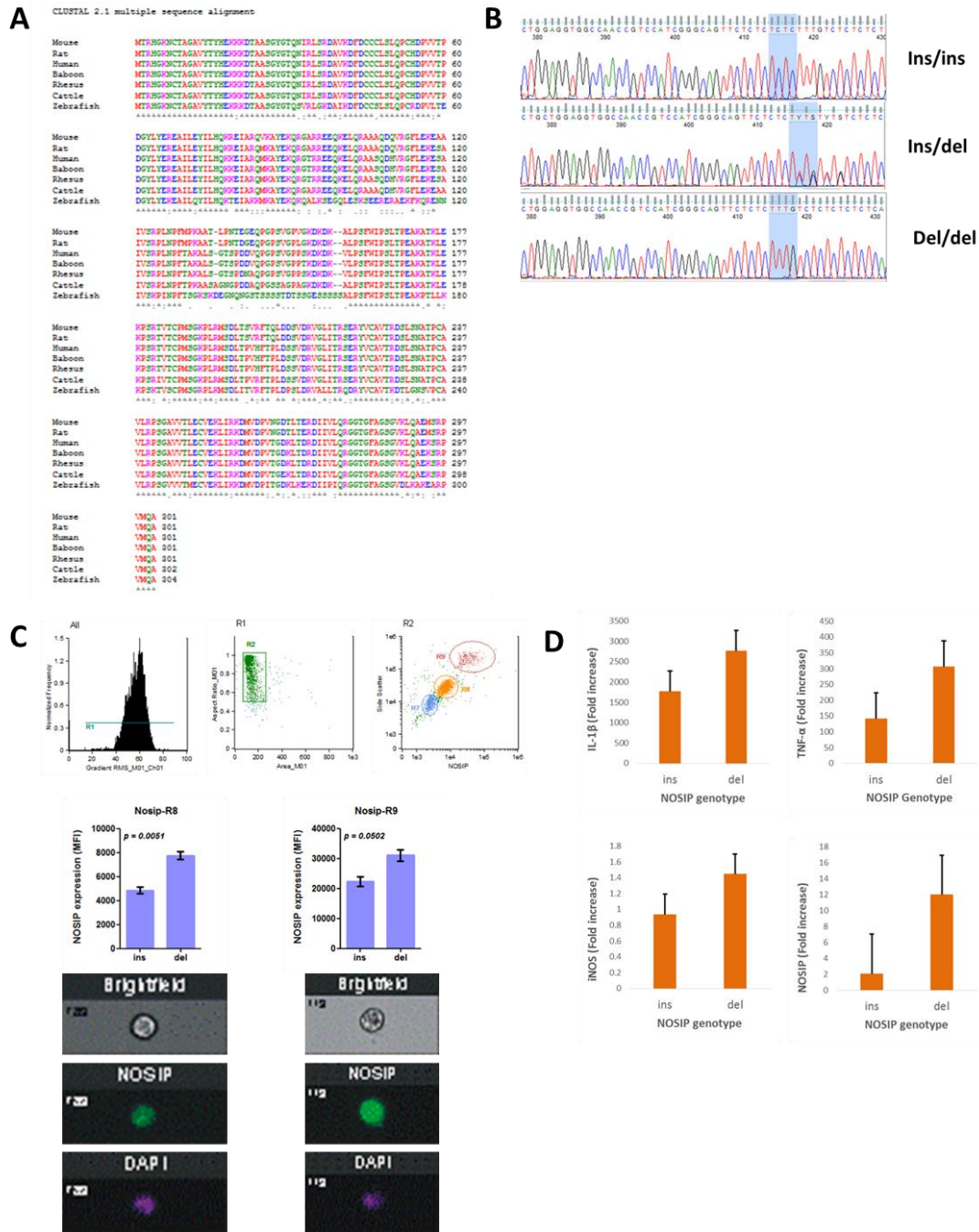
Figure 2. A novel mutation in NOSIP gene leads to increased intracellular protein expression. (A) Multiple sequence alignment of NOSIP protein among different species showing high conservateion of amino acid sequence. (B) Chromatogram showing a four nucleotide (TCTC) deletion downstream of exon 1 coding sequence. Top – ins/ins; middle – ins/del; and bottom – del/del. (C) Imaging cytometry analysis of intracellular NOSIP expression showing significantly elevated levels of NOSIP protein as a consequence of

