Electronic Supplementary Information

Graphene oxide activates B cells with upregulation of granzyme B expression: evidence at the single-cell level for its immune-modulatory properties and anticancer activity

Marco Orecchioni,[1]# Laura Fusco,[2,3,4] # Raghvendra Mall,[5]# Valentina Bordoni,[1] Claudia Fuoco,[6] Darawan Rinchai,[2] Shi Guo,[7] Raquel Sainz,[7,§] Martina Zoccheddu,[1] Cansu Gurcan,[8] Acelya Yilmazer,[8] Barbara Zavan,[9] Cécilia Ménard-Moyon,[7] Alberto Bianco,[7] Wouter Hendrickx,[2,10]* Davide Bedognetti,[2,10,11]* and Lucia Gemma Delogu[1, 3]*

[1]*Department of Chemistry and Pharmacy University of Sassari, Sassari, Italy;*
[2] *Department of Immunology, Cancer Program, Sidra Medicine, Education City, Doha, Qatar;*
[3]*Department of Biomedical Sciences, University of Padua, Padua, Italy;*
[4]*Department of Chemical and Pharmaceutical Sciences, University of Trieste, Trieste, Italy;*
[5]*Qatar Computing Research Institute (QCRI) Hamad Bin Khalifa University (HBKU), Doha, Qatar.*
[6]*Department of Biology, University of Rome Tor Vergata, Rome, Italy;*
[7]*CNRS, Immunology, Immunopathology and Therapeutic Chemistry, UPR3572, University of Strasbourg, ISIS, 67000 Strasbourg, France;*
[8]*Department of Biomedical Engineering, Faculty of Engineering, Ankara University, Ankara, Turkey;*
[9]*Department of Medical Sciences, University of Ferrara, Ferrara, Italy;*
[10]*College of Health and Life Sciences, Hamad Bin Khalifa University, Doha, Qatar*
[11]*Dipartimento di Medicina Interna e Specialità Mediche, Università degli Studi di Genova, Genova, Italy*

*Corresponding authors: luciagemma.delogu@unipd.it; dbedognetti@sidra.org; whendrickx@sidra.org

# These authors contributed equally to this work
§ Current address: Instituto de Catálisis y Petroleoquímica (CSIC) c/ Marie Curie 2, 28049 Madrid, Spain

