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Yerba mate (*Ilex paraguariensis)* inhibits lymphocyte activation *in vitro*

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Abstract text goes here. The abstract should be a single paragraph that summarises the content of the article

Yerba mate (YM) has been shown to have anti-inflammatory properties in several studies. However, this effect has been found mainly in obesity-related in inflammation. The aim of this work was to study the effect of YM in cultured peripheral blood mononuclear cells to see whether it has anti-inflammatory properties. We stimulated peripheral blood mononuclear cells in vitro with phytohemagglutinin in the presence of yerba mate and determined their activation measuring the expression of CD25 by flow cytometry. We observed that YM treatment produced a dose-dependent reduction in PBMC activation (CD25 positive cells) when they were stimulated with PHA. This effect was also observed in T cells (CD3 positive) subpopulation. Microarray analysis revealed the differential expression of 128 genes in YM-treated cells. According to a protein-protein interaction database, these genes were highly connected and they are involved in inflammatory response. In summary, it was demonstrated that YM produces a reduction in the amount of activated cells under the stimulation of PHA. Therefore, it might be used in diseases with an inflammatory component.

Introduction

Yerba mate (YM) or mate is a tea-like beverage made from dried leaves of *Ilex paraguariensis* and it is widely consumed in South America, mainly in Argentina, Brazil, Paraguay and Uruguay. In the last years, the interest in YM properties and its effect in the organism has grown considerably in the scientific community, as shown by the increasing number of publications in PubMed (1).

It has been reported that YM has various beneficial biological activities including antioxidant and anti-inflammatory properties (2–4), regulation of adipogenesis (5), weight reduction and anti-obesity properties (4,6,7) and it reduces the blood lipid levels and atherosclerosis risk factors(8,9). All these effects of YM in the organism have been conferred to its bioactive compounds, such as polyphenols (chlorogenic acid and tannins), xanthines (caffeine and theobromine), purine alkaloids, flavonoids, amino acids, vitamins, minerals and saponins (1,10).

Conversely, many epidemiological papers have reported a correlation between YM drinking and cancer incidence, e.g., esophagux, larynx, oral cavity or bladder cancer. However, this association is not clear due to confounding factors such as the temperature effect of YM drink, tobacco and alcohol consumption or malnutrition. Some systematic reviews performed in the last years seem to confirm that hot YM and high consumption are related to higher cancer risk(11,12).

Anti-inflammatory properties of YM have been studied in several pathological conditions in different tissues. For instance, in acute lung inflammation produced by cigarette smoke exposure in mice, YM extract was able to reduce leukocyte infiltrates in pulmonary tissue and macrophage and neutrophil counts in bronchoalveolar lavage fluid (13). YM also seems to be a natural treatment for psoriasis and it has been reported to be able to reduce pro-inflammatory mediators in plasma, liver and adipose tissue in animals who had underwent a high fat diet (4,7). However, few studies have assessed the effect of YM in peripheral blood leukocytes and evaluate its anti-inflammatory properties in blood. Some authors have evaluated the toxicity of YM in peripheral lymphocytes, at different concentrations obtaining unclear results (14,15).

To our knowledge, there is not any report assessing the effects that YM have on peripheral blood leucocytes apart from its toxicity. Hence, in this work we investigate the influence of YM in the activation of peripheral blood mononuclear cells *in vitro* and we show that it decreases their activation in a dose-dependent manner.

Materials and methods

PBMC isolation and culture

Peripheral blood (16ml) was collected from healthy subjects after receiving informed consent by venipuncture in sodium heparin

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tubes (Vacutainer, Becton Dickinson). PBMCs were isolated using the Ficoll-Hypaque density gradient method within 30 minutes of sampling. Cells were cultured at a density of 10^6 cells/ml in 96-well flat-bottom plates in RPMI 1640 medium supplemented with 10% foetal bovine serum, 10,000 U/ml penicillin and 10,000 µg /ml streptomycin. Activation of cells was induced stimulating them with phytohemagglutinin (PHA) (Gibco, Thermo Fisher) at 0.1% and 0.5%. Cells were incubated for 72h at 37ºC and 5% CO_2.

