



# Analysis of the Effect of Intestinal Ischemia and Reperfusion on the Rat Neutrophils Proteome

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Intestinal ischemia and reperfusion injury is a model system of possible consequences of severe trauma and surgery, which might result into tissue dysfunction and organ failure. Neutrophils contribute to the injuries preceded by ischemia and reperfusion. However, the mechanisms by which intestinal ischemia and reperfusion stimulate and activate circulating neutrophils is still not clear. In this work, we used proteomics approach to explore the underlying regulated mechanisms in Wistar rat neutrophils after ischemia and reperfusion. We isolated neutrophils from three different biological groups; control, sham laparotomy, and intestinal ischemia/reperfusion. In the workflow, we included iTRAQ-labeling quantification and peptide fractionation using HILIC prior to LC-MS/MS analysis. From proteomic analysis, we identified 2,045 proteins in total that were grouped into five different clusters based on their regulation trend between the experimental groups. A total of 417 proteins were found as significantly regulated in at least one of the analyzed conditions. Interestingly, the enzyme prediction analysis revealed that ischemia/reperfusion significantly reduced the relative abundance of most of the antioxidant and pro-survival molecules to cause more tissue damage and ROS production whereas some of the significantly up regulated enzymes were involved in cytoskeletal rearrangement, adhesion and migration. Clusters based KEGG pathways analysis revealed high motility, phagocytosis, directional migration, and activation of the cytoskeletal machinery in neutrophils after ischemia and reperfusion. Increased ROS production and decreased phagocytosis were experimentally validated by microscopy assays. Taken together, our findings provide a characterization of the rat neutrophil response to intestinal ischemia and reperfusion and the possible mechanisms involved in the tissue injury by neutrophils after intestinal ischemia and reperfusion.

**Keywords:** ischemia reperfusion, neutrophils, proteomics, systemic inflammatory response, LC-MS/MS

## INTRODUCTION

The intestine is the most sensitive organ to ischemia and reperfusion (IR) injury. This injury can result from various clinical situations, such as intestinal obstruction, acute mesenteric ischemia, incarcerated hernia, small intestine transplantation, neonatal necrotizing enterocolitis, trauma, and shock, taking the patient to relentless clinical syndromes, and even death (Mojzis et al., 2001; Mallick et al., 2004; Guneli et al., 2007). It is clear now that the reperfusion following ischemia leads to significantly greater mucosal intestinal injury as compared to the ischemia alone (Crissinger and Granger, 1989) whereas development of the systemic inflammatory response syndrome (SIRS) and multiple organ failure (MOF) can be the final consequences of IR (Ceppa et al., 2003).

Among the polymorphonuclear leukocytes (PMNs), neutrophils are the first line of defense against bacterial and fungal infections (Kaufmann, 2008). However, a large number of studies showed that IR injury is mainly because of PMN and endothelial cell (EC) interactions in reperfused tissues (Massberg et al., 1998; Kumar et al., 2009; Kvietyts and Granger, 2012). Normally, a multistep process of neutrophil recruitment to the site of infection requires three types of adhesion receptors, like integrins, selectins, and adhesion receptors of the immunoglobulin superfamily (Rao et al., 2007).

It has been shown that P- and E-selectins are over expressed in a mouse model after intestinal ischemia/reperfusion and that inhibiting P-selectin decreased neutrophil rolling and adhesion, reducing the injury (Riaz et al., 2002). Another IR model showed decreased neutrophil rolling and adherence by blocking of P- and L-selectins (Kubes et al., 1995). The up-regulation of adhesion molecules on the endothelial surface was observed following ischemia/reperfusion injury (IRI) that can result in diapedesis of neutrophils, further contributing to muscle dysfunction (Hierholzer et al., 1999). Similarly, in small intestine, an increase in expression of inflammatory mediators like tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), cyclooxygenase-2 (COX-2) and intercellular adhesion molecule-1 (ICAM-1), have been observed after IR with increase in neutrophil infiltration (Watanabe et al., 2014). Another study demonstrated systemic serum level elevation of the CC chemokines along with XC chemokines in a model of intestinal ischemia, hence leading to greater PMNs activation and tissue injury (Jawa et al., 2013). Each of these signals interact with specific receptors expressed on the plasma membrane of

PMNs with an overlapping array of signal transduction pathways leading to functional responses such as rearrangement of the actin cytoskeleton (Luerman et al., 2010).

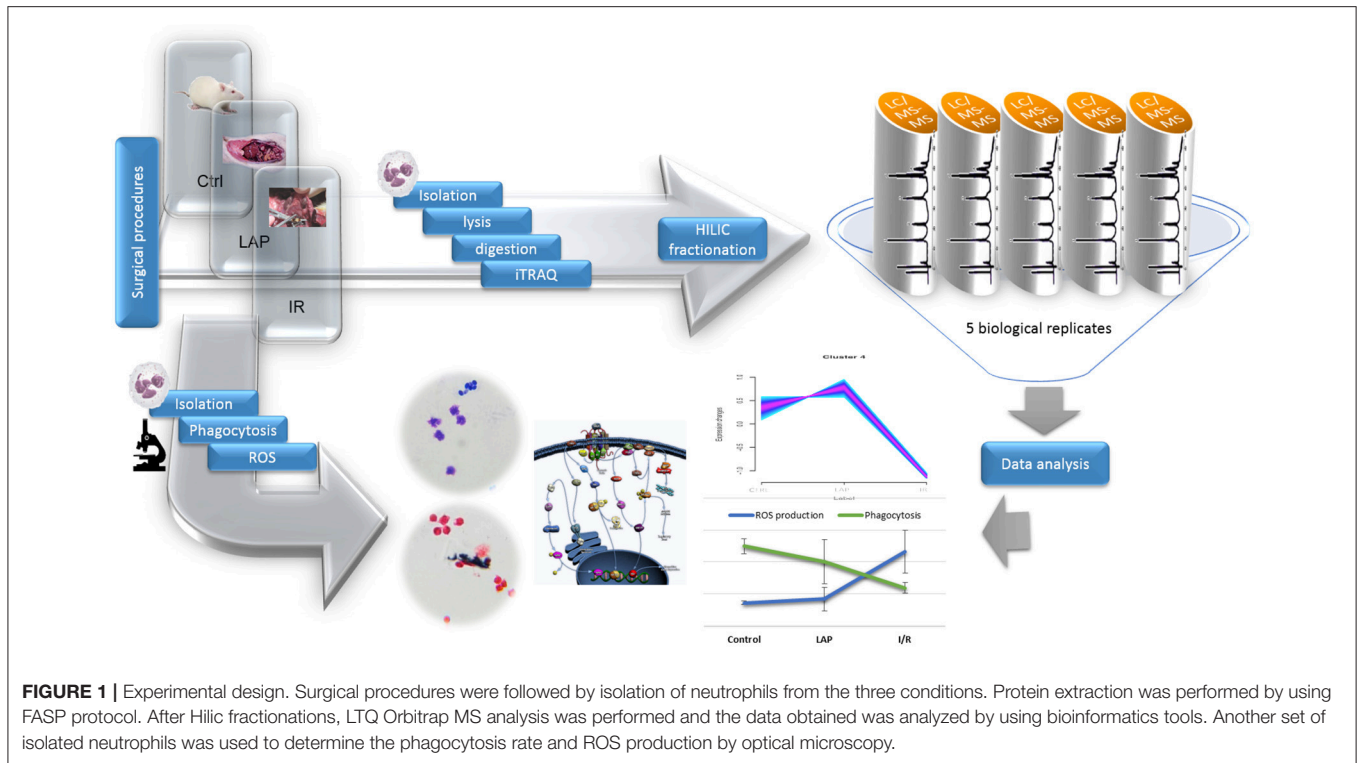
A gradient of chemoattractant signals arising from the dying tissues helps in the recruitment of PMNs (McDonald et al., 2010) that secrete a large number of factors like reactive oxygen species, chemokines, cytokines, lipid mediators, and proteases (Rodrigues and Granger, 2010). However, molecular mechanisms, enzymes, and pathways by which PMNs participate in the IR injury are not fully understood. The current understanding of the interconnections among the many signal transduction pathways that regulate neutrophil activation is incomplete. Proteomics research has improved the understanding of the neutrophil biology in the past, and few publications describe the effect of the inflammatory response on the PMNs proteome (Morris et al., 2008). There is one proteomic study on the response to intestinal ischemia and reperfusion, however limited on molecules expressed by the intestinal epithelium, and there is lack of understanding regarding the PMNs response against IR. The main objective of this study is to explore the effect of IR on the PMNs in rats using mass spectrometry based proteomics (Hurst et al., 1998). We analyzed the neutrophil proteome from three different biological conditions, including control, sham laparotomy and intestinal ischemia/reperfusion. Proteomic analysis of neutrophils after ischemia and reperfusion revealed that neutrophils down regulate the expression of different antioxidant, pro-survival molecules. Significantly up regulated oxidoreductases and down regulated transferases can interfere in the integrin signaling pathway, lipid metabolism and reactive oxygen species (ROS) generation, leading to the local and remote tissues injury. Furthermore, our analysis shows the regulation of different proteins and pathways required for neutrophil adhesion, directional migration, and phagocytosis after intestinal ischemia and reperfusion. Down regulation of important enzymes from LTB<sub>4</sub> synthesis pathway opens some questions that need further analysis. Functional assays revealing increased ROS production and decreased phagocytosis after ischemia/reperfusion are coherent with the proteomics findings. We anticipate that these findings will provide trustworthy basis for further deep analysis in neutrophil biology.