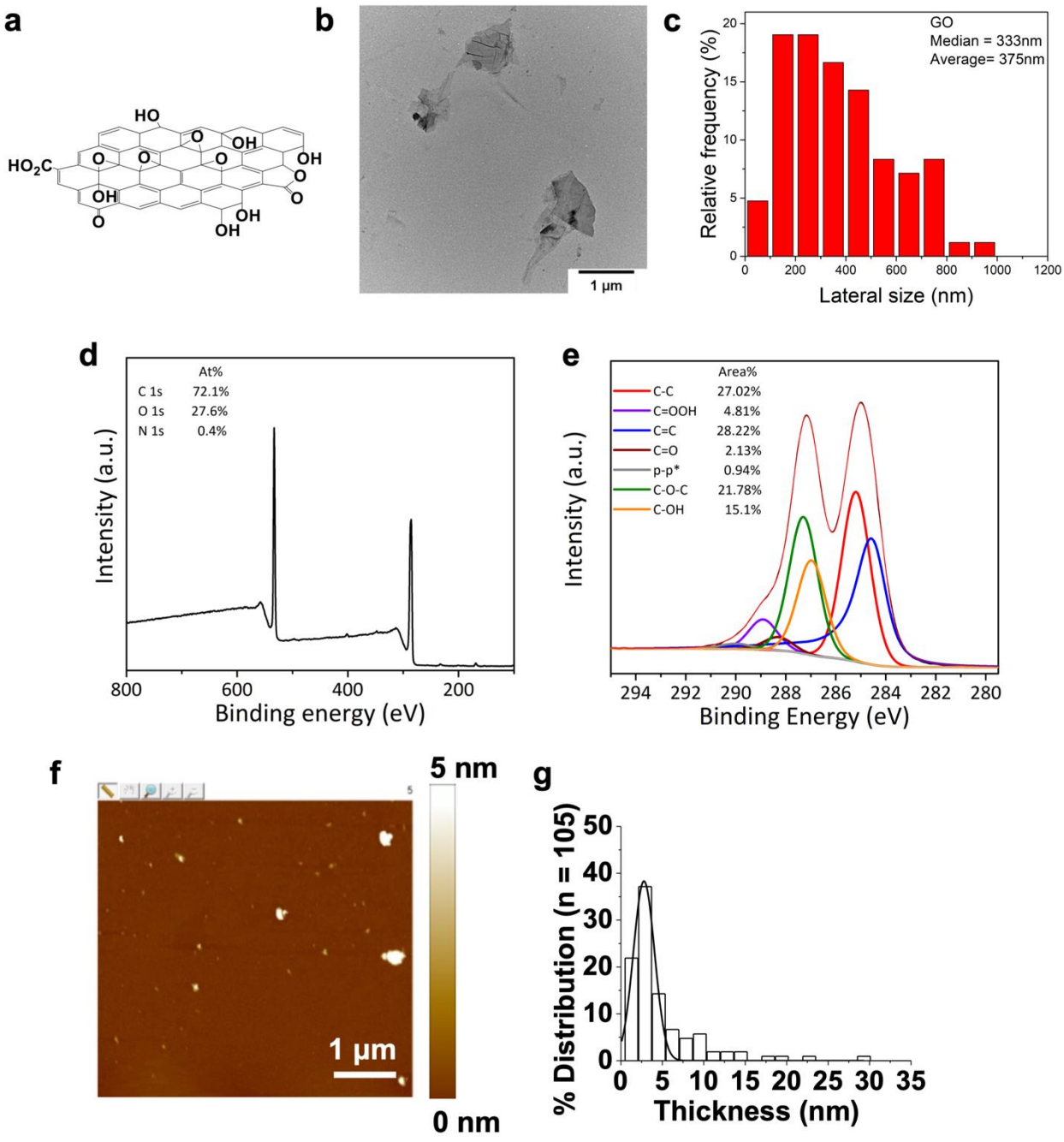

Figure S1. Characterization of GO. Molecular structure (**a**), TEM image (**b**), size distribution (**c**), XPS survey (**d**), C1s spectrum (**e**), AFM image (**f**) and thickness distribution (0.4 nm – 29.4 nm) (**g**).