Yerba mate preparation

The roasted YM extract was prepared fresh each day by dissolving lyophilised instant mate tea (Leao Jr, Curitiba-PR, Brazil) in distilled water, using a homogeniser. Then, it was filtered using a 0.22 µm filter before adding to PBMC culture. The extract contained quinic acid (25.1 ± 0.1 ug/ml), caffeoyl glucose (34.4 ± 2.5 µg/ml), caffeyolquinic acid isomers (crypto-chlorogenic acid, neo-chlorogenic acid, 3,4-dicaffeoylquinic acid and 3,5-dicaffeoylquinic acid) (216 ± 3.5 µg/ml), caffeic acid (5.1 ± 0.3 µg/ml), feruloyquinic acid (21.7 ± 0.2 µg/ml), caffeoylshikimic acid isomers (36.0 ± 0.5 µg/ml), rutin (145 ± 4.3 µg/ml), dicaffeoylquinic acid isomers (878 ± 96.7 µg/ml) and dicaffeoylshikimic acid isomers (4.6 ± 0.3 µg/ml). The analysis of the yerba mate extract was performed by liquid chromatography as described previously (6).

First, different concentrations of YM were used to test its toxicity, ranging from 0 to 1500 µg/ml. Afterwards, to test its effect in PBMC activation, 25 µg/ml and 100 µg/ml of YM were used. This experiment was run twice and two technical replicates were included in all the experiments.

Flow cytometry

After 72 h of incubation, cultured PBMCs were harvested, washed with PBS and incubated with antibodies for 20 minutes at room temperature. Afterwards, cells were washed with PBS and analysed in a Guava EasyCyte 8HT flow cytometer (Millipore) using the InCyte software v2.2.2. Cell viability was assessed with 7-aminoactinomycin D (7-AAD) (Molecular Probes), FITC-conjugated anti-human CD3 was used to detect T lymphocytes and the activation was determined measuring the expression of CD25 in cell membrane (PE-conjugated anti-human CD25, BD Pharmingen™). T cells and activated cells were measured gated on live lymphocytes (7AAD- cells).

RNA isolation and microarray hybridization

RNA was isolated from PBMCs activated with 0.5% PHA, from both with and without 100 µg/ml YM treatment, using the miRNeasy Mini Kit (Qiagen) following the manufacturer's instructions. RNA concentration was measured using a NanoDrop ND-1000 spectrophotometer. 130 ng of total RNA was used as starting material. RNA samples were hybridized to the GeneChip Human Transcriptome Array 2.0 (Affymetrix) following the manufacturer's protocol in order to analyse gene expression. Each condition was run in duplicates in the Genomic Platform of Biodonostia Institute (San Sebastian, Spain). The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE84407).

Data analysis

Flow cytometry data were analysed using Excel and RStudio v0.99.486 running R version 3.2.2. Microarray data were normalized using SST-RMA algorithm in Affymetrix Expression Console v.1.4.1. Three filtering steps were applied to select the most informative genes with smallest variation. First, RefSeq genes were selected in order to have information about well annotated genes. Second, a detection filter was applied as follows: the probes having an intensity signal above that of the background probes were considered as detected. Therefore, all the probes that were not detected in the four arrays were removed. Furthermore, probes having a discordant detection call between the two replicates (i.e. detected in one replicate and not-detected in the other) were also discarded. Finally, those probes having a variation coefficient higher than 10% between the two technical replicates were removed.

Then, the expression fold-change was calculated for each probe between 100 µg/ml YM-treated samples and non-treated samples and probes having a fold-change equal to or higher than 2 (both directions) were considered as differentially expressed genes.

With the resulting differentially expressed genes, we searched for protein-protein interactions using STRING database (16). Afterwards, KEEG pathway enrichment analysis was performed with the differentially expressed gene list, taking as the background the human genome.

Results

Cell survival

First, we wanted to assess the effect of YM in cell survival *in vitro*. To do that, we added a range of YM concentration to the culture and measure the cell viability after three days in culture. We observed that when high YM concentrations were used, (>200 µg/ml) only 15% or less of the cells were alive after three days in culture. The addition of 100 µg/ml YM resulted in higher percentage of live cells, ranging from 40 to 70%, while below this concentration nearly 90% of the PBMCs were alive (Figure 1).

