Neutrophils, the first line of defense, are recruited earliest to the site of infection, inflammation or injury **(Nathan C, 2006)**. Neutrophil internalize and destroy infectious agents by the sequential formation of phagosomes, recruitment of lysosomes and various types of granules to release proteolytic enzymes/ microbicidal peptides, and free radical formation, to mediate the killing of endocytosed pathogens **(Babior *et al*, 2002; Segal, 2005)**. Neutrophil, an active player in inflammatory pathologies, thus pose a challenge of being a classical friend or foe.

NO, a potent free radical regulates a variety of physiological and pathological processes. Neutrophils produce free radicals toward an immune response so as to effectively combat invading pathogens. The ability of NO to promote neutrophil free radical generation has been extensively documented **(Sethi *et al*, 1999; Patel *et al*, 2009)**. In the present study effect of NO on the free radical generation in neutrophil was investigated by DCF-DA and DMPO nitrone adduct formation. DMPO measured both intracellular and extracellular free radical production, while DCF assessed intracellular free radical generation **(Marchesi *et al*, 1999)**. Neutrophil showed concentration (1μM-1mM) **(Figure 7A)** and time dependent (up to 15 min.) **(Figure 7B)** increase in free radical generation following NO treatment. Neutrophil incubated with DETA-NO and DMPO exhibited a time dependent (up to 3hrs.) formation of DMPO-nitrone adducts, which was abolished in the presence of NAC **(Figure 8)**.

Brinkmann *et al*, (2004) have shown a novel mechanism to eliminate invading pathogens by NETs formation. NETs are actively released from stimulated neutrophil, with granular proteins and do not exhibit nuclear degradation as seen in the late apoptotic stages **(Fuchs *et al*, 2007)**. Presence of NET components had also been noticed in various inflammatory and infectious conditions such as malaria **(Baker *et al*, 2008)**, sepsis **(Clark *et al*, 2007)** and colitis **(Morohoshi *et al*, 2006)** which are accompanied by high NO/RNS availability. Large amount of NO due to iNOS induction in systemic inflammatory response syndrome **(Spack *et al*, 1997)** and hypotensive sepsis **(Kawashima *et al*, 2007)** is well documented. iNOS in the migrated neutrophil also mediate acute lung injury during sepsis **(Shelton *et al*, 2007)**. LPS induced increase in NO and iNOS mRNA expression seems to be TLR4-dependent **(Frost *et al*, 2004)**, while NO also augments TLR4 expression **(Eswarappa *et al*, 2008)**. Platelet TLR4 mediated

neutrophil activation and NETs formation has also been reported **(Clark *et al*, 2007)**. **Nagy *et al*, (2007)** have demonstrated role of NO in inflammation, even tubulovesicular extension formation in human neutrophil suspension within 20 min of the addition of DETA-NO were also reported **(Galkina *et al*, 2005)**. Free radicals were considered as the stimulating factor to form NETs in response to interleukin-8 (IL-8), lipopolysaccharide (LPS) or phorbol myristate acetate (PMA). In the present study, we have demonstrated NETs release in response to DETA-NO **(Figure 9C)** as shown by elastase and DNA labeling. Hence, we propose role of NO in NETs release by augmenting free radical generation.

Neutrophil generate ROS due to the activation of NADPH-oxidase, and MPO. During NADPH oxidase activation, electrons are transferred from cytoplasmic NADPH to oxygen on the phagosomal side of the membrane, generating superoxide along with a range of other reactive oxygen species, essential for microbes killing **(Mark *et al*, 1998)**. NADPH oxidase also regulate tyrosine phosphorylation-dependent pathways to modulate host defense responses such as phagocytosis and, via NF-κB, expression of cytokines and chemokines that further modulate the inflammatory response **(Fialkow *et al*, 2007)**. MPO catalyzes the formation of hypochlorous acid (HOCl) in the presence of halide such as Cl^- **(Halliwell *et al*, 1995)**. Involvement of NADPH oxidase and MPO in free radical generation and their derived radicals in NETs formation was confirmed by using neutrophil from CGD patients **(Fuchs *et al*, 2007)** and MPO deficient subjects, which failed to generate NETs **(Metzler *et al*, 2010)**. However, role and presence of NOS in free radical and NETs release was not explored.