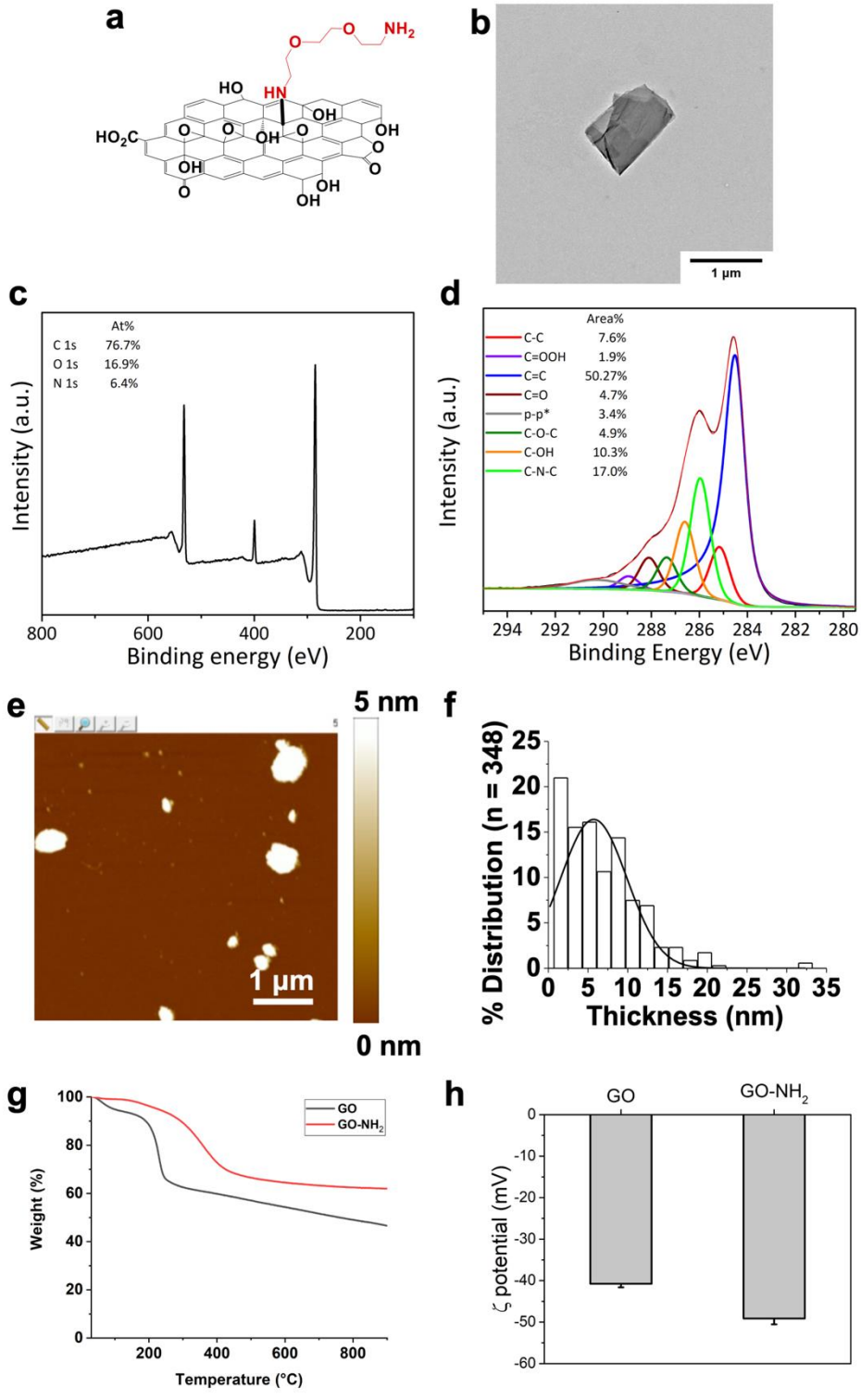

Figure S2. Characterization of GONH2. Molecular structure (**a**), TEM image (**b**), XPS survey (**c**), C1s spectrum (**d**), AFM image (**e**) and thickness distribution (0.4 nm – 32.4 nm) (**f**). TGA (**g**) and zeta potential in water (**h**). The values of zeta potential are indicative of a good colloidal stability. Although the value of zeta potential should increase after the reaction with TEG, the