## RESULTS

### Protein Identification and Relative Abundance Profile

For the large-scale proteomics analysis of rat neutrophils, samples were collected from three experimental groups (Figure 1) including control, laparotomy and intestinal ischemia/reperfusion groups. Proteins from each experimental group with five biological replicates were iTRAQ labeled for relative quantification analysis. To find the significant changes in protein abundance among three groups, unique peptides with high confidence identification and their respective iTRAQ reporter ion intensity values were analyzed by using R. For a detailed description of the methods, see the **Supplementary Methods** section. Mass spectrometry

**Abbreviations:** CAMP, Cathelicidin antimicrobial peptide; DTT, Dithiothreitol; EC number, Enzyme commission number; FASP, Filter aided sample prep; FDR, False discovery rate; fMLP, N-Formyl-Met-Leu-Phe; GFP-PH-Akt, Green fluorescent protein probe for the PH domain of protein kinase B; HCD, higher energy collisional dissociation; HILIC, Hydrophilic interaction chromatography; IR, Ischemia and reperfusion; iTRAQ, Isobaric tag for relative and absolute quantitation; KEGG, Kyoto Encyclopedia of Genes and Genomes; LC-MS/MS, Tandem mass spectrometry liquid chromatography; Lcn2, Lipocalin-2; LTB<sub>4</sub>, Leukotriene B<sub>4</sub>; LTQ, Linear trap quadrupole; MOF, multiple organ failure; PCA, Principal Component Analysis; PMNs, polymorphonuclear leukocytes; PtdIns(3,4,5)P<sub>3</sub>, Phosphatidylinositol (3,4,5)-trisphosphate; ROS, Reactive oxygen species; SDS, Sodium dodecyl sulfate; SHP-1, SH2-containing tyrosine-specific protein phosphatase; SIRS, systemic inflammatory response syndrome; TEAB, Triethylammonium bicarbonate; WASE, Wiskott-Aldrich syndrome protein; XC and CC, families of chemokines.



analysis resulted in the identification of 2045 proteins (**Supplementary Table S1**).

After the data analysis, proteins were grouped into clusters according to their abundance profile among the three conditions. To assign the proteins according to their abundance in the best number of clusters, two validation indices, Xie-Beni index (Xie and Beni, 1991) and minimal centroid distance (Schwammler and Jensen, 2010), were applied and proteins were assigned to five different clusters based on their regulation trend (**Figure 2**, **Supplementary Table S2**). Expression changes are shown as z scores (i.e., relative abundances normalized by mean and standardized by dividing by the standard deviation). The colors correspond to the so-called membership values (values in the range [0,1]), representing the degree of how much a protein belongs to the nearest cluster. Only proteins with memberships above 0.5 are shown. A subset of 417 proteins was significantly regulated (Limma and rank products  $q$ -value < 0.05) in at least one of the analyzed conditions within the five clusters. A standard PCA analysis was performed to check the similarity among different samples.

### Go slim Analysis and Enzyme Activity Prediction for Total Rat Neutrophil Proteome

Protein classification was performed by GO *slim* analysis using ProteinCenter (Thermo Scientific) as a platform. The resultant cellular component, biological process and molecular function terms for the five clusters are shown in **Supplementary Figure S1**. Molecular function analysis revealed

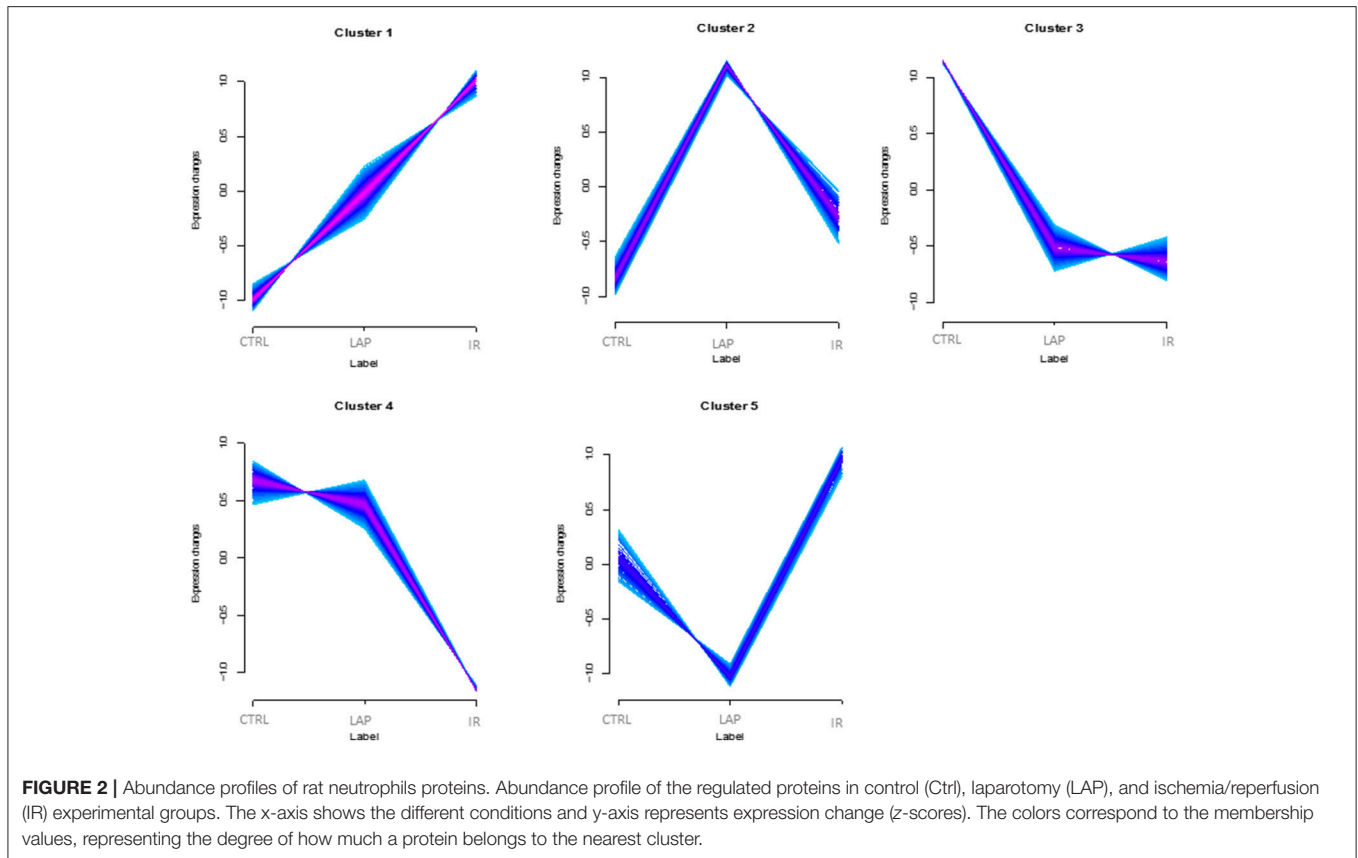
that about 33% of the neutrophil proteome has predicted enzymatic activity. It is composed of 18% oxidoreductases (EC:1), 27% transferases (EC:2), 38% hydrolases (EC:3), 3% lyases (EC:4), 5% isomerases (EC:5) and 9% ligases (EC:6) as predicted activity, as shown in **Figure 3A**. The overall distribution of the enzymes across five clusters is illustrated in **Figure 3B** whereas **Table 1** represents the significantly regulated proteins in at least two conditions among 5 clusters.

### Major Functional Classes of the Neutrophil Proteome Regulated by IR

For the functional classification of the significantly regulated identified proteins, KEGG and Wiki pathways analyses were done and the enriched pathways are listed in **Table 2**. Most of the enriched pathways found were immune-related indicating the effect of intestinal ischemia and reperfusion on the neutrophil function. Phagocytosis was found significantly down regulated in IR as shown in **Table 2**, **Figure 4**.

### Verification of ROS Production and Phagocytosis

To validate such findings we performed a ROS production and phagocytosis assays by incubating neutrophils from the three groups with *Saccharomyces cerevisiae* yeast cells. Phagocytic activity was significantly decreased in IR group ( $p$ -value < 0.05) compared to control and LAP (**Figure 4**). Only about 23.90% of cells phagocytosed in IR group while control and LAP presented 50 and 40.7% phagocytosis rates respectively.



## DISCUSSION

Speculating about correlations between cluster profiles and physiological conditions, cluster 1 suggests possible markers that would correlate to the injury severity, since protein abundances progressively increase with the severity of the surgical event. Clusters 2 and 5 suggest proteins that change the sense of regulation when the condition changes from mild to more intense surgical procedure and, in similar ways, cluster 3 detected proteins that respond similarly to any intensity of trauma, and proteins regulated only by intestinal ischemia and reperfusion were clustered in 4.

The five clusters where our quantified proteins were grouped did not enrich for specific cellular localization. According to **Supplementary Figure S1A**, most of the significantly regulated proteins from clusters 1, 3, and 4 were annotated to cytoplasm, extracellular, and nucleus. Cytosol and organelle lumen had proteins from cluster 1 whereas ribosome proteins were exclusively annotated to cluster 1. Mostly membrane proteins were grouped in cluster 4 that is down regulated in IR as compared to the control and laparotomy groups.

Proteins related to metabolic process from cluster 1 and 4 showed enrichment (**Supplementary Figure S1B**) whereas, the highest number of proteins related to metabolic processes belong to cluster 1 which is continuously up regulated in laparotomy and IR as compared to the control group. Gene Ontology (GO) terms distribution for the molecular functions

(**Supplementary Figure S1C**) of all the regulated proteins from clusters 1, 3, and 4 showed annotation to catalytic activity, binding proteins whereas cluster 1 represented a high amount of protein enrichment for RNA binding and structural molecule activity. Proteins from cluster 1 and 4 showed highest diversity in molecular functions among the clusters.