Effect of YM in PBMC activation

In order to determine whether the presence of YM in culture could have any effect in PBMC activation, cells were incubated with 25 and 100 µg/ml of YM and 0.1% and 0.5% PHA to induce their activation. As shown in figure 2A, the percentage of live cells in this experiment is around 85-90% for cells with no YM treatment or 25 µg/ml of YM. Conversely when 100 µg/ml of YM was added, this value dropped to nearly 60%. It can also be observed that the stimulation with PHA at 0.5% slightly reduced the percentage of live cells. We also measured the T cell population present in our culture after three days and we saw that neither PHA nor YM significantly changed the percentage of T cell population in the culture (Figure 2B). Finally, we assessed the activation of the whole PBMC population and T cell subset specifically under the stimulation with PHA and noticed an YM dose-dependent reduction of activated cells in both studied populations, both with 0.1 and 0.5% of PHA. Nevertheless, when a high percentage of cells were activated, as seen with 0.5% of PHA, 100 µg/ml of YM was needed to get a significant reduction in activated cell population (Figure 2 C-D).

Microarray results

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In order to identify the genes that were involved in the inhibitory effect of YM we analysed the gene expression pattern of PBMC treated with 100 µg/ml YM and compared to the gene expression pattern obtained from non-treated PBMC, both under the stimulation with 0.5% PHA. After applying all the filtering steps (selection of RefSeq genes, detection call and removing the probes having a variation coefficient ≥ 10) 14883 probes remained for further analysis. We identified 128 differentially expressed genes (101 overexpressed and 27 underexpressed) between samples treated with 100 µg/ml YM and non-treated ones (Supplementary table 1). Among altered genes, there were 5 miRNA genes and 7 genes coding small nucleolar RNAs.

Protein-protein interactions of the remaining 116 protein-coding genes revealed that there is an enrichment in the interactions among both upregulated (125 observed/29 expected interactions; p=0) and downregulated genes (5 observed/1 expected interactions; p=9.37 x 10^{-4}) (Figure 3). On the other hand, applying a KEGG pathway enrichment analysis of overexpressed genes, we identified several immune-related pathways being the most enriched *cytokine-cytokine receptor interaction* and *chemokine signalling pathway*. Furthermore, the *antigen processing and presentation* pathway is enriched among underexpressed genes (Table 1).

Discussion

YM has demonstrated several beneficial effects among which anti-inflammatory properties have been discovered, mainly in obesity-related inflammation. With this study, we wanted to test whether YM was able to produce an anti-inflammatory effect in peripheral blood leukocytes, thus being also a candidate to be studied in inflammatory or autoimmune diseases.

First, we studied the *in vitro* toxicity of YM for PBMC and found that beyond 100 µg/ml cell death was considerably increased. Below this concentration, cell death was not affected and the addition of YM at 100 µg/ml produced variable results, suggesting that this is the threshold at which YM becomes somehow toxic. Previous reports studying cytotoxicity in human lymphocytes concluded that YM at 10 µg/ml and beyond (range 10-1000 µg/ml) produced higher rate of cell death (15). However, the YM used in this study was prepared as an infusion, brewed up for 10 minutes after boiling, in contrast to the mate powder solution that we used. Therefore, the higher toxicity shown by Wnuk *et al* could be attributed to the mate preparation mode, especially due to the high temperatures of the water used to prepare the infusion. In fact, the consumption of high temperature beverage drinking, including mate tea, has been associated to increased risk for esophageal cancer highlighting that the effect of the temperature is quite remarkable (17,18). Moreover, differences in the phenolic composition and anti-oxidant capacity have been reported among green and roasted yerba mate infusion (19), suggesting that the properties of different types of YM could be slightly different, and thus, also its toxicity for lymphocytes.