positive charge associated to the amines is likely neutralized by the new hydroxyl groups generated by the epoxide ring opening reaction. The lower value might be also due to the different dispersibility of the two materials.

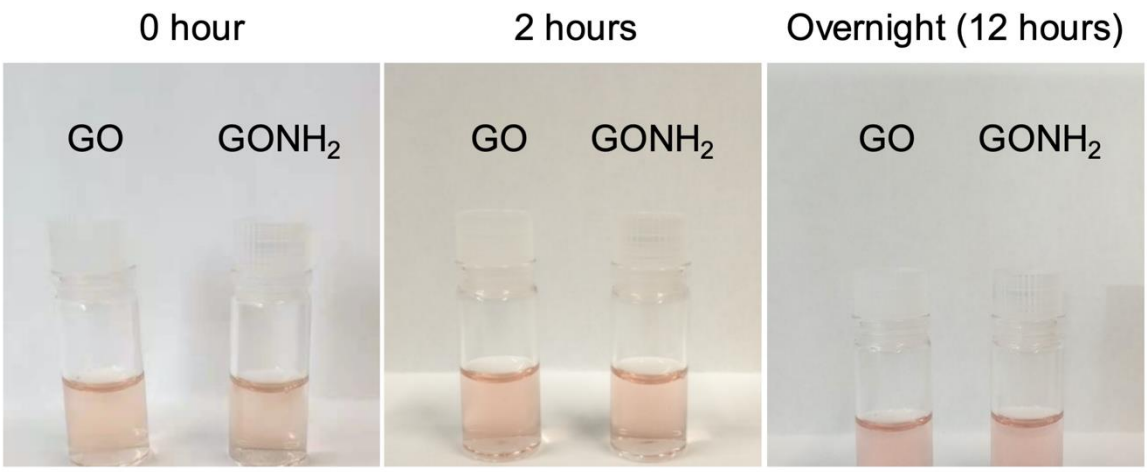

Figure S3. Stability of GO and $GONH_2$ in cell culture media. Optical image of GO and $GONH_2$ (50 µg/mL) in cell culture media at time zero and after incubation of 2 h and overnight.