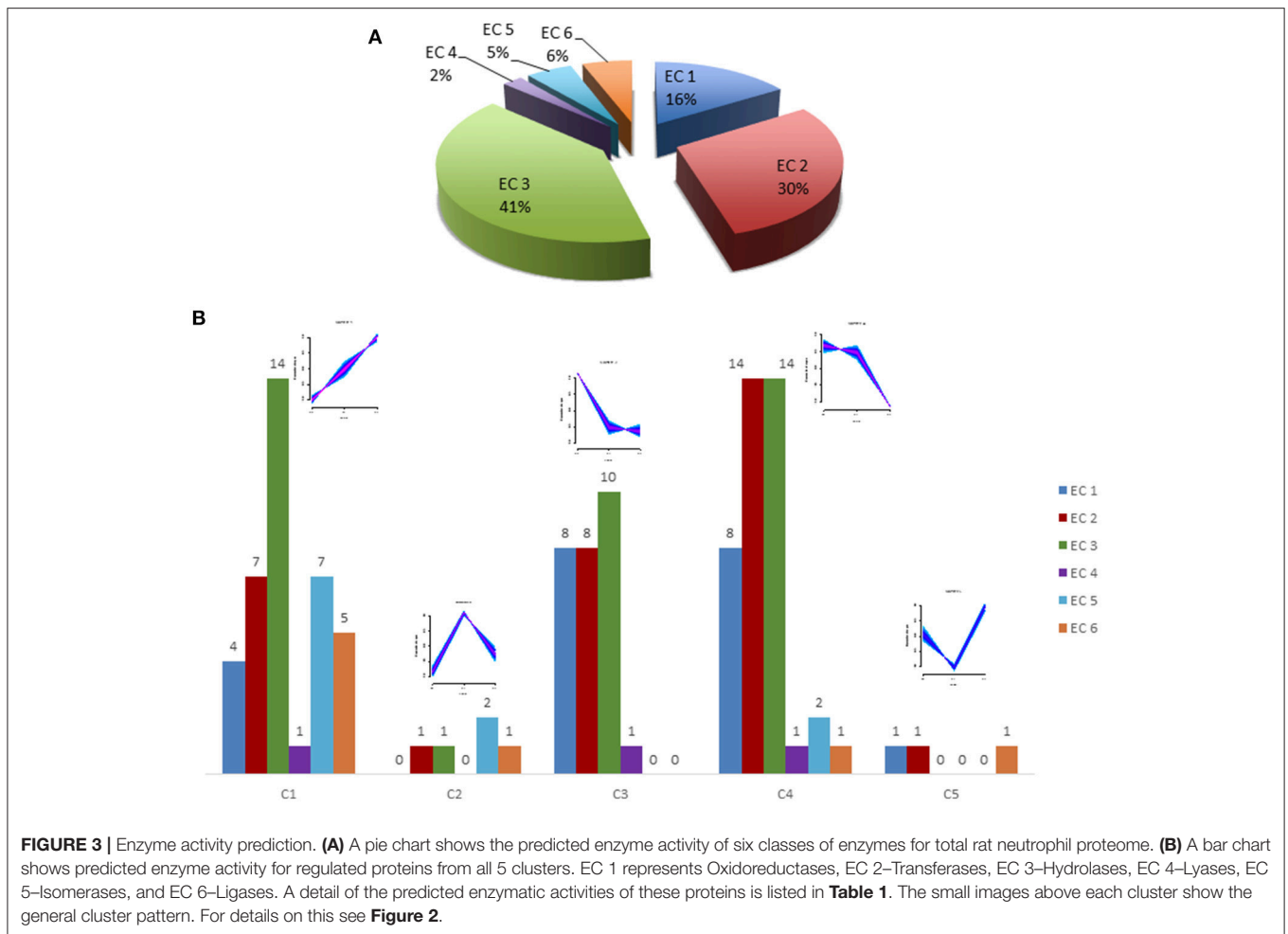
After identifying a large number of proteins, the data were analyzed in Uniprot to predict the enzyme activity for the identified neutrophil proteome. It has been clear from the literature that different enzymes play an important role in neutrophils. The enzyme activities encountered varies for each cluster (**Figure 3B**).

### Predicted Enzyme Activity for Cluster 1 Proteins

These enzymes are found progressively up regulated in neutrophils from the control to the laparotomy to the ischemia/reperfusion group suggesting that the intensity of regulation may be proportional to the severity of the inflammatory process.

#### Oxidoreductases

The quantitative analysis revealed significantly up regulated oxidoreductases in cluster 1, among which we found superoxide dismutase (SOD). Normally during inflammation, SOD regulates ROS concentration and reactive nitrogen species however high levels of extracellular SOD activity resulted in reduced innate



**FIGURE 3 |** Enzyme activity prediction. **(A)** A pie chart shows the predicted enzyme activity of six classes of enzymes for total rat neutrophil proteome. **(B)** A bar chart shows predicted enzyme activity for regulated proteins from all 5 clusters. EC 1 represents Oxidoreductases, EC 2–Transferases, EC 3–Hydrolases, EC 4–Lyases, EC 5–Isomerases, and EC 6–Ligases. A detail of the predicted enzymatic activities of these proteins is listed in **Table 1**. The small images above each cluster show the general cluster pattern. For details on this see **Figure 2**.

immune response of neutrophils (Break et al., 2012). Increased level of DHFR was observed in our quantitative analysis whereas in peripheral blood leucocytes, mainly neutrophils, of cancer patients' higher expression of DHFR has been associated with leukocytosis (Iqbal et al., 2000). Other significantly up regulated oxidoreductases of cluster 1 include Prostaglandin reductase 1 (PtGR-1), and fatty acyl-CoA reductase 1 (**Table 1**). PtGR-1 is a nitroalkene reductase and its overexpression in HEK293T cells promoted inactivation of nitroalkane, inhibition of hemeoxygenase HO-1 and finally abrogated tissue protection (Vitturi et al., 2013). It has been shown that inducing HO-1 prior to IRI results in a significant decrease of intestinal tissue injury (Wasserberg et al., 2007).

### Transferases

N-myristoyltransferase (NMT1) catalyzes protein myristoylation process that is essential for leukocyte growth and development, found elevated in activated neutrophils along with increase in lifespan (Kumar et al., 2011). The majority of significantly regulated transferases are oligosaccharyltransferases involved in N-Linked protein glycosylation. Since pro-inflammatory stimuli modify glycan profiles on cell surfaces, the overexpression of such enzymes can lead to an increased adhesion of

activated neutrophils to endothelial cells (Sriramarao et al., 1993). S-adenosylmethionine (SAM) acts as a donor for methyltransferases that is involved in methylation of DNA, RNA, proteins, and lipids. SAM has important implications in antigen-induced immune responses (Wu et al., 2005).

### Hydrolases

Most of the regulated hydrolases are annotated to DNA helicases and one RNA helicase. Other statistically regulated hydrolases, relevant to the inflammation were Caspase-1, involved in the inflammasome pathway, and Cathepsin D. Increased activity of cathepsin D in myocardial ischemic neutrophils was found to be associated with increased ROS production (Miriya et al., 2013). Both the Caspase-1 and Cathepsin D were significantly found up regulated in this study showing high inflammasome pathway activation and ROS production.

### Predicted Enzyme Activity for Cluster 4 Proteins

Another group of transferases, oxidoreductases, and hydrolases present predominant regulation in neutrophils from intestinal ischemia/reperfusion in cluster 4 as shown in **Figure 3B**.