presence of deletion allele as assessed by unpaired $t-test$. (n = 4 for ins/ins and ins/del category, n = 1 for del/del category). (D) Total RNA was extracted from human whole blood left untreated or stimulated with LPS at 100 ng/ml concentration. Isolated RNA was converted to complementary DNA (cDNA) which was subjected to quantitative real-time PCR analysis.

Table 1. Genotype distribution of *NOSIP* (TCTC ins/del) polymorphism and its association with mortality in sepsis

Genotype	Survivors (n = 36)	Nonsurvivors (n = 13)	
ins/ins	19 (53%)	3 (23%)	
ins/del	14 (39%)	5 (38.5%)	
del/del	3 (8%)	5 (38.5%)	

Genotype	*P*-value	Odds ratio	95% C.I.
ins/ins vs. ins/del	0.4362	2.462	0.4615 to 11.09
ins/ins vs. del/del	**0.0159**	10.56	1.611 to 69.15

Note: Association between NOSIP genotype and mortality to sepsis was assessed by Fisher's exact test. A p-value < 0.05 was considered statistically significant.

Table 2. Inverse association of plasma nitrite with inflammatory factors in a mouse model of endotoxemia and human sepsis

		Human		Mouse	
Factor	**By factor**	**R**	**p-value**	**R**	**p-value**
Nitrite	IL-1β	-0.17808	0.03805	-0.63321	>0.0001
Nitrite	IL-6	-0.06230	n.s.	-0.29015	0.03006
Nitrite	IL-8	-0.18577	0.03036	N.A.	N.A.
Nitrite	IL-9	-0.16614	0.05322	N.A.	N.A.
Nitrite	IL-12 (p70)	0.08337	n.s.	-0.32543	0.01438
Nitrite	MIP-1α	-0.25125	0.00317	-0.62772	>0.0001
Nitrite	MIP-1β	-0.14636	n.s.	-0.61086	>0.0001
Nitrite	TNF-α	-0.10051	n.s.	-0.72785	>0.0001