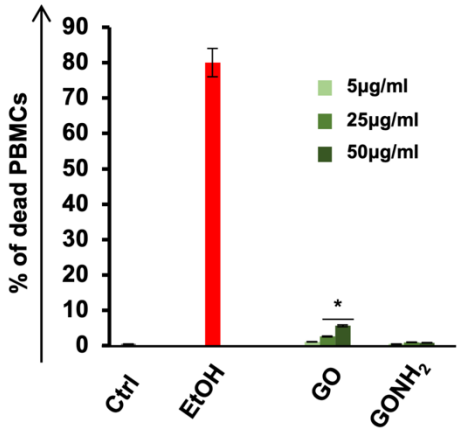

Figure S4. Cell viability assays on human peripheral blood mononuclear cells (PBMCs) treated with GO or $GONH_2$. Purified PBMCs were incubated with GO or $GONH_2$ for 24 h or left untreated (Ctrl). Ethanol was used a positive control. Percentage of late apoptotic and necrotic cells was assessed by staining with an amine-reactive dye (7AAD) after incubation with 5, 25, and 50 µg/mL). All experiments were performed in triplicate and shown as means ±SD. *p<0.05; by one-way ANOVA with Bonferroni post tests and Tukey's multiple comparisons test.

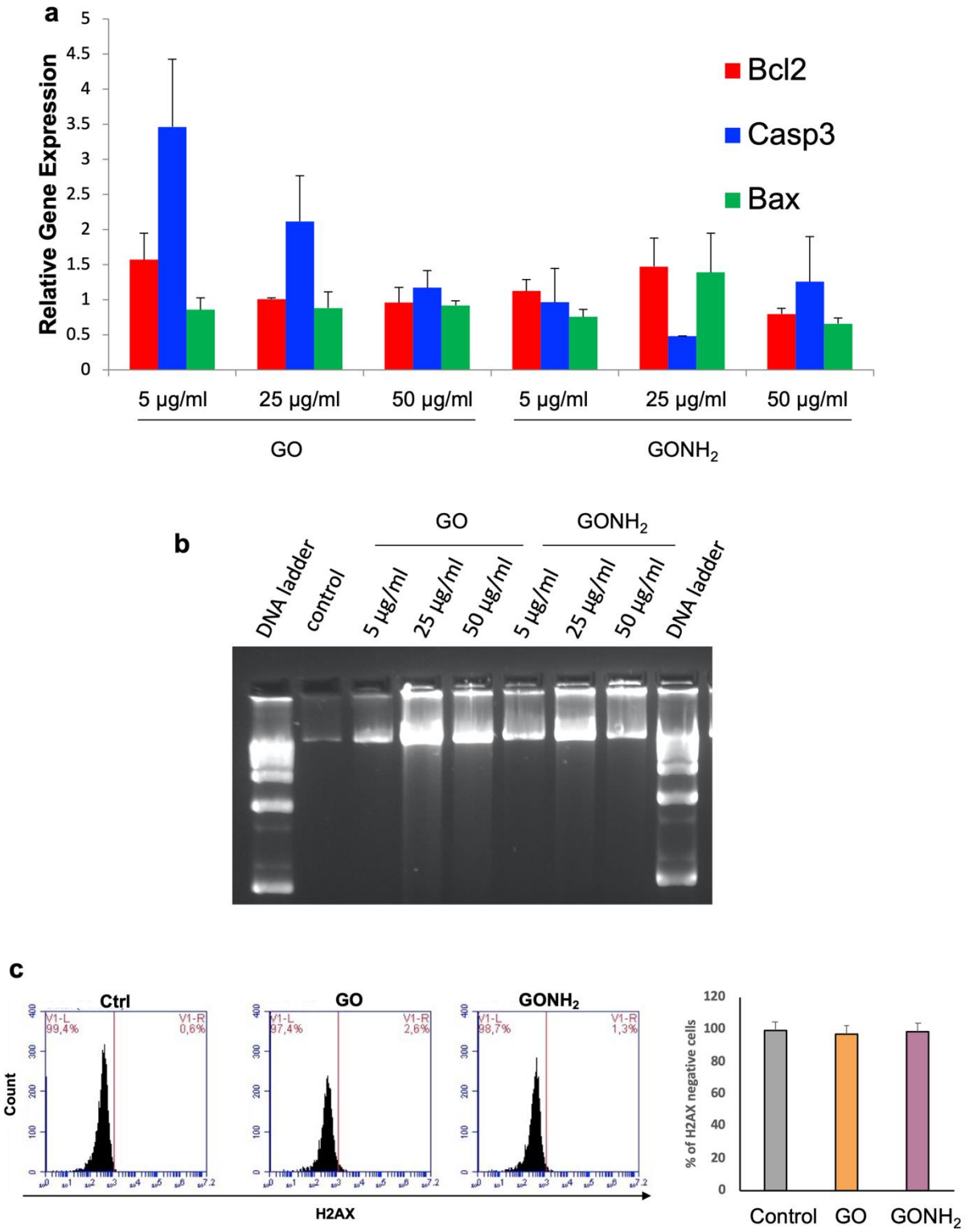

Figure S5. Genomic toxicity evaluation. PBMCs were incubated with 5, 25 or 50 µg/mL of GO or $GONH_2$ for 24 h, or left untreated (Ctrl), and the impact on genotoxicity was evaluated on human PBMCs (**a**). Real time qPCR analysis of Bcl2, *Caspase1*, and *Bax* expression normalized to *Gapdh* expression and untreated control applying the delta delta Ct method. (**b**) Cell pellets were used in DNA fragmentation assay. Samples were run on agarose gel electrophoresis. (**c**) Treated cells were stained with H2AX antibody to determine the levels of H2AX activation in response to DNA damage. Stained cells (10.000 counts) were run on flow cytometer (BD Accuri). Data are presented as mean ± ST.D. of three independent experiments. ****p<0.001*