**TABLE 1** | Enzyme activities prediction for the significantly regulated proteins.

| Enzyme description   | EC number     | Protein IDs | Gene symbol | Cluster no. | LAP vs. Ctrl Log2 ratio | IR vs. Ctrl Log2 ratio | IR vs. LAP Log2 ratio |
|--|---------------|-------------|-------------|-------------|-------------------------|------------------------|-----------------------|
| <b>OXIDOREDUCTASES</b>   |               |             |             |             |                         |                        |                       |
| superoxide dismutase [Cu-Zn]   | EC 1.15.1.1   | P07632      | Sod1        | 1           | 0.108233771             | 0.130217203            | 0.021983432           |
| Fatty acyl-CoA reductase 1 isoform 1   | EC 1.2.1.n2   | Q922J9      | Far1        | 1           | 0.335933388             | 0.471008515            | 0.135075127           |
| Prostaglandin reductase 1  | EC 1.3.1.48   | P97584      | Ptgr1       | 1           | 0.151344167             | 0.63932175             | 0.487977582           |
| Dihydrofolate reductase  | EC 1.5.1.3    | Q920D2      | Dhfr        | 1           | 0.202589021             | 0.26928123             | 0.06669221            |
| NAD-dependent malic enzyme, mitochondrial precursor                                    | EC 1.1.1.38   | Q99KE1      | Me2         | 3           | -0.125334931            | -0.211535203           | -0.086200272          |
| Eosinophil peroxidase precursor  | EC 1.11.1.7   | P49290      | Epx         | 3           | -1.461573896            | -1.86665744            | -0.405083544          |
| Glutathione peroxidase 1   | EC 1.11.1.9   | P04041      | Gpx1        | 3           | -0.131894129            | -0.236200267           | -0.104306138          |
| Protein-methionine sulfoxide oxidase MICAL1  | EC 1.14.13.-  | D3ZBP4      | Mical1      | 3           | -0.132806847            | -0.172661531           | -0.039854685          |
| Leukotriene-B(4) omega-hydroxylase 2   | EC 1.14.13.30 | Q3MID2      | Cyp4f18     | 3           | -0.314624374            | -0.54501604            | -0.230391666          |
| Metalloreductase STEAP4  | EC 1.16.1.-   | Q4V8K1      | Steap4      | 3           | -0.634298418            | -0.633934112           | 0.000364306           |
| Peroxisomal acyl-coenzyme A oxidase 1  | EC 1.3.3.6    | P07872      | Acox1       | 3           | -0.153657032            | -0.11360617            | 0.040050862           |
| NADPH-cytochrome P450 reductase  | EC 1.6.2.4    | P00388      | Por         | 3           | -0.14668384             | -0.203604695           | -0.056920854          |
| GDP-L-fucose synthase  | EC 1.1.1.271  | P23591      | Tsta3       | 4           | -0.053953695            | -0.146615802           | -0.092662107          |
| Myeloperoxidase precursor  | EC 1.11.2.2   | P11247      | Mpo         | 4           | -0.010129062            | -0.152429094           | -0.142300032          |
| Arachidonate 15-lipoxygenase   | EC 1.13.11.33 | Q02759      | Alox15      | 4           | -0.591830552            | -1.210220167           | -0.618389614          |
| Xanthine dehydrogenase/oxidase   | EC 1.17.1.4   | P22985      | Xdh         | 4           | -0.049187813            | -0.100438443           | -0.05125063           |
| Aldehyde dehydrogenase family 3 member B1  | EC 1.2.1.5    | Q5XI42      | Aldh3b1     | 4           | -0.044890506            | -0.14087961            | -0.095989104          |
| acyl-CoA dehydrogenase family member 9, mitochondrial                                  | EC 1.3.99.-   | Q8JZN5      | Acad9       | 4           | 0.094393882             | -0.091932542           | -0.186326425          |
| NAD(P)H dehydrogenase [quinone] 1  | EC 1.6.5.2    | P05982      | Nqo1        | 4           | -0.094854334            | -0.270101397           | -0.175247063          |
| Thioredoxin reductase 1, cytoplasmic   | EC 1.8.1.9    | O89049      | Txnrd1      | 4           | -0.001836217            | -0.119040483           | -0.117204265          |
| L-lactate dehydrogenase C chain  | EC 1.1.1.27   | P19629      | Ldhc        | 5           | -0.156311009            | 0.132653839            | 0.288964848           |
| <b>TRANSFERASES</b>  |               |             |             |             |                         |                        |                       |
| Non-specific lipid-transfer protein  | EC 2.3.1.176  | P11915      | Scp2        | 1           | 0.197153822             | 0.41885748             | 0.221703658           |
| Glycopeptide N-tetradecanoyltransferase 1  | EC 2.3.1.97   | O70310      | Nmt1        | 1           | 0.054980313             | 0.204797239            | 0.149816926           |
| Dolichyl-diphosphooligosaccharide-protein glycosyltransferase 48 kDa subunit precursor | EC 2.4.99.18  | O54734      | Ddost       | 1           | 0.093155294             | 0.086662               | -0.006493294          |
| Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 1 precursor      | EC 2.4.99.18  | P07153      | Rpn1        | 1           | 0.058894484             | 0.175025097            | 0.116130613           |
| Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit DAD1             | EC 2.4.99.18  | P61806      | Dad1        | 1           | 0.115180081             | 0.180992851            | 0.065812769           |
| S-adenosylmethionine synthase isoform type-2   | EC 2.5.1.6    | P18298      | Mat2a       | 1           | 0.037481668             | 0.159209384            | 0.121727716           |
| Serine/threonine-protein kinase VRK1 isoform a   | EC 2.7.11.1   | Q80X41      | Vrk1        | 1           | 0.091142922             | 0.205754141            | 0.114611219           |
| Microsomal glutathione S-transferase 1   | EC 2.5.1.18   | P08011      | Mgst1       | 2           | 0.234880268             | 0.203988231            | -0.030892038          |
| Glutathione S-transferase P  | EC 2.5.1.18   | P04906      | Gstp1       | 3           | -0.168833409            | -0.247907738           | -0.079074329          |
| Phosphatidylinositol 5-phosphate 4-kinase type-2 alpha                                 | EC 2.7.1.149  | Q9R0I8      | Pip4k2a     | 3           | -0.143480491            | -0.17807905            | -0.034598559          |
| Protein-tyrosine kinase 2-beta   | EC 2.7.10.2   | P70600      | Ptk2b       | 3           | -0.084516425            | -0.157783059           | -0.073266634          |
| Serine/threonine-protein kinase OSR1   | EC 2.7.11.1   | Q6P9R2      | Oxsr1       | 3           | -0.118920844            | -0.165596042           | -0.046675198          |
| Serine/threonine-protein kinase 17B  | EC 2.7.11.1   | Q91XS8      | Stk17b      | 3           | -0.135846465            | -0.179465752           | -0.043619286          |
| Serine/threonine-protein kinase 4  | EC 2.7.11.1   | Q9JI11      | Stk4        | 3           | -0.050646666            | -0.090317152           | -0.039670486          |
| 5'-AMP-activated protein kinase catalytic subunit alpha-1                              | EC 2.7.11.1   | P54645      | Prkaa1      | 3           | -0.101888187            | -0.111546268           | -0.009658081          |
| Protein kinase C delta type  | EC 2.7.11.13  | P09215      | Prkcd       | 3           | -0.120430329            | -0.183010542           | -0.062580213          |
| 3-ketoacyl-CoA thiolase, mitochondrial   | EC 2.3.1.16   | P13437      | Acaa2       | 4           | 0.082421511             | -0.127697255           | -0.210118766          |
| Lysophosphatidylcholine acyltransferase 2  | EC 2.3.1.23   | Q8BYI6      | Lpcat2      | 4           | -0.181813638            | -0.5111139267          | -0.329325629          |
| 1,4-alpha-glucan-branching enzyme  | EC 2.4.1.18   | Q9D6Y9      | Gbe1        | 4           | -0.042286018            | -0.148930328           | -0.10664431           |
| Uridine phosphorylase 1  | EC 2.4.2.3    | P52624      | Upp1        | 4           | -0.083242608            | -0.392550565           | -0.309307957          |

(Continued)

TABLE 1 | Continued

| Enzyme description   | EC number    | Protein IDs | Gene symbol | Cluster no. | LAP vs. Ctrl Log2 ratio | IR vs. Ctrl Log2 ratio | IR vs. LAP Log2 ratio |
|--|--------------|-------------|-------------|-------------|-------------------------|------------------------|-----------------------|
| Hypoxanthine-guanine phosphoribosyltransferase                   | EC 2.4.2.8   | Q64531      | Hprt1       | 4           | -0.024144311            | -0.083055294           | -0.058910983          |
| Hexokinase-3   | EC 2.7.1.1   | P27926      | Hk3         | 4           | -0.016624745            | -0.117983675           | -0.10135893           |
| 6-phosphofructokinase, liver type                                | EC 2.7.1.11  | P12382      | Pfkl        | 4           | -0.071999766            | -0.176198903           | -0.104199137          |
| N-acetylgalactosamine kinase                                     | EC 2.7.1.157 | Q5XIG6      | Galk2       | 4           | -0.038659611            | -0.179037398           | -0.140377787          |
| Pyruvate kinase PKM  | EC 2.7.1.40  | P11980      | Pkm         | 4           | -0.055693459            | -0.118552564           | -0.062859105          |
| Tyrosine-protein kinase Lyn isoform A                            | EC 2.7.10.2  | Q07014      | Lyn         | 4           | -0.020298629            | -0.15028629            | -0.129987661          |
| Tyrosine-protein kinase Fgr                                      | EC 2.7.10.2  | Q6P6U0      | Fgr         | 4           | -0.046943698            | -0.136581547           | -0.089637849          |
| Ribosomal protein S6 kinase alpha-3                              | EC 2.7.11.1  | P18654      | Rps6ka3     | 4           | -0.073325148            | -0.182621537           | -0.109296389          |
| Pyruvate dehydrogenase kinase, isozyme 3                         | EC 2.7.11.2  | Q922H2      | Pdk3        | 4           | -0.04791905             | -0.361413111           | -0.313494062          |
| 3-mercaptopyruvate sulfurtransferase                             | EC 2.8.1.2   | P97532      | Mpst        | 4           | -0.083073726            | -0.202096667           | -0.119022941          |
| Interferon-induced, double-stranded RNA-activated protein kinase | EC 2.7.11.1  | Q63184      | Eif2ak2     | 5           | -0.360888899            | -0.108859607           | 0.252029292           |
| <b>HYDROLASES</b>  |              |             |             |             |                         |                        |                       |
| Palmitoyl-protein thioesterase 1 precursor                       | EC 3.1.2.22  | P45479      | Ppt1        | 1           | 0.24879684              | 0.347257042            | 0.098460201           |
| Eukaryotic translation initiation factor 3 subunit F             | EC 3.4.19.12 | Q9DCH4      | Eif3f       | 1           | 0.108275952             | 0.155827424            | 0.047551472           |
| Caspase-1  | EC 3.4.22.36 | P43527      | Casp1       | 1           | 0.103221535             | 0.121577846            | 0.01835631            |
| Cathepsin D  | EC 3.4.23.5  | P24268      | Ctsd        | 1           | 0.133927025             | 0.311194503            | 0.177267478           |
| N-acylethanolamine-hydrolyzing acid amidase precursor            | EC 3.5.1.-   | Q5KTC7      | Naaa        | 1           | 0.085215327             | 0.277437413            | 0.192222086           |
| DNA replication licensing factor MCM2                            | EC 3.6.4.12  | P97310      | Mcm2        | 1           | 0.056502266             | 0.187656256            | 0.13115399            |
| DNA replication licensing factor MCM3                            | EC 3.6.4.12  | P25206      | Mcm3        | 1           | 0.150264288             | 0.218667178            | 0.06840289            |
| DNA replication licensing factor MCM4                            | EC 3.6.4.12  | P49717      | Mcm4        | 1           | 0.126913574             | 0.250998827            | 0.124085253           |
| DNA replication licensing factor MCM5                            | EC 3.6.4.12  | P49718      | Mcm5        | 1           | 0.105563621             | 0.199463388            | 0.093899767           |
| DNA replication licensing factor MCM6                            | EC 3.6.4.12  | P97311      | Mcm6        | 1           | 0.076101678             | 0.193375561            | 0.117273883           |
| DNA replication licensing factor MCM7                            | EC 3.6.4.12  | Q61881      | Mcm7        | 1           | 0.064784112             | 0.346338741            | 0.281554628           |
| Chromodomain-helicase-DNA-binding protein 4                      | EC 3.6.4.12  | Q6PDQ2      | Chd4        | 1           | 0.032541132             | 0.099751668            | 0.067210535           |
| Eukaryotic initiation factor 4A-I isoform 1                      | EC 3.6.4.13  | P60843      | Eif4a1      | 1           | 0.128043156             | 0.286460547            | 0.158417392           |
| Probable ATP-dependent RNA helicase DDX5                         | EC 3.6.4.13  | Q61656      | Ddx5        | 1           | 0.110607888             | 0.189602631            | 0.078994744           |
| Myeloblastin   | EC 3.4.21.76 | Q61096      | Prtn3       | 2           | 0.478989991             | -0.295825849           | -0.77481584           |
| Neutral cholesterol ester hydrolase 1                            | EC 3.1.1.-   | B2GV54      | Nceh1       | 3           | -0.170985165            | -0.281472967           | -0.110487802          |
| Eosinophil cationic protein precursor                            | EC 3.1.27.-  | P70709      | Ear11       | 3           | -1.094986988            | -1.472034709           | -0.377047722          |
| Protein phosphatase 1F   | EC 3.1.3.16  | Q9WVR7      | Ppm1f       | 3           | -0.15635207             | -0.217872783           | -0.061520713          |
| Inositol monophosphatase 1                                       | EC 3.1.3.25  | P97697      | Impa1       | 3           | -0.13409359             | -0.123614044           | 0.010479545           |
| Mast cell carboxypeptidase A                                     | EC 3.4.17.1  | P21961      | Cpa3        | 3           | -2.502754553            | -2.166354088           | 0.336400465           |
| Mast cell protease 1 preproprotein                               | EC 3.4.21.39 | P09650      | Mcpt111     | 3           | -2.243417189            | -1.066062508           | 1.177354681           |
| Chymase precursor  | EC 3.4.21.39 | P50339      | Cma1        | 3           | -1.929537438            | -0.974073498           | 0.95546394            |
| Protein-arginine deiminase type-4                                | EC 3.5.3.15  | O88807      | Pad14       | 3           | -0.072190624            | -0.125052572           | -0.052861949          |
| Ectonucleoside triphosphate diphosphohydrolase 1                 | EC 3.6.1.5   | P97687      | Entpd1      | 3           | -0.486430431            | -0.642852938           | -0.156422508          |
| Putative helicase MOV-10 isoform b                               | EC 3.6.4.13  | P23249      | Mov10       | 3           | -0.562804813            | -0.622111451           | -0.059306638          |
| Acyl-protein thioesterase 2                                      | EC 3.1.2.-   | Q9QYL8      | Lypla2      | 4           | -0.101689793            | -0.218873152           | -0.117183359          |
| Receptor-type tyrosine-protein phosphatase C isoform 4 precursor | EC 3.1.3.48  | P04157      | Ptpcr       | 4           | -0.097829627            | -0.215167164           | -0.117337537          |
| Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 1         | EC 3.1.3.86  | P97573      | Inpp5d      | 4           | -0.027078682            | -0.067062757           | -0.039984075          |
| Beta-hexosaminidase subunit beta precursor                       | EC 3.2.1.52  | Q6AXR4      | Hexb        | 4           | -0.031166072            | -0.116565045           | -0.085398973          |
| Leukotriene A-4 hydrolase  | EC 3.3.2.6   | P24527      | Lta4h       | 4           | 0.196955814             | -0.205664033           | -0.402619848          |
| Aminopeptidase N precursor                                       | EC 3.4.11.2  | P15684      | Anpep       | 4           | -0.058052056            | -0.136747257           | -0.078695201          |