Present study found attenuation in NO induced free radical generation and NETs formation in the presence of DPI (NADPH-oxidase and NOS inhibitor) and ABAH (MPO inhibitor) suggesting involvement of NADPH-oxidase, MPO and NOS **(Figure 10)**. Moreover, migration of p47 phox to the membrane fraction in NO treated cells confirmed the role of NADPH-oxidase **(Figure 11)** and has opened the new avenue to explore the possible interaction of NOS with NADPH oxidase components. Physical interaction between iNOS and Rac2 was observed in LPS stimulated murine macrophages **(Kuncewiczk *et al*, 2001)**. In the present study we screened Rac2 as putative interacting partner of iNOS by performing 2D SDS-PAGE **(Figure 12)**, which

we have subsequently confirmed by the co-immunoprecipitation **(Figure 13 lane1)** and immunocytochemical **(Figure 14)** experiments. We were however unable to detect any interaction between nNOS and Rac2 by using the similar experimental conditions which demonstrated interaction between iNOS and Rac2 **(Figure 13 lane4). Kuncewiczk *et al*, (2001)** have shown that the oxygenase domain (N-terminal) of iNOS interacted with Rac2. Whereas, in case of neutrophil nNOS N-terminal contains PDZ domain (not present in iNOS), which might have hindered nNOS and Rac2 interaction. Furthermore, we observed the interaction between iNOS and Rac2 in the cytosol fraction in resting cells **(Figure 15A)** and in the membrane fraction in PMA stimulated cells **(Figure 15B),** demonstrating migration of Rac2 with iNOS from cytosol to the membranes in activated neutrophil. Association of Rac2 with iNOS seems to support superoxide anion and NO generation in the close proximity of each other, possibly to facilitate the formation of peroxynitrite, a potent microbicidal agent. Furthermore, NO could also be oxidised to nitrite, which might also be oxidized by hypochlorous acid to generate nitrogen dioxide (NO_2) and nitryl chloride (NO_2Cl), potent reactive nitrogen species through MPO dependent reactions **(Eiserich *et al*, 1998)**.

Further studies were carried out to assess the presence of NOS (nNOS and iNOS) and Rac2 in NETs. Recently **Lim *et al* (2011),** have reported the role of Rac2 in NETs formation via involvement of ROS and NOS, but they did not check the presence of NOS and Rac2 proteins in NETs. We however failed to detect nNOS **(Figure 16A)**, iNOS **(Figure 16B)** and Rac2 **(Figure 16C)** in NETs, which seems to be due to the degradation of these proteins by the proteases and granule proteins present in NETs.

NADPH oxidase and MPO have been well characterized in neutrophil, however NOS is still least explored. We therefore aimed to explore the molecular characteristics of NOS isoforms so as to understand its role in neutrophil functions. Besides this, neutrophil and NO are the important participants in inflammatory conditions, which seem to be associated with many of human pathologies **(Sethi *et al*, 2000)**. Hence, assessment of the NOS molecular characteristics in human neutrophil and their precursor cells was considered important to get better insight into the complexity of the pathological conditions attributed to NO and neutrophil. Presence of nNOS, eNOS and iNOS in peripheral neutrophil has been documented (**Cedergren *et al*, 2003; Frutos *et al*, 2001;**

Henric *et al*, 2010). In most of these studies only one or two isoforms were characterized at either RNA or protein level. However conflicting reports exist in the literature regarding their functional status in neutrophil **(Yan *et al*, 1994; Salvemini *et al*, 1989; Greenberg *et al*, 1998)**. Moreover, endogenous NO level and NOS expression have not been explored so far in the bone marrow neutrophil precursor cells. The present study thus elucidated NOS characteristics in neutrophil and explored the presence of NOS isoforms for the first time in human neutrophil precursor cells.