Furthermore, we have shown that YM has a dose-dependent inhibitory effect in cultured PBMCs, measured by the expression of CD25 (IL2RA (the interleukin 2 (IL2) receptor alpha chain) in the cell surface. . CD25 is one of the receptor proteins that shows upregulation in proliferating lymphocytes and it is used to evaluate their activation (20).   To our knowledge, this is the first study analysing the effect that YM has in PBMC upon mitogen-induced activation. Nonetheless, other herbs, beverages or foods such as green tea, red wine or cocoa have shown immunomodulatory effects as well (21–23). Several authors have investigated the effect of epigallocatechin-3-gallate (EGCG) in immune cells. EGCG accounts for 50 to 80% of total catechins present in green tea and any effect found in this herb is attributed to this compound (24,25). As far as its effect is concerned, Wu et al demonstrated that EGCG reduced the expression of CD25 in stimulated PBMC but found an accumulation of IL-2, pointing to EGCG as a blocking agent for the interaction between IL-2 and its receptor, thus inhibiting its downstream signalling. Therefore, the authors suggest that EGCG supplementation could be beneficial for those people having an abnormally excessive T cell function such as autoimmune and inflammatory disorders (25). Furthermore, similarly to what we observed with YM, they found a toxic effect of EGCG at high concentration, highlighting the importance of the dose of such molecules to obtain a beneficial effect. Moreover, several authors have concluded that EGCG reduces the proliferation capacity of both murine and human-derived lymphocytes (24–26). Anderson et al reported that walnut-derived polyphenols inhibit lymphocyte proliferation when they were stimulated with PHA (27). Likewise, YM could have a similar inhibitory effect in proliferation capacity of lymphocytes, given that it is rich in polyphenols, and thus, it might be interesting to check this in detail in further studies. Microarray analysis revealed an overexpression of genes involved in chemokine signalling pathways and cytokine-cytokine receptor interaction in the YM treated PBMCs. Moreover, there are additional enriched pathways among overexpressed genes related to the development of an immune response, such as NOD-like receptor signalling pathway, TNF and NFκB signalling pathway, processes related to leukocyte migration and natural killer cell mediated cytotoxicity, processes that are necessary for the inflammatory response to occur. The upregulation of genes involved in the inflammatory response could be a compensatory effect of the inhibition of CD25 surface marker, trying to maintain the response to the PHA stimulus. Likewise, it could be an additional effect of YM. In fact, it has previously been shown that YM can have opposite effects in different cytokines (28).   On the other hand, among downregulated genes, there are three genes involved in antigen processing and presentation, suggesting that YM treatment impairs this process by inhibiting the expression of those genes. It is worth to highlight that IL23R is decreased after YM treatment. This receptor plays a role in the development of TH17 cells, which are involved in autoimmunity (29–31). Thus, YM treatment could be beneficial for autoimmune processes by reducing the expression of IL23R. Therefore, functional studies will be needed in order to establish the exact effect of YM in human lymphocytes.

In the present study we have described that YM has an inhibitory effect in the activation of lymphocytes *in vitro*. Nonetheless, it would be very interesting to see whether this reduction in CD25 expression is also observed *in vivo*. To our knowledge, such a study has not been published yet. In order to achieve a biological effect in the organism, YM has to be absorbed and its different compounds have to be transported to organs or bloodstream. With this objective, de Oliveira et al conducted a study to analyse the biodistribution and metabolization of yerba mate in rats, concluding that absortion as well as metabolization begins in stomach. According to their data, the most abundant compound found in plasma was caffeic acid and this was present early (0.5, 1 and 2h) after ingestion of YM (32).Moreover, other compounds metabolized

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by enzymes and intestinal microbiota appear 4h after ingestion. As far as we know, there is not any study in humans analysing mate compounds or metabolites in plasma. Therefore, assuming a similar biodistribution as described by de Oliveira et al., we could hypothesize that circulating lymphocytes from mate consumers would be exposed mainly to caffeic acid. It would be of great interest to see whether circulating lymphocytes of people with an inflammatory disease exhibit a decrease in CD25 expression upon YM consumption. In addition, it has been described that the hydrolysis of the extract of Ilex paraguariensis improves it bioavailability and its anti-oxidant capacity in rats (33). Therefore, in case YM is prescribed as a treatment or supplement for patients with inflammatory diseases, the way it is prepared or consumed is something that should be kept in mind, in order to improved its absortion and as a consequence, its effect in the organism.

Furthermore, knowing that caffeic acid is the main molecule found in plasma after YM consumption, it would be worth analysing whether it is able to produce the same inhibitory effect as YM extract. In fact, some studies have analysed the effect of the whole YM extract as well as its bioactive compounds alone, concluding that bioactive compounds are able to produce the same effect as the whole extract, even if higher doses are needed. This observation suggest that a synergistic effect among different phenolic compounds occurs (5,34–36). Therefore, it would be interesting to assess the effect of different phytochemical of YM extract in lymphocytes, given that they have shown different inhibitory capacity of inflammation in macrophages (36).