(Continued)

TABLE 1 | Continued

| Enzyme description   | EC number    | Protein IDs | Gene symbol | Cluster No. | LAP vs. Ctrl Log2 ratio | IR vs. Ctrl Log2 ratio | IR vs. LAP Log2 ratio |
|--|--------------|-------------|-------------|-------------|-------------------------|------------------------|-----------------------|
| Leucyl-cystinyl aminopeptidase isoform 1                       | EC 3.4.11.3  | P97629      | Lnpep       | 4           | 0.024568668             | -0.161756158           | -0.186324826          |
| Dipeptidyl peptidase 1 precursor                               | EC 3.4.14.1  | P80067      | Ctsc        | 4           | -0.030607236            | -0.175121607           | -0.144514371          |
| Gamma-glutamyltransferase 5                                    | EC 3.4.19.13 | Q9QWE9      | Ggt5        | 4           | -0.024176962            | -0.279898505           | -0.255721543          |
| Neutrophil elastase precursor                                  | EC 3.4.21.37 | Q3UP87      | Elane       | 4           | 0.216634577             | -0.233671599           | -0.450306176          |
| Signal peptidase complex catalytic subunit SEC11A              | EC 3.4.21.89 | P42667      | Sec11a      | 4           | -0.053486912            | -0.112319915           | -0.058833002          |
| Diphosphoinositol polyphosphate phosphohydrolase 2             | EC 3.6.1.52  | Q8R2U6      | Nudt4       | 4           | -0.159124629            | -0.324161988           | -0.165037359          |
| Vesicle-fusing ATPase  | EC 3.6.4.6   | P18708      | Nsf         | 4           | -0.034479238            | -0.137419351           | -0.102940112          |
| Dynamin-2  | EC 3.6.5.5   | P39052      | Dnm2        | 4           | -0.002743949            | -0.079469533           | -0.076725584          |
| <b>LYASES</b>  |              |             |             |             |                         |                        |                       |
| 40S ribosomal protein S3                                       | EC 4.2.99.18 | P62908      | Rps3        | 1           | 0.196114129             | 0.350698421            | 0.154584292           |
| DNA-(apurinic or apyrimidinic site) lyase                      | EC 4.2.99.18 | P43138      | Apex1       | 3           | -0.085613485            | -0.139340232           | -0.053726747          |
| Argininosuccinate lyase  | EC 4.3.2.1   | P20673      | Asl         | 4           | -0.045119034            | -0.12824703            | -0.083127997          |
| <b>ISOMERASES</b>  |              |             |             |             |                         |                        |                       |
| Peptidyl-prolyl cis-trans isomerase B                          | EC 5.2.1.8   | P24368      | Ppib        | 1           | 0.050687673             | 0.110150995            | 0.059463322           |
| Peptidyl-prolyl cis-trans isomerase FKBP3                      | EC 5.2.1.8   | Q62446      | Fkbp3       | 1           | 0.099918153             | 0.244218789            | 0.144300636           |
| Peptidyl-prolyl cis-trans isomerase D                          | EC 5.2.1.8   | Q6DGG0      | Ppid        | 1           | 0.050889252             | 0.096319227            | 0.045429975           |
| Protein disulfide-isomerase                                    | EC 5.3.4.1   | P04785      | P4hb        | 1           | 0.070606559             | 0.218578766            | 0.147972206           |
| Protein disulfide-isomerase A3                                 | EC 5.3.4.1   | P11598      | Pdia3       | 1           | 0.085046824             | 0.153754172            | 0.068707347           |
| Protein disulfide-isomerase A4                                 | EC 5.3.4.1   | P38659      | Pdia4       | 1           | 0.072096175             | 0.155111366            | 0.083015192           |
| Protein disulfide-isomerase A6                                 | EC 5.3.4.1   | Q63081      | Pdia6       | 1           | 0.108211498             | 0.208593967            | 0.100382469           |
| Peptidyl-prolyl cis-trans isomerase F, mitochondrial precursor | EC 5.2.1.8   | P29117      | Ppif        | 2           | 0.581369784             | 0.210543386            | -0.370826398          |
| Macrophage migration inhibitory factor                         | EC 5.3.2.1   | P30904      | Mif         | 2           | 0.461822345             | -0.148221794           | -0.610044139          |
| Ribulose-phosphate 3-epimerase                                 | EC 5.1.3.1   | Q8VEE0      | Rpe         | 4           | -0.174094827            | -0.354098654           | -0.180003827          |
| NAD(P)H-hydrate epimerase                                      | EC 5.1.99.6  | B0BNM1      | Apoa1bp     | 4           | -0.03634563             | -0.110532371           | -0.074186741          |
| <b>LIGASES</b>   |              |             |             |             |                         |                        |                       |
| Aspartate-tRNA ligase, cytoplasmic                             | EC 6.1.1.12  | P15178      | Dars        | 1           | 0.047262581             | 0.090565223            | 0.043302641           |
| Leucine-tRNA ligase, cytoplasmic                               | EC 6.1.1.4   | Q8BMJ2      | Lars        | 1           | 0.107642729             | 0.217467451            | 0.109824722           |
| E3 ubiquitin-protein ligase NEDD4                              | EC 6.3.2.-   | Q62940      | Nedd4       | 1           | 0.086895543             | 0.239777618            | 0.152882074           |
| Ubiquitin-conjugating enzyme E2 L3                             | EC 6.3.2.19  | P68037      | Gm10705     | 1           | 0.02016907              | 0.091055143            | 0.070886073           |
| Asparagine synthetase [glutamine-hydrolyzing]                  | EC 6.3.5.4   | P49088      | Asns        | 1           | 0.166112474             | 0.242415172            | 0.076302699           |
| Glycine-tRNA ligase  | EC 6.1.1.14  | Q510G4      | Gars        | 2           | 0.119496174             | 0.008715768            | -0.110780406          |
| Nicotinate phosphoribosyltransferase                           | EC 6.3.4.21  | Q6XQN1      | Naprt1      | 4           | 0.091298572             | -0.136451197           | -0.227749769          |
| Methionine-tRNA ligase, cytoplasmic isoform 2                  | EC 6.1.1.10  | Q68FL6      | Mars        | 5           | -0.056877927            | 0.129932982            | 0.186810909           |

## Oxidoreductases

There are some important oxidoreductases regulated in this cluster. Arachidonate 15-lipoxygenase was found significantly down regulated. It has been known that arachidonate 12/15-lipoxygenases (12/15-LOX) forms hydroperoxy eicosatetraenoic acids (HPETEs) from arachidonic acid that subsequently produces eicosanoids. Leukocytes highly expresses 15-LOX but little is known about its role in neutrophils (Nadel et al., 1991). Xanthine oxidase (Xdh) and thioredoxin reductase 1 (TXNRD1) are cellular defense enzymes against oxidative stress. Significantly down regulation of these antioxidants after IR can be an important step in modulating the neutrophil response to oxidative stress (Vorbach et al., 2003; Biterova et al., 2005). The most extensively studied primary granule enzyme of neutrophils

is myeloperoxidase (MPO), found significantly regulated in cluster 4 by our quantitative analysis (Table 1). Recently an increase in MPO along with ICAM-1 and P-selectin expressions in neutrophils has been found in a model of small intestinal ischemia in rats (Gan et al., 2015).