Neutrophils seem to significantly contribute in the circulating levels of NO/metabolites **(Salvemini *et al*, 1989)** and play a key role in regulating blood pressure **(Morton *et al*, 2008)**, relaxation of aortic rings **(Rimele *et al*, 1988)**, inhibiting platelet aggregation **(Dikshit *et al*, 1993)** and are also involved in antimicrobial function **(Malawista *et al*, 1992)**. Inter-species differences in terms of NOS expression and enzymatic NO production have been reported **(Greenberg *et al*, 1998; Miles *et al*, 1995)**. In the present study, qualitative and quantitative expression of NOS isoforms was explored in the human neutrophil by RT-PCR, real time PCR and immunoprecipitation. RT-PCR data indicated constitutive expression of nNOS and iNOS, but did not detect eNOS expression **(Figure 18A)** by using cDNAs prepared from oligo dT or by using random primers. **Frutos *et al*, (2001)** had reported eNOS expression in neutrophil by using Western and Northern blot analysis. Real time PCR analysis **(Figure 18B)** and immunoprecipitation **(Figure 18C)** studies also indicated higher expression of nNOS over iNOS. Existing reports in the literature suggested presence of nNOS in human neutrophil **(Gatto *et al*, 2000; Henric *et al*, 2010)**. **Greenberg *et al*, (1998)** reported nNOS in neutrophil at mRNA level but failed to detect at the protein level. Moreover, **Gatto *et al*, (2000)** reported over expression of neutrophil nNOS mRNA and protein in PD patients. Expression of iNOS was also reported in human neutrophil **(Amin *et al*, 1995; Jablonska *et al*, 2005)**. Most of the methods used in the NOS expression studies are reliable but vary in their sensitivity and also have inherent drawbacks. The results obtained demonstrated constitutive and predominant expression of nNOS over iNOS by using highly sensitive methods. Microarray experiments however failed to detect NOS transcripts in healthy human neutrophil suggesting its expression might be very low in

neutrophil. The present study has observed that NOS expression in the healthy human neutrophil was 10^4 folds less than the β-actin (house keeping gene).

Based on WB and RT-PCR experiments, nNOS and iNOS in neutrophil seems to be full-length. nNOS having PDZ domain at its N- terminal interacts with several other PDZ domain containing proteins, which play an important role in the targeting of proteins to specific membrane compartments and their assembly into supramolecular complexes. As nNOS in neutrophil also possess a PDZ domain, it is also likely to interact with PDZ domain-interacting proteins in neutrophil to regulate some important functions. The results obtained thus warrant further investigations to identify the nNOS-interacting proteins in neutrophil.

Furthermore, the relative NO generation potential was investigated by using DAF-2DA. Half life of NO being less is readily converted to an oxidized metabolite N_2O_3 by reacting with nitrite. Since DAF also reacts with N_2O_3, non-enzymatic generation of N_2O_3 might also contribute to the formation of fluorescent product. DAF-2DA however provided an approximation of NO generation potential in neutrophil. In circulating neutrophil, DAF labeling data clearly indicated role of nNOS in NO generation **(Figure 18F)**. Selectivity of Vinyl-L-NIO for nNOS (500 fold) over iNOS and 1400W for iNOS (30 fold) over nNOS **(Vallance *et al*, 2010)** has conferred advantage in determining the isoform specific role of NOS towards NO generation. Recently, **Michael *et al*, (2010)** used Vinyl-L-NIO, nNOS inhibitor to establish nNOS dependent transnitrosylation of nuclear proteins, where GAPDH provided nuclear access for NO. Furthermore, evaluation of NOS expression, activity and NO generation under diverse pathological conditions is of great relevance since excessive production of NO is detrimental to tissue injury in various diseases many of which also involve active participation of neutrophil.

NOS isoforms in neutrophil precursor cells separated on discontinous Percoll density gradient were further characterized. It was observed that nNOS/ iNOS expression and their catalytic activity increased with neutrophil maturation **(Figure 18)**. Recently, **Henric *et al* (2010)**, demonstrated involvement of nNOS-derived NO in autocrine dendritic cell (DC) maturation and DC-mediated induction of T cell activation. In yet another study, nNOS activity and its mRNA level were increased during neuronal cell

differentiation in human neuroblastoma cell line following trans-retinoic acid (RA)-induced neuronal differentiation **(Nagl *et al*, 2009).** Level of NO as measured by DAF-2DA was also found to be increased during neutrophil maturation **(Figure 18E).** **Peunova *et al* (2007)** have reported regulatory role of NO in neurogenesis via S-nitrosylation of RhoA. More studies are needed to ascertain the molecular mechanism involved in NO mediated neutrophil maturation. Furthermore, it will be important to evaluate NOS expression and NO generation in diseases associated with neutrophilia and neutropenia. Recent report from this lab has established the role of NO and nitrite in modulating HL-60 (neutrophil precursor promyelocytic cell line) cell proliferation by cdk2 nitrosylation and apoptosis by mitochondrial dysfunction **(Kumar *et al*, 2010; Kumar *et al*, 2011).** Specific role of NOS isoforms in modulating cell cycle and maturation of bone marrow derived neutrophil also needs to be ascertained, which is currently ongoing in this lab.