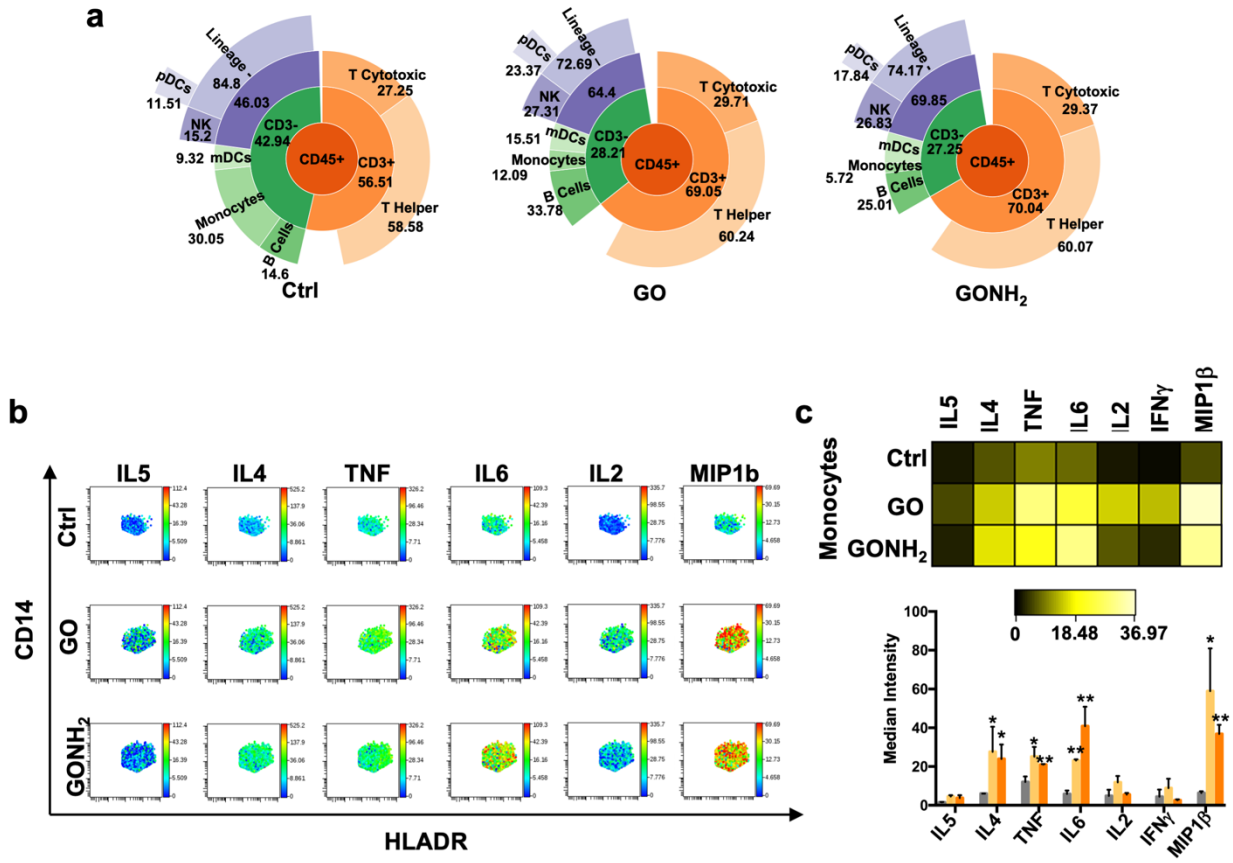

Figure S6. Classical monocytes response to GO or $GONH_2$ incubation. (**a**) Representative immune cell composition analysis with sunburst after treatment with GO or $GONH_2$. **(b)** Single-cell characterization of gated CD14+ classical monocytes after GO or $GONH_2$-treatment (viSNE analysis). Plots show the use of viSNE to obtain a comprehensive single-cell view of 6 cytokines expression. (**c)** Representative heat map and bar graph of median expression intensity of IL5, IL4, IL6, IL2, IFNg, TNF, and MIP1b on classical monocytes. All experiments were performed in triplicate and shown as means ±SD. Statistical differences: *p<0.05; **p<0.01; ***p<0.001 (One-way ANOVA with Bonferroni's post tests and Tukey's multiple comparisons test).

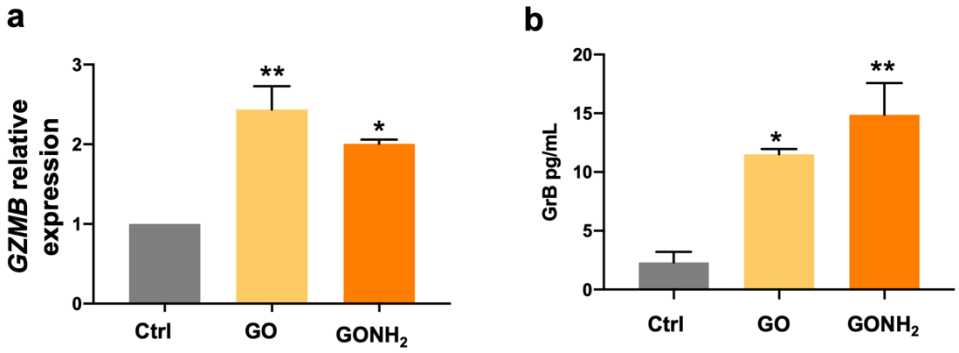

Figure S7. GO and $GONH_2$-induced GrB expression and secretion in B cell activation assays. **a)** *GZMB* expression after treatment with GO or $GONH_2$ at fixed concentration (50 µg/mL) or left untreated (Ctrl) determined by RT qPCR normalized to *GAPDH* applying the $2^{-\Delta\Delta CT}$ method. **b**) GrB proteihn secretion after treatment with GO or $GONH_2$ at fixed concentration (50 µg/mL) or left untreated (Ctrl) determined by cytokine bead array (CBA).
All experiments were performed in triplicate and shown as means ±SD. Statistical differences