## Transferases

Most down regulated transferases have function in phospholipid metabolism, carbohydrate degradation (glycolysis) and nucleotide metabolism. The tyrosine-protein kinases Lyn/Fgr belong to the Src family of kinases that negatively regulate integrin-signaling pathway. Neutrophils deficient of these kinases had enhanced respiratory burst, secondary granule release, and a hyper-adhesive phenotype due to reduced



**TABLE 2** | Major functional classification.

| Protein ID  | Gene symbol | Protein name   | Cluster | LAP vs. Ctrl Log2 ratio | IR vs. Ctrl Log2 ratio | IR vs. LAP Log2 ratio |
|---|-------------|--|---------|-------------------------|------------------------|-----------------------|
| <b>KEGG: Spliceosome (<i>rawP</i> = 1.13e-05; <i>adjP</i> = 5.09e-05)</b>                               |             |  |         |                         |                        |                       |
| P63017  | Hspa8       | Heat shock 70kDa protein 8   | 1       | 0.045693617             | 0.106185806            | 0.06049219            |
| O35900  | Lsm2        | LSM2 homolog, U6 small nuclear RNA associated ( <i>S. cerevisiae</i> )           | 1       | 0.067225314             | 0.155666556            | 0.088441241           |
| O35326  | Srsf5       | Serine/arginine-rich splicing factor 5   | 1       | 0.144719584             | 0.239436591            | 0.094717008           |
| Q8VE97  | Srsf4       | Serine/arginine-rich splicing factor 4   | 1       | 0.048158793             | 0.118255729            | 0.070096936           |
| Q61656  | Ddx5        | DEAD (Asp-Glu-Ala-Asp) box helicase 5  | 1       | 0.110607888             | 0.189602631            | 0.078994744           |
| P60335  | Pcbp1       | Poly(rC) binding protein 1   | 1       | 0.104001931             | 0.13900654             | 0.035004608           |
| <b>KEGG: antigen processing and presentation (<i>rawP</i> = 2.26e-05; <i>adjP</i> = 8.72e-05)</b>       |             |  |         |                         |                        |                       |
| P63017  | Hspa8       | Heat shock 70kDa protein 8   | 1       | 0.045693617             | 0.106185806            | 0.06049219            |
| P06761  | Hspa5       | Heat shock protein 5   | 1       | 0.10315848              | 0.165189029            | 0.06203055            |
| P11499  | Hsp90ab1    | Heat shock protein 90 alpha (cytosolic), class B member 1                        | 1       | 0.062540962             | 0.274247984            | 0.211707022           |
| P82995  | Hsp90aa1    | Heat shock protein 90, alpha (cytosolic), class A member 1                       | 1       | 0.034297768             | 0.130094309            | 0.095796541           |
| P11598  | Pdia3       | Protein disulfide isomerase family A, member 3                                   | 1       | 0.085046824             | 0.153754172            | 0.068707347           |
| <b>KEGG: NOD-like receptor signaling pathway (<i>rawP</i> = 4.45e-05; <i>adjP</i> = 0.0002)</b>         |             |  |         |                         |                        |                       |
| P43527  | Casp1       | Caspase 1  | 1       | 0.103221535             | 0.121577846            | 0.01835631            |
| P11499  | Hsp90ab1    | Heat shock protein 90 alpha (cytosolic), class B member 1                        | 1       | 0.062540962             | 0.274247984            | 0.211707022           |
| P82995  | Hsp90aa1    | Heat shock protein 90, alpha (cytosolic), class A member 1                       | 1       | 0.034297768             | 0.130094309            | 0.095796541           |
| Q66HD0  | Hsp90b1     | Heat shock protein 90, beta, member 1  | 1       | 0.12678686              | 0.188938948            | 0.062152088           |
| <b>Wikipathways: TNF-alpha NF-kB signaling pathway (<i>rawP</i> = 9.40e-07; <i>adjP</i> = 2.98e-06)</b> |             |  |         |                         |                        |                       |
| P50878  | Rpl4        | Ribosomal protein L4   | 1       | 0.143724339             | 0.304591572            | 0.160867233           |
| P49718  | Mcm5        | Minichromosome maintenance complex component 5                                   | 1       | 0.105563621             | 0.199463388            | 0.093899767           |
| P11499  | Hsp90ab1    | Heat shock protein 90 alpha (cytosolic), class B member 1                        | 1       | 0.062540962             | 0.274247984            | 0.211707022           |
| P68040  | Gnb211      | Guanine nucleotide binding protein (G protein), beta polypeptide 2 like 1        | 1       | 0.198001407             | 0.364800338            | 0.166798931           |
| P82995  | Hsp90aa1    | Heat shock protein 90, alpha (cytosolic), class A member 1                       | 1       | 0.034297768             | 0.130094309            | 0.095796541           |
| P21533  | Rpl6        | Ribosomal protein L6   | 1       | 0.100057017             | 0.30453836             | 0.204481343           |
| Q61881  | Mcm7        | Minichromosome maintenance complex component 7                                   | 1       | 0.064784112             | 0.346338741            | 0.281554628           |
| P62281  | Rps11       | Ribosomal protein S11  | 1       | 0.180726804             | 0.335684506            | 0.154957702           |
| <b>Wikipathways: MAPK signaling pathway (<i>rawP</i> = 0.0033; <i>adjP</i> = 0.0074)</b>                |             |  |         |                         |                        |                       |
| P63017  | Hspa8       | Heat shock 70kDa protein 8   | 1       | 0.045693617             | 0.106185806            | 0.06049219            |
| P43527  | Casp1       | Caspase 1  | 1       | 0.103221535             | 0.121577846            | 0.01835631            |
| P06761  | Hspa5       | Heat shock protein 5   | 1       | 0.10315848              | 0.165189029            | 0.06203055            |
| P13668  | Stmn1       | Stathmin 1   | 1       | 0.10969688              | 0.252735553            | 0.143038673           |
| <b>Wikipathways: IL-2 signaling pathway (<i>rawP</i> = 0.0047; <i>adjP</i> = 0.0089)</b>                |             |  |         |                         |                        |                       |
| P68040  | Gnb211      | Guanine nucleotide binding protein (G protein), beta polypeptide 2 like 1        | 1       | 0.198001407             | 0.364800338            | 0.166798931           |
| P82995  | Hsp90aa1    | Heat shock protein 90, alpha (cytosolic), class A member 1                       | 1       | 0.034297768             | 0.130094309            | 0.095796541           |
| P52631  | Stat3       | Signal transducer and activator of transcription 3 (acute-phase response factor) | 1       | 0.040131681             | 0.155755431            | 0.115623749           |
| <b>KEGG: Fc gamma R-mediated phagocytosis (<i>rawP</i> = 2.74e-06; <i>adjP</i> = 6.17e-05)</b>          |             |  |         |                         |                        |                       |
| Q8BH43  | Wasf2       | WAS protein family, member 2   | 4       | -0.046564123            | -0.154032936           | -0.107468813          |
| P04157  | Ptpnc       | Protein tyrosine phosphatase, receptor type, C                                   | 4       | -0.097829627            | -0.215167164           | -0.117337537          |
| Q07014  | Lyn         | V-yes-1 Yamaguchi sarcoma viral related oncogene homolog                         | 4       | -0.020298629            | -0.15028629            | -0.129987661          |
| P39052  | Dnm2        | Dynamin 2  | 4       | -0.002743949            | -0.079469533           | -0.076725584          |
| P97573  | Inpp5d      | Inositol polyphosphate-5-phosphatase D   | 4       | -0.027078682            | -0.067062757           | -0.039984075          |
| <b>KEGG: regulation of actin cytoskeleton (<i>rawP</i> = 0.0001; <i>adjP</i> = 0.0005)</b>              |             |  |         |                         |                        |                       |
| Q8BH43  | Wasf2       | WAS protein family, member 2   | 4       | -0.046564123            | -0.154032936           | -0.107468813          |
| P11835  | Itgb2       | Integrin, beta 2   | 4       | 0.039783401             | -0.134544799           | -0.1743282            |
| P27601  | Gna13       | Guanine nucleotide binding protein (G protein), alpha 13                         | 4       | 0.027347849             | -0.171688468           | -0.199036317          |
| Q5SQX6  | Cyfp2       | Cytoplasmic FMR1 interacting protein 2   | 4       | -0.077078437            | -0.151342467           | -0.07426403           |

(Continued)