As discussed above, nNOS, the most abundant isoform in human neutrophil seems to be the major source of NO generation during the basal stage and iNOS could be considered to be further enhanced during pathological conditions. Expression and catalytic activity of iNOS was less as compared to nNOS, possibly because healthy donors were used in this study. Previous study from this lab demonstrated higher iNOS catalytic activity and more iNOS distribution than nNOS in rat neutrophil **(Saini *et al*, 2006).** Furthermore we have observed upregulation of nNOS expression during human neutrophil maturation which is contrary to our previous finding in rat neutrophil precursor cells exhibiting upregulation in iNOS **(Kumar *et al*, 2010)** indicating a species specific difference in expression, activity and localization of NOS isoform in rat and human neutrophil.

Alternative splicing is one of the most important mechanisms to generate a large number of mRNA and protein isoforms by changing the structure of transcripts and their encoded proteins. Nevertheless, use of alternative promoters is a versatile mechanism that can influence gene expression by various means. In the present study we have detected the presence of neuronal nNOS cluster in human neutrophil **(Figure 20).** nNOS expressed in a variety of human tissues, exhibit a complex transcriptional regulation with the presence of nine alternative first exons (1a-1i) resulting in nNOS transcripts with

differing 5'-untranslated regions **(Boissel *et al*, 2003).** The 5' ends of the mRNA species from various tissues show different first exons alternately spliced to a common second exon. Different exon1 have been demonstrated that correspond to the 5' flanking/promoter regions accounting for different regulatory mechanisms. nNOS are widely expressed in different tissues, in cerebral cortex and hippocampus (1d, 1f and 1g), skin (1b, 1d, 1f and 1g), heart (1d and 1g) , tibia muscle (1d) and in testis (1f, 1g, 1k) having various transcripts of exon 1. Among the cell lines, neuroepithelial cell line A 673 (1f, 1g, 1k), neuroblastoma cell line SKMNC (1f, 1g, 1k), keratinocyte cell line HaCaT (1f,1g,1i ,1k) have been found to express different exon 1i. These transcripts have implications in different pathological conditions. In many tissues and cells exons 1d, 1f, and 1g were expressed in parallel and accounted for the majority of nNOS transcripts. We have observed expression of exon 1i in human PMN nNOS, however expression of exon 1d, f and g was not detected **(Figure 20C)**. Various transcription factors such as SP1, Ik/ NFAT, USF, MyoD, MzF1, NrF, AP2 and AP1 have been reported to bind the exon 1i promoter **(Bros *et al*, 2006).** Expression of different transcripts is regulated by various stimuli. Exon 1f can be stimulated by nerve growth factor **(Rife *et al*, 2000)**, while phorbol myrystate activates the promoter of exon 1g. Regulatory polymorphisms of NOS1 contribute to the genetic risk for diseases. Marker association analysis showed that the exon 1c promoter polymorphism was linked to schizophrenia (SCZ) **(Reif *et al*, 2006)**. In hypertrophic pyloric stenosis, expression of exon 1d is down regulated due to the SNP in promoter region. CREB plays an important role in enhancing expression of nNOS by activating transcription of exon 1f/1g. Epidermal growth factor up regulate the expression of NOS 1i+1k in keratinocyte by increasing its stability **(Boissel *et al*, 2004)**. A 89-nucleotide alternatively spliced exon located in the 5-untranslated region between exon 1 variants and a common exon 2. This alternatively spliced exon forms a stem-loop secondary structure and acts as a potent translational repressor **(Newton *et al*, 2003)**.

NO has also been recognized as modulator of several important functions of blood cells. Hence, we also explored expression of NOS isoforms at the molecular and biochemical level to further assess NO generation ability in human blood cells including PBMCs (monocytes and lymphocytes), platelets and RBCs.