Conclusions

In summary, we have reported that high concentrations of YM added to PBMCs *in vitro* are toxic, while lower concentrations of YM do not produce cell death. Moreover, we have shown a YM dose dependent decrease in CD25 expression in cell surface of both the whole PBMC population and T cell subset upon PHA stimulation. Our results lay the ground for further studies aiming at exploringthe exact mechanism by which YM produces its effect and they suggest that YM might be useful in diseases with an inflammatory component.

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Figures and Tables

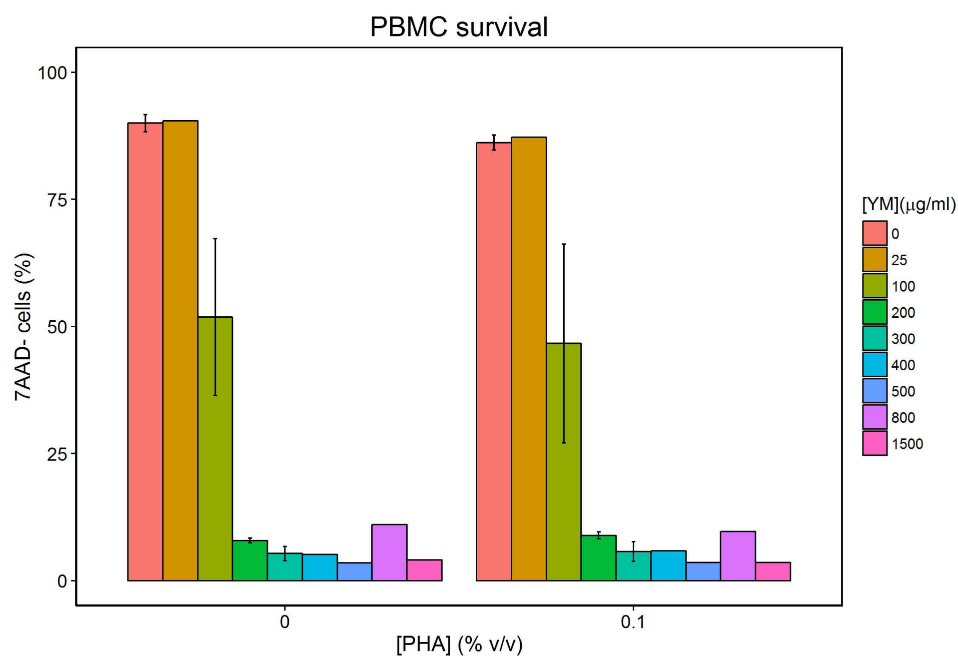

Figure 1. Cell survival after three days of incubation measured by 7AAD under different YM concentrations with and without PHA. Bars indicate the mean of 7AAD negative population. For [YM]= 0 and 100 µg/ml, n=3; For [YM]= 200 and 300 µg/ml, n=2. The rest of the condition n=1 (bars show the mean of duplicates). Error bars show the standard error.