TABLE 2 | Continued

| Protein ID  | Gene symbol | Protein name  | Cluster | LAP vs. Ctrl Log2 ratio | IR vs. Ctrl Log2 ratio | IR vs. LAP Log2 ratio |
|---|-------------|---|---------|-------------------------|------------------------|-----------------------|
| Q62812  | Myh9        | Myosin, heavy chain 9, non-muscle   | 4       | -0.032033077            | -0.082815246           | -0.050782169          |
| <b>KEGG: Chemokine signaling pathway (rawP = 7.05e-05; adjP = 0.0005)</b>                       |             |   |         |                         |                        |                       |
| P04897  | Gnai2       | Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 2 | 4       | 0.013197352             | -0.127231852           | -0.140429204          |
| P62994  | Grb2        | Growth factor receptor bound protein 2  | 4       | -0.034963374            | -0.080293598           | -0.045330224          |
| Q07014  | Lyn         | V-yes-1 Yamaguchi sarcoma viral related oncogene homolog                                | 4       | -0.020298629            | -0.15028629            | -0.129987661          |
| Q6P6U0  | Fgr         | Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog                           | 4       | -0.046943698            | -0.136581547           | -0.089637849          |
| Q9CXP8  | Gng10       | Guanine nucleotide binding protein (G protein), gamma 10                                | 4       | 0.027942431             | -0.231202992           | -0.259145422          |
| <b>Biological process: leukotriene biosynthetic process (rawP = 3.87e-06; adjP = 0.0010)</b>    |             |   |         |                         |                        |                       |
| Q02759  | Alox15      | Arachidonate 15-lipoxygenase  | 4       | -0.591830552            | -1.210220167           | -0.618389614          |
| P20291  | Alox5ap     | Arachidonate 5-lipoxygenase activating protein  | 4       | -0.023100206            | -0.204507854           | -0.181407647          |
| P24527  | Lta4h       | Leukotriene A4 hydrolase  | 4       | 0.196955814             | -0.205664033           | -0.402619848          |
| Q9QWE9  | Ggt5        | Gamma-glutamyltransferase 5   | 4       | -0.024176962            | -0.279898505           | -0.255721543          |
| <b>Biological process: response to bacterium (Rawp = 8.09e-05; AdjP = 0.0015)</b>               |             |   |         |                         |                        |                       |
| P51437  | Camp        | Cathelicidin antimicrobial peptide  | 5       | -0.158024975            | -0.007959577           | 0.150065397           |
| P42225  | Stat1       | Signal transducer and activator of transcription 1                                      | 5       | -0.13682191             | 0.04853719             | 0.1853591             |
| Q63184  | Eif2ak2     | Eukaryotic translation initiation factor 2-alpha kinase 2                               | 5       | -0.360888899            | -0.108859607           | 0.252029292           |
| <b>Biological process: response to reactive oxygen species (rawP = 1.90e-05; adjP = 0.0016)</b> |             |   |         |                         |                        |                       |
| P04906  | Gstp1       | Glutathione S-transferase pi 1  | 3       | -0.168833409            | -0.247907738           | -0.079074329          |
| P70600  | Ptk2b       | PTK2B protein tyrosine kinase 2 beta  | 3       | -0.084516425            | -0.157783059           | -0.073266634          |
| P09215  | Prkcd       | Protein kinase C, delta   | 3       | -0.120430329            | -0.183010542           | -0.062580213          |
| P43138  | Apex1       | APEX nuclease (multifunctional DNA repair enzyme) 1                                     | 3       | -0.085613485            | -0.139340232           | -0.053726747          |
| P54645  | Prkaa1      | Protein kinase, AMP-activated, alpha 1 catalytic subunit                                | 3       | -0.101888187            | -0.111546268           | -0.009658081          |
| P11672  | Lcn2        | Lipocalin 2   | 3       | -0.538858464            | -0.554555684           | -0.01569722           |
| P04041  | Gpx1        | Glutathione peroxidase 1  | 3       | -0.131894129            | -0.236200267           | -0.104306138          |

rawP, p-value from hypergeometric test.

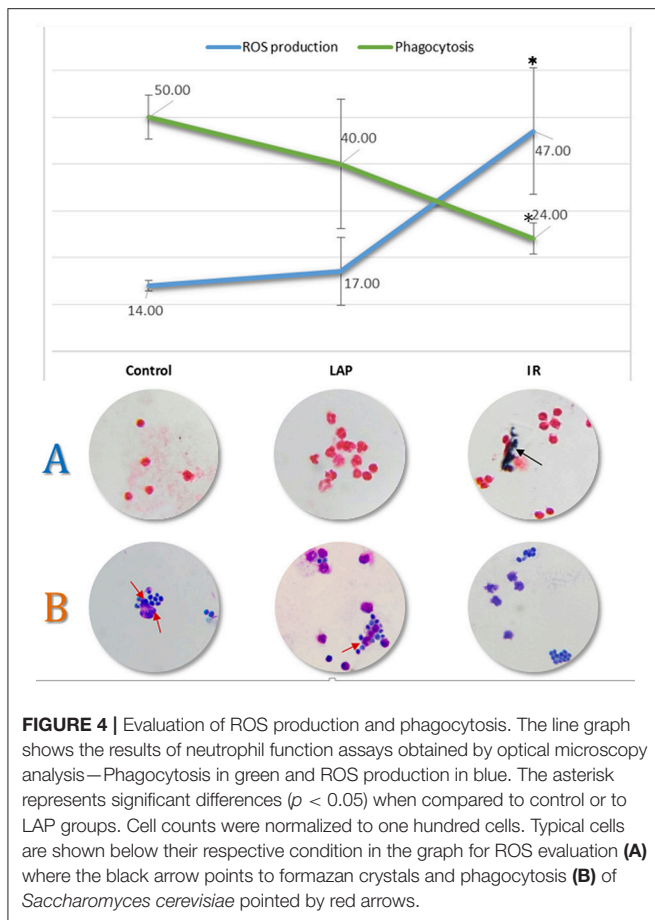
adjP, p-value adjusted by the multiple test adjustment.

mobilization of SHP-1 (Pereira and Lowell, 2003). Many metabolic enzymes including hexokinase, phosphofructokinase and pyruvate kinase were found significantly down regulated in cluster 4 in this study, changes that have also been previously observed during neutrophil activation (Huang et al., 2002). Pyruvate kinase down regulation can lead to partial inhibition of glycolysis at the last step and enhance the synthesis of lipids and nucleic acids, however neutrophils deficient in PK also showed immunodeficiency (Burge et al., 1976). At the first step of glycolysis, Hexokinase (HK3) has 70–80% activity in granulocytes while its down regulation impairs neutrophil differentiation (Federzoni et al., 2012). Although significantly regulated, the transposition of these results to functional inferences needs validation due to the presence of multiple proteoforms of such enzymes.

## Hydrolases

Some of the important hydrolases were found significantly down regulated like P97573, Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase (SHIP). Neutrophil with loss of SHIP showed defective cell migration, loss of polarity upon cell adhesion and increased adhesion due to Akt activation and higher PtdIns(3,4,5)P3 (Mondal et al., 2012). Leukotrienes (LTs) are implicated in a wide variety of inflammatory disorders and are

produced in arachidonic acid (Kutmon et al., 2015) cascade in immune cells. Two bifunctional hydrolases of AA cascade were found regulated in cluster 4. One is leukotriene A-4 hydrolase (LTA4H) involved in leukotriene B4 biosynthesis from LTB<sub>4</sub> (Liu and Yokomizo, 2015) and in aminopeptidase activity by breakdown and clearance of Proline-Glycine-Proline (PGP), a neutrophil chemoattractant (Snelgrove et al., 2010). The other bifunctional hydrolase found down regulated, Gamma-glutamyltransferase 5 (GGT5), participates in glutathione metabolism and in leukotriene D4 biosynthesis from LTC<sub>4</sub> and down regulation of these enzymes can lead to accumulation of leukotriene products (Liu and Yokomizo, 2015) along with PGP and affect neutrophil biology with influx of neutrophils into the tissue and air spaces (Paige et al., 2014). Another interesting hydrolase is Dynamin-2/Dynamin GTPase (DNM), which is involved in microtubules production, binding and hydrolyzation of GTP. Recently, a study showed that inhibition of dynamin impaired the membrane fusion/fission events and resulted in production of highly adhesive cellular secretory protrusions called cytonemes that help neutrophils in long-range contacts with other cells or bacteria after adhesion. Down regulation of dynamin can be an important step in cytoneme production leading to increase in adhesion (Galkina et al., 2005).



An overall evaluation of the enzymes regulated in cluster 1 suggests that neutrophils would progressively increase the ROS production and self-protection against ROS (like SOD, DHFR, Cathepsin-D), decrease tissue protection e.g. PtGR1, increase neutrophil lifespan such as NMT1 and promote adhesion (oligosaccharyl transferases). Such progression appears to be proportional to the injury severity. The regulated enzymes in cluster 4 also suggest an intense effect of IR on the oxidative stress mechanism (including XO, TXNRD1, MPO, Lyn, Fgr, and GGT5) and leukotriene metabolism (as LTA4H and GGT5).

To validate the results regarding ROS production, we performed an NBT test that allows the visualization of blue formazan crystals inside cells that produced ROS. We compared the formazan crystals formation in control, LAP and IR surgical groups. Yellow and water-soluble NBT is reduced to blue formazan crystals by ROS produced by activated neutrophils (Baehner and Nathan, 1968). The exposure of neutrophils to intestinal IR induced a significantly higher ( $p < 0.05$ ) amount of formazan (Figure 4), used as a marker of NADPH oxidase activity: 46.5% of the cells contained extensive formazan formation while LAP and control showed 16.9% and 14.4% cells respectively, probably due to baseline production. These results support the hypothesis that neutrophils contribute to the ischemic oxidative stress as shown in (Jaeschke et al., 1992;

Arumugam et al., 2004) and may be related to the regulation of antioxidant molecules after IR found in cluster 4, as well as the regulation of SOD, DHFR, and Cathepsin-D found in cluster 1.