PBMCs constitute important component of both vascular and immune systems. NO essentially influences both the systems, a detailed insight into the status of NOS might thus be useful to understand diverse pathologies involving PBMCs. NO release from human PBMCs was first demonstrated by **Salvemini *et al* (1989),** which was subsequently identified as mediator of their microbicidal and tumoricidal actions, as well as a regulator (enhancer and suppressor) of T-cell functions **(Liew *et al*, 1995).** Real time PCR analysis and immunoprecipitation studies indicated constitutive expression of cNOS and iNOS in PBMCs, monocytes and lymphocytes. Higher expression of iNOS was found in PBMCs as compared to eNOS and nNOS **(Figure 23B and C),** while monocytes **(Figure 23E and F)** and lymphocytes **(Figure 23H and I)** expressed more amount of eNOS and iNOS respectively. Number of lymphocytes in PBMCs is much more than monocytes, we therefore detected higher expression of iNOS in total PBMCs. Existing reports in the literature demonstrate presence of iNOS and cNOS transcripts in monocytes obtained from severe acute pancreatitis patients complicated with systemic inflammatory response syndrome **(Tanjoh *et al*, 2007).** Moreover an inverse relationship was observed between the expression of iNOS and cNOS in monocytes, macrophages and monocytic cell lines upon stimulation with LPS/IFN-γ **(Reiling *et al*, 1994).** Upregulation of iNOS in monocytes from pulmonary tuberculosis patients have also been reported **(Wang *et al*, 2001).** Germinal center T cells and follicular dendritic cells did not express eNOS mRNA. Presence of eNOS protein was however confirmed in T cells from peripheral blood and in various germinal center cells of frozen tonsillar sections **(Reiling *et al*, 1996).** Thyroid hormones upregulate iNOS gene expression downstream to PKC-zeta in murine tumor T cells **(Arco *et al*, 2006).** T cell activation induces formation of reactive oxygen intermediates and Ca^{+2} -dependent NO production **(Nagy *et al*, 2003).** Furthermore, NO generation in PBMCs, monocytes and lymphocytes was measured by using DAF-2DA, exhibiting NO formation in the presence of substrate and cofactors, which was significantly inhibited by L-NAME, the NOS inhibitor **(Figure 23D, G and J).** Understanding of the differential regulation of NOS expression and catalysis in PBMCs might be helpful to evolve strategies against graft versus host responses following transplantation, tumor surveillance, inflammation and infectious diseases. Results obtained by real time PCR and IP/WB indicated the differences in NOS ratio to

β-actin (10^6 folds in the case of real time PCR and 25 folds in IP/WB). This could be due to the differences in the sensitivity of the methods used, mRNA stability or protein degradation, which needs to be ascertained in PBMCs subsets under physiologolical and pathological conditions.

NOS isoforms interact with a wide array of scaffolding proteins, positive and negative catalytic regulators, signaling intermediates to membrane receptors which might affect both its trafficking and NO generation potential **(Kavya *et al*, 2006).** nNOS having PDZ domain interacts with several other PDZ domain containing proteins. Western blot analysis with antibodies specifically directed against the N-terminal region of nNOS which bears the PDZ domain encoded by exon 2, revealed a full length nNOS protein of 160 kDa in the PBMCs, monocytes and lymphocytes **(Figure 23C, F and I).** iNOS and eNOS in PBMCs were also full length proteins of 130 kDa. Based on our observations, future endeavors need to be directed to assess the relative expression of NOS isoforms, their subcellular localization, trafficking, nitration and nitrosylation of important proteins in different pathological conditions where PBMCs play an active role in disease onset and progression.

Platelets are the most crucial element in regulating hemostasis. Human platelets express both calcium independent iNOS and predominantly eNOS **(Mehta *et al*, 1995)**, in cytosolic **(Muruganandam and Mutus 1994)** and membrane **(Sase and Michel 1995)** fractions detected by Western blot analysis. Platelet derived NO exhibits anti-aggregatory effects to inhibit platelet activation as a feedback regulation, NO generation in the resting platelets remains a doubtful issue as compared to activated ones. Enhancement in NO release from platelets during aging and atherothrombotic diseases has been evidenced, interestingly NO generation potential from activated platelets of the aged individuals was found to decline as compared to young volunteers, suggesting towards the pro-aggregatory properties of NO **(Goubareva *et al*, 2007)**. However basal level and expression of NOS was comparable in aged and young individuals. Although an increment in the phosphorylation of eNOS among the aged possibly suggested specific signaling events regulating NOS catalysis. Basal NOS expression and its catalytic activity in resting platelets is therefore worth exploring. We detected expression of iNOS and eNOS at both transcript and protein level by RT-PCR **(Figure 25A)**, real time PCR

analysis **(Figure 25B)** and immunoprecipitation/ Western blot analysis **(Figure 25C)** in resting platelets. Results obtained indicate that resting platelets express both iNOS and eNOS in a constitutive manner, which supports previous observations **(Sase and Michel 1995)**. However nNOS expression was also detected by real time PCR **(Figure 25B)**, but its expression level was very less as compared to iNOS and eNOS. **Chen and Mehta (1996)** have shown an increment in calcium independent iNOS activity in response to cytokines. Since platelets have little capacity of protein synthesis, it could be speculated that iNOS in platelets is ready to use and its inactivation is overcome by the cytokine stimulation due to the presence of its mRNA in these cells.