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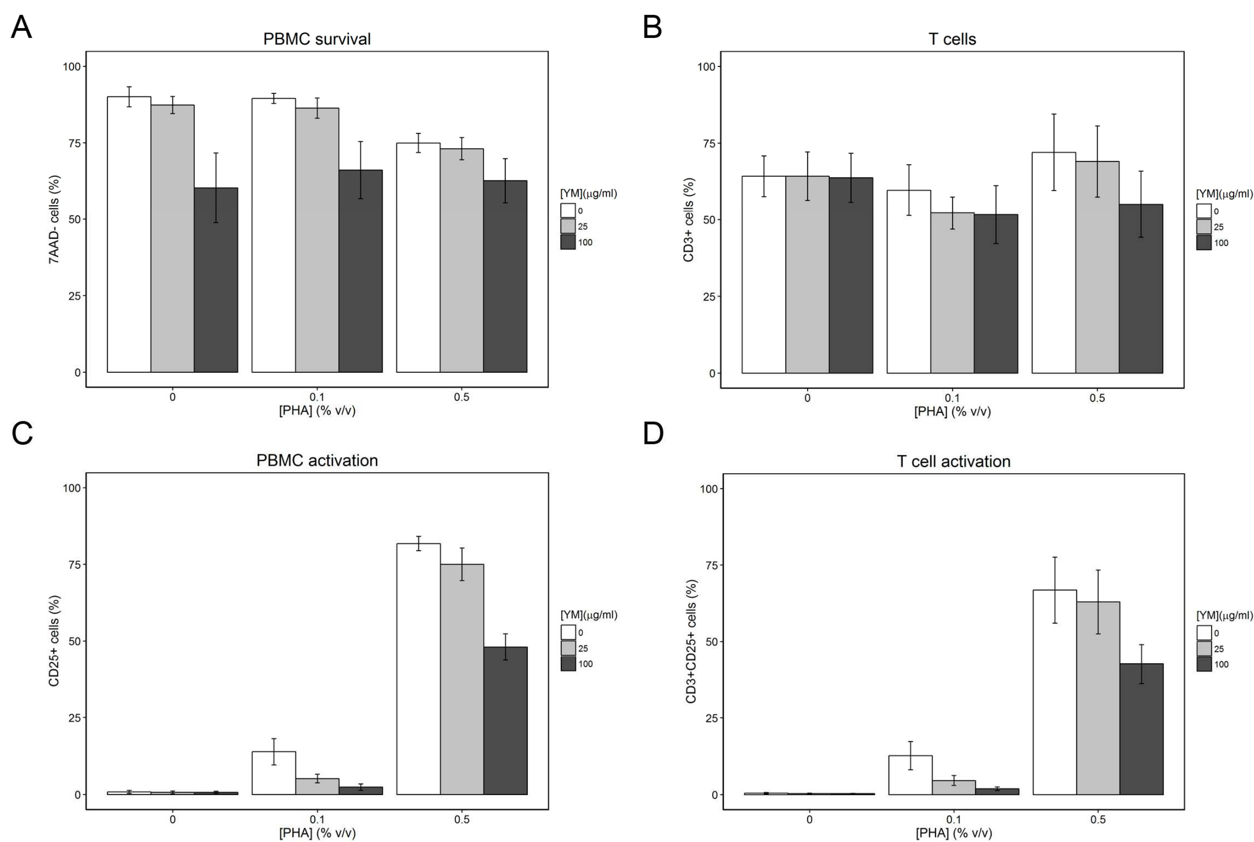

Figure 2. Results from the activation experiment after three days in culture. A: PBMC survival treated with YM under PHA stimulation. B: T cell population in culture in each condition measured by the presence of the CD3 marker in cell surface. C: The activation of the whole PBMC population measured by the expression of CD25 antigen in cell surface. D: Activation of T cells present in the culture measure by the expression of CD25 antigen among CD3 positive cells. Bars show the mean value of each marker and the error bars indicate the standard error. This experiment was performed twice (n=2).

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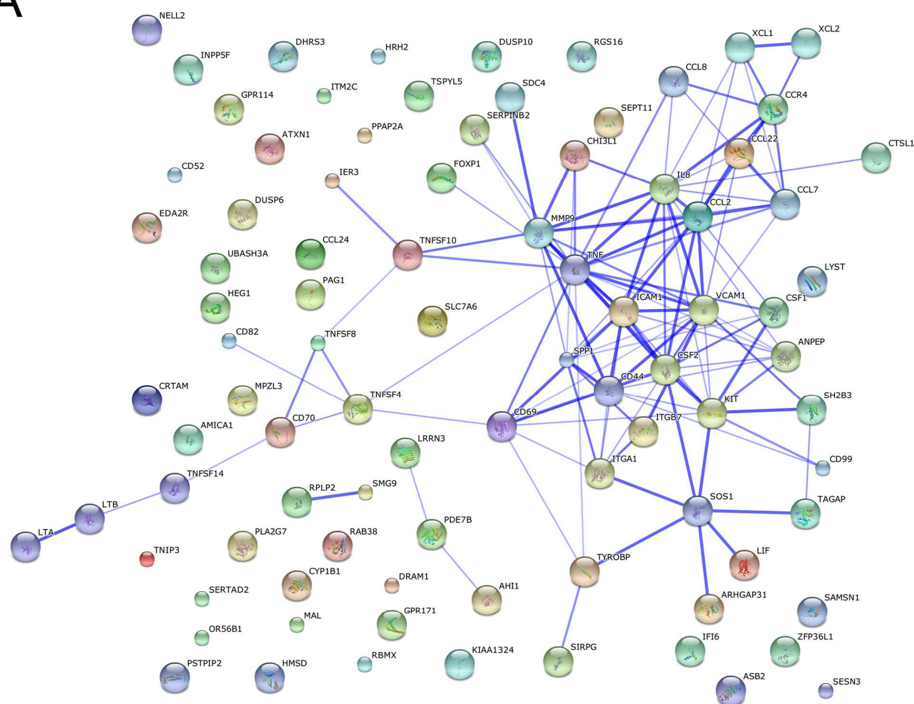