## Major Functional Classes of Neutrophil Proteome

For the functional classification of the significantly regulated identified proteins, KEGG pathways, and Wikipathways databases were used as a reference knowledge base to understand various signaling mechanisms and pathways (Zhang and Wiemann, 2009; Kutmon et al., 2015). Differentially regulated proteins were mapped to the *Rattus norvegicus* genome as reference set for enrichment analysis using the online analysis WebGestalt (Wang et al., 2013). Most of the enriched pathways are immune-related indicating the effect of intestinal ischemia and reperfusion on the neutrophil function.

Five proteins from cluster 1 were found to be involved in antigen processing and presentation. They are Hspa8 (heat shock 70kDa protein 8), Hspa5 (heat shock protein 5), Hsp90aa1/Hsp90ab1 [heat shock protein 90, alpha (cytosolic), class A member 1/class B member 1] and Pdia3 (protein disulfide isomerase family A, member 3). Antigen processing and presentation is a well-known phenomenon performed by antigen-presenting cells, including dendritic cells, B cells, macrophages, and thymic epithelial cells, signaling to antigen-specific T cells in order to generate effective adaptive immune responses (Roche and Furuta, 2015). It has been found recently that mouse neutrophils express MHC class II and co-stimulatory molecules like CD80 and CD86, and can prime antigen-specific T cells in an MHC class II-dependent manner (Abi Abdallah et al., 2011). Interestingly the identification of up regulation of the identified proteins in our data set suggests that intestinal ischemia and reperfusion increases antigen processing and presentation process in neutrophils.

TNF-alpha/NF-kB signaling pathway was significantly enriched with eight proteins from cluster 1. TNF-alpha is a multifunctional proinflammatory cytokine, which can induce a wide range of apoptosis and cell survival genes as well as inflammation and immunity-related genes by the NF-kB signaling pathway (Barnes and Karin, 1997; Blackwell and Christman, 1997). It has been shown that up-regulation of TNF-alpha/NF-kB signaling pathway after ischemia and reperfusion induces inflammation and tissue injury by the activated neutrophils (Kin et al., 2006). The identification of up-regulation of eight proteins in this pathway expands the previous results that showed up-regulation of this pathway in activated neutrophils after intestinal ischemia and reperfusion causing more injury to the bystander tissues by ROS production (Tang et al., 2011).

Three proteins with significant up-regulation from cluster 1 were grouped in IL-2 signaling pathway with significant enrichment. They include Gnb2l1 [Guanine nucleotide binding protein (G protein), beta polypeptide 2 like 1], Hsp90aa1 [Heat shock protein 90, alpha (cytosolic), class A member 1], and Stat3 (Signal transducer and activator of transcription 3). Jablons et al. (1990) demonstrated that *in vivo* administration of IL-2

in humans with advanced cancers suppresses Fc $\gamma$ R expression (CD16) and chemotaxis in neutrophils whereas another study had opposite results and was performed *in vitro* to check the direct effect of IL-2 on the neutrophils functions (Girard et al., 1996). Our results are in accordance with the *in vivo* analysis as our results show up-regulated IL-2 signaling pathway proteins concomitantly with a down regulation of Fc $\gamma$ R signaling proteins after IR. Fc gamma R-mediated phagocytosis down regulation was found in cluster 4 (Table 2) and is further discussed below.

Here Fc gamma R mediated phagocytosis, regulation of actin cytoskeleton and chemokine signaling pathways were found down regulated in IR, as described by the cluster 4 profile. All the down regulated proteins in these pathways could lead to dramatically slower cell migration, improper polarization, transendothelial migration (TEM), and phagocytosis. Studies have reported that WASF requirement for initial spike in actin polymerization correlates with directional sensing. Zhang et al. (2006) observed reduced adhesion followed by reduced migration toward fMLP along with reduced TEM in WASF-deficient neutrophils associated with defective  $\beta$ 2-integrin clustering. In addition, WASF may play multiple roles in chemotaxis (Kumar et al., 2012).

Disruption of SHIP1\INPP5D, a primary inositol phosphatase causes accumulation of [a PtdIns(3,4,5)P3 probe] and F-actin polymerization across the cell membrane in neutrophils and as a result the neutrophils become flattened and display irregular polarization and less cell migration (Nishio et al., 2007).

We observed down regulation (cluster 4) of Leukotriene synthesis pathways represented by important enzymes like 15 lipoxygenase, 5 lipoxygenase activating protein (FLAP), and LTA<sub>4</sub> hydrolase. Leukotrienes (LTs) are inflammatory mediators causing, for example, phagocyte chemotaxis and increased vascular permeability. LTB<sub>4</sub> is a potent chemotactic agent produced by almost all types of immune cells, especially by neutrophils, and its overproduction leads to certain pathological conditions such as lung edema (Pace et al., 2004) and inflammatory bowel disease (Singh et al., 2003). Few studies have shown the effect of down regulation of 5-LO however, one study reported an increased neutrophil infiltration and TNF expression within the myocardial infarction area of 5-LO deficient mice. However inhibition of 5-lipoxygenase did not affect IR related injury of the wild type mice (Adamek et al., 2007). We have also found down regulation of important enzymes of LTB<sub>4</sub> synthesis pathway in PMNs after intestinal IR. Similarly, platelets could naturally inhibit the LTB<sub>4</sub> synthesis in neutrophils through their spontaneous interactions with these cells. The inhibitory factor can be adenosine as identified in ligand-operated interactions of platelets with neutrophils, but the platelet-derived product responsible for down regulation of neutrophil lipid mediators release and generation remains to be identified. This could have a significant impact on the homeostatic process of inflammation (Chabannes et al., 2003).

It is clear that intestinal obstruction (Sagar et al., 1995) and ischemia (Meddah et al., 2001) cause mucosal injury with a subsequent increase of mucosal permeability and bacterial translocation. Therefore, the up regulation of antimicrobial proteins in cluster 5 like CAMP and Lcn2 observed after IR

could be related to protection against bacterial infection and modulation of oxidative stress (Chakraborty et al., 2012).

To validate such findings we performed a phagocytosis assay by incubating neutrophils from the three groups with *Saccharomyces cerevisiae* yeast cells. Phagocytic activity was significantly decreased in the IR group ( $p$ -value < 0.05) compared to control and LAP (Figure 4). Only about 23.90% of cells phagocytosed in IR group while control and LAP presented 50 and 40.7% phagocytosis rates, respectively. The mechanisms for the decreased neutrophils phagocytosis have not been explained yet, but might be related to the down regulation (cluster 4) of SHIP and the five proteins that belong to the Fc gamma R-mediated phagocytosis pathway (Detmers et al., 1987; Ravetch and Kinetic, 1991).

As a result of oxidative stress, most of the tissues undergo various anti-oxidative defense mechanisms. In our study, we found various anti-oxidant proteins in neutrophils that have been significantly regulated after IR. It includes glutathione reductase and glutathione S-transferase (GST). GSTs and its isoenzyme activities increase in response to oxidative stress due to lipid peroxidation resulting from superoxide production (Vasieva, 2011). Glutathione S transferase 1 (GSTP1), is a detoxification enzyme and regulator of cell signaling in response to growth factors, hypoxia, stress, and other stimuli in human hepatocellular carcinoma (HCC) (Kou et al., 2013) but its role in neutrophils is not clear. Down regulation of the response to reactive oxygen species was observed in cluster 3 after IR in neutrophil. Their major role in inflammatory and immune responses has long been thought to be phagocytosis and killing of bacteria via the generation of ROS and release of lytic enzymes stored in granules (Root and Cohen, 1981). Especially ROS, since they are potentially toxic essential molecules in the killing of bacteria. PMNs are thought to be exposed to ROS produced by themselves and by other inflammatory cells, and to suffer from resulting damage, such as DNA cleavage, protein modifications, and lipid peroxidation. The ROS-mediated damage to intracellular molecules is considered to be limited by cellular antioxidant enzymes, such as SODs (superoxide dismutase), Glutathione-s-transferase (Hurst et al., 1998) and GPx (glutathione peroxidase) (Arai et al., 1999). Down regulation of Glutathione-s-transferase and peroxidase could limit PMNs role in inflammatory and immune responses such as phagocytosis and killing of bacteria, as confirmed by our functional assays and previously mentioned in the literature (Hattori et al., 2005).

## CONCLUDING REMARKS

Conclusively, our proteomic approach revealed that intestinal ischemia/reperfusion causes the down regulation of important antioxidants together with the up regulation of enzymes involved in ROS production. This could result in reactive oxygen species accumulation observed by many researchers and confirmed herein. From enzyme classification, cluster

based KEGG pathways analysis and phagocytosis assays, we observed the changes in neutrophils motility, phagocytosis, directional migration, and cytoskeletal machinery activation after ischemia and reperfusion. Moreover, regulated pathways and enzymes suggest the influence of IR on the carbohydrate and lipid metabolism and a possible correlation to bacterial translocation, but such findings need further studies.

Collectively, our MS-based quantitative proteomic analysis illustrates the significance of comparative proteomic strategies applied to the neutrophils in different surgical groups, showing results supported by functional assays and consistent with the literature, and lay the basis for further profound studies in neutrophil biology.

## ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Ethics committee at the Faculty of Medicine, University of São Paulo. The protocol was approved

by the Ethics committee at the Faculty of Medicine, University of São Paulo, registered under protocol number 8186.

## AUTHOR CONTRIBUTIONS

WF and PR: Study idea and design, data analysis, manuscript writing. MT, SA: sample prep, proteomics analyses, manuscript writing. BF: surgical assays. IL, KB, and MC: functional assays, manuscript writing. SS, VS: data analysis, manuscript writing.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmolb.2018.00089/full#supplementary-material>

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