NO derived from RBCs is speculated to mediate vasorelaxation. **Crawford *et al* (2004)** reported that NO derived from RBCs in sepsis patients forms S-nitrosohemoglobin to promote vasorelaxation mediated through cGMP production. Presence and functionality of NOS in RBC has been widely debated with reports in support **(Chen *et al*, 1998; Mehta 2000)** of a functional NOS system and also those opposing it **(Kang *et al*, 2000)**. We have detected expression of iNOS and eNOS at both transcript and protein level by RT-PCR **(Figure 26A)**, real time PCR analysis **(Figure 26B)** and immunoprecipitation/ Western blot analysis **(Figure 26C)** in RBCs. Results obtained indicate that RBCs express both iNOS and eNOS in a constitutive manner, which is in agreement to the previous observations **(Kang *et al*, 2000)** who failed to detect the NOS catalytic activity. According to their assessment L-arginine to L-citrulline conversion a measure of NOS catalysis, was due to arginase activity, since the catalytic activity was not inhibited by NOS inhibitors. This was contrary to the reports of **Chen and Mehta, (1998) and Yang *et al*, (1996),** who demonstrated functional NOS system by using same catalytic method and also assessed by utilizing a sensitive bioassay method of platelet aggregation inhibition.

RBCs the most abundant circulating blood corpuscles (nearly 92% of whole blood cells) expressed minimal NOS. Presence of NOS in RBCs was considered a vestige of their precursors **(Mehta *et al*, 2000)**, the functional implication of NO generation thus remained obscure in RBCs. The main function of RBC is to carry oxygenated blood and SNO-Hb (due to interaction of hemoglobin and NO) from lungs to tissues as well as to take out deoxygenated blood from tissues to lungs. Since RBCs contain less NOS/NO, it

might enhance the ability of RBC to remove NO from the environment, a process critical for the regulation of blood pressure and oxygen delivery **(Kang *et al*, 2000)**. Platelets in the circulation (nearly 7.5% of whole blood cells) are also regulated primarily by NO originating from endothelial cells and neutrophil, and it is hypothesized that platelet derived NO might have insignificant role **(Tymvios *et al*, 2009)**. Platelets are enucleated cells, levels of pre formed transcripts are therefore very less in number, which might be the reason for the detection of low levels of NOS transcript. Previous report suggested that neutrophil are able to generate NO at a rate of 10-100 nmoles/5min/10^6 cells, comparable to the endothelial cells, contributing much to the amount of NO in circulation **(Salvemini *et al*, 1989)**. neutrophil are also the first line of defense and kill the pathogens by forming free radicals such as superoxide and NO. This could be the reason for the presence of higher levels of NOS transcripts in these cells. Hence, in spite of its low abundance in blood (nearly 0.1% of whole blood cells), NO derived from neutrophil relaxes aortic rings **(Rimele *et al*, 1988)**, inhibits platelet aggregation **(Dikshit *et al*, 1993)** and is also involved in antimicrobial functions. PBMCs constituting monocytes (nearly 0.015% of whole blood cells) and lymphocytes (nearly 0.05% of whole blood cells) are an important component of both vascular and immune systems. Expression of NOS isoforms mainly iNOS and eNOS in lymphocytes and monocytes may be to enhance their microbicidal and tumoricidal actions as well as regulating T-cell functions **(Liew, 1995)**.

Real time PCR data exhibit the copy number of NOS isoforms to reflect their relative abundance in blood cells. In the present study, among the blood cells, nutrophil seems to be the forerunners closely followed by monocytes and lymphocytes (PBMCs), with platelet and RBCs bringing up to the rear. This was in accordance with NOS localization (by transmission electron microscopy), NO generation (by DAF-2DA labeling in flow cytometry) and NOS catalysis (by [H^3]-L-arginine to [H^3]-l-citrulline conversion assay) data generated from this lab previously **(Saluja, 2008)**. NOS expression and NO generation status in the various circulating cells seems to be largely determined by the contribution of NO in regulating their functional responses rather than by the absolute number of these cells.