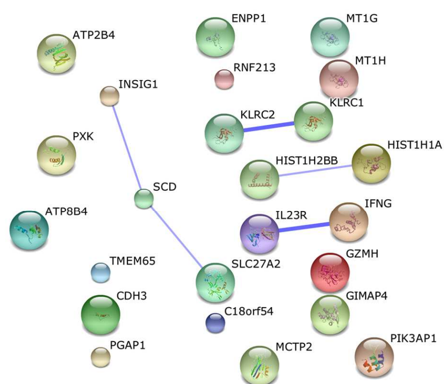

Figure 3. Protein-protein interaction network. A: overexpressed genes. B: underexpressed genes.

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Table 1. KEGG pathway enrichment results of differentially expressed genes between YM-treated vs non-treated samples.

Pathway	Number of genes	p-value	FDR	Genes in pathway
		Overexpressed genes		
Cytokine-cytokine receptor interaction	21	5.30E-21	1.52E-18	TNFSF10,LTA,CCL22,KIT,EDA2R,CCL8,CSF1,TNFSF4,IL8,CCL24,CCR4,TNF,TNFSF8,XCL2,XCL1,CCL7,CSF2,CCL2,TNFSF14,LTB,CD70
Rheumatoid arthritis	8	3.73E-09	5.35E-07	LTB,TNF,IL8,CSF2,ICAM1,CTSL1,CSF1,CCL2
Chemokine signaling pathway	10	6.05E-09	5.79E-07	CCL24,SOS1,CCL22,CCR4,XCL2,XCL1,CCL7,CCL8,CCL2,IL8
TNF signaling pathway	8	2.17E-08	1.56E-06	LTA,TNF,CSF2,ICAM1,CSF1,CCL2,VCAM1,MMP9
Hematopoietic cell lineage	7	9.40E-08	5.40E-06	TNF,CD44,KIT,ANPEP,CSF2,CSF1,ITGA1
NF-kappa B signaling pathway	7	1.20E-07	5.72E-06	LTA,TNFSF14,LTB,TNF,IL8,ICAM1,VCAM1
Malaria	5	1.75E-06	7.18E-05	IL8,ICAM1,CCL2,TNF,VCAM1
Natural killer cell mediated cytotoxicity	6	1.72E-05	6.18E-04	TNFSF10,SOS1,TYROBP,TNF,CSF2,ICAM1
ECM-receptor interaction	5	3.66E-05	1.17E-03	SDC4,SPP1,ITGB7,CD44,ITGA1
Cell adhesion molecules (CAMs)	5	3.37E-04	9.67E-03	CD99,ICAM1,VCAM1,SDC4,ITGB7
Proteoglycans in cancer	6	4.04E-04	9.77E-03	SDC4,MMP9,CTSL1,SOS1,TNF,CD44
African trypanosomiasis	3	4.08E-04	9.77E-03	ICAM1,TNF,VCAM1
Transcriptional misregulation in cancer	5	7.91E-04	1.71E-02	DUSP6,CSF2,ITGB7,IL8,MMP9
Influenza A	5	8.35E-04	1.71E-02	TNFSF10,TNF,ICAM1,CCL2,IL8

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Amoebiasis	4	1.15E-03	2.20E-02	TNF,CSF2,SERPINB2,IL8
Leukocyte transendothelial migration	4	1.57E-03	2.81E-02	CD99,ICAM1,VCAM1,MMP9
NOD-like receptor signaling pathway	3	1.93E-03	3.26E-02	TNF,IL8,CCL2
Fc epsilon RI signaling pathway	3	3.08E-03	4.92E-02	SOS1,TNF,CSF2
		Underexpressed genes		
Antigen processing and presentation	3	6.18E-05	1.78E-02	KLRC2,KLRC1,IFNG

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