Present study demonstrates role of NO in the modulation of human neutrophil free radical generation and NETs formation. Neutrophil and NO are the important participants in inflammatory pathologies, which seem to be associated with many of the human diseases. Hence, studies were carried out to further assess NOS isoforms at the molecular level and their NO generation potential among platelets, RBCs, PBMCs and neutrophil and also in neutrophil precursor cells. Moreover, we also identified Rac2 as an important interacting iNOS interacting protein in neutrophil for the first time.

Free radicals and NETs are important arsenals of neutrophil. We evaluated role of NO in free radical generation and NETs release. A concentration (1μM-1mM) and time (up to 15 min) dependent augmentation in free radical generation was observed in the presence of NO donor, DETA-NO. The involvement of enzymatic free radicals was checked by using inhibitors of NADPH oxidase/ NOS (DPI), MPO (ABAH) and also NAC, a free radical scavenger, in flow cytometry as well as in DMPO nitrone adducts formation measured by Western blotting. Since NO mediated free radical generation was reduced by NADPH oxidase, NOS and MPO inhibitors, it implied role of NADPH oxidase, NOS and MPO. p47 phox a cytosolic component of NADPH oxidase in the resting neutrophil, migrated to plasma membrane in response to DETA-NO, in a time dependent manner up to 3hrs confirming the role of NADPH oxidase activation in NO mediated free radical generation. Furthermore, Rac2 an important Rho GTPase family protein was found to interact with iNOS in the neutrophil as explored by co-immunoprecipitation and co-labeling experiments. We observed interaction between iNOS and Rac2 in the cytosolic fraction in the resting cells, while in PMA stimulated cells, their interaction was evident in the membrane fraction, suggesting migration of Rac2 from cytosol to the membrane in PMA treated cells. Association of Rac2 with iNOS might help to generate superoxide anion and NO in the phagosomes, in a close proximity so as to facilitate formation of peroxynitrite for the microbicidal action. NETs formation was also observed in response to NO donor in human neutrophil, which was significantly reduced by DPI and ABAH, suggesting involvement of NADPH oxidase, NOS and MPO. NO mediated NETs generation has important implications for various inflammatory conditions.

NADPH oxidase and MPO are well characterized in neutrophil, however NOS was so far least explored. Hence the present study also characterized NOS isoforms at the molecular level so as to understand its role in modulating neutrophil functions. We found that human neutrophil constitutively expressed nNOS and iNOS as assessed using real time PCR and immunoprecipitation followed by Western blotting. eNOS expression could not be detected under the experimental conditions used. nNOS in human neutrophil possessed PDZ domain (Exon-2, N-terminal), which was confirmed by RT-PCR using exon-2 specific primer and Western blotting using N-terminal specific antibody. Results of the present study indicate that neuronal exon 1i cluster was present in human neutrophil nNOS, this promoter has been documented to contain binding site for various transcription factors that are activated during inflammatory conditions. Further studies were undertaken to explore the status of nNOS and NO in immature (from bone marrow) and mature neutrophil (from blood) in normal subjects. Level of NO was increased significantly during neutrophil maturation which was confirmed by using DAF-2DA, a NO binding fluorescent dye. Real time PCR and Western blotting also revealed upregulation of nNOS during neutrophil maturation.

Moreover, NOS molecular characterization expression studies were also undertaken in other human blood cells viz RBCs, platelets, monocytes and lymphocytes by real time PCR and immunoprecipitation followed by Western blotting. Quantitative analysis showed significantly higher expression of eNOS over iNOS and nNOS in monocytes, RBCs and platelets. However, highest level of iNOS expression was evident in resting human lymphocytes.

In conclusion, present study highlights the role of NO in modulating free radical generation, NETs release and distinct molecular characteristics of NOS isoforms in human neutrophil. Moreover, Rac2 was identified as an important interacting partner of iNOS in human neutrophil, which seems to facilitate NO generation in close vicinity to superoxide radical generation in the activated cell. We also assessed presence of NOS isoforms in other human blood cells, which expressed all the three isoforms in variable proportion. Based on these observations, future endeavors need to be directed to assess the relative expression of NOS isoforms and identification of their interacting proteins,

which will have important bearing in various pathological conditions associated/ documented to have an association with these cells.