Note: Plasma nitrate+nitrite was estimated by a commercially available kit. Cytokines in plasma was measured by Bioplex suspension array system. Only cytokines having negative association with nitrite are shown. The correlation values displayed were calculated by constructing a nXn scatterplot matrix and scoring for bivariate associations between each pair of factors.
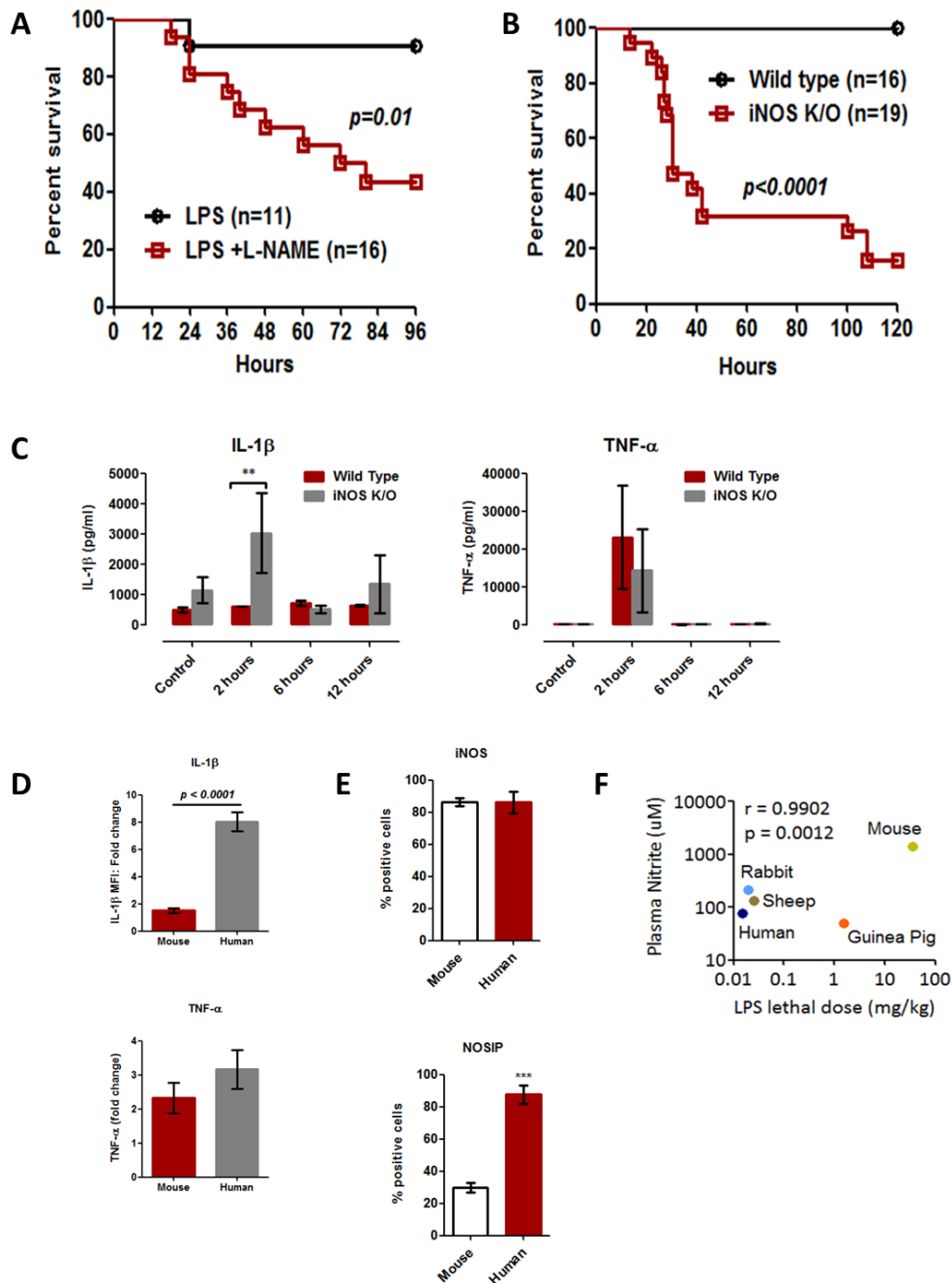
Figure 3. Deficiency of nitric oxide increases LPS – mediated toxicity by increased IL-1β production. (A) Treatment of BALB/C mice with a nonselective NOS inhibitor renders the animals more susceptible to LPS – mediated endotoxic shock in comparison to untreated mice. L-NAME was injected at 100 mg/kg once every 24 hours. LPS was injected at 2 mg/kg body weight. (B) iNOS-/- C57BL/6 mice and their wild-type littermates were injected with 15 mg/kg LPS and mortality was scored for 120 hours. (C) The animals (n = 4 in each group)

were weighed and LPS was injected at 15 mg/kg intraperitoneally. For assessment of cytokine production, animals were sacrificed at the indicated time points and isolated plasma was subjected to a multiplex cytokine assay. Significance levels were assessed by two-way ANOVA with Bonferroni's multiple comparison test. (*p<0.05, **p<0.01) (D) Mouse (n = 4) and human (n = 5) whole blood were left untreated or treated with 1 μg/ml LPS for 2 hours along with Brefeldin A. Following stimulation, cells were fixed, permeabilized, washed and stained with flurochrome-conjugated antibodies for analysis on a flow cytometer. Only monocytes were analysed. (E) Intracellular expression of iNOS and NOSIP in human and mouse circulating monocytes showing higher expression of NOSIP in human monocytes as assessed by unpaired $t - test$. (n = 6) for both mouse and human. *** p<0.0001. (F) Maximum plasma nitrite produced in a cohort of individuals was plotted on the y-axis against absolute lethal dose of LPS on the x-axis. In case of humans, dose required for endotoxic shock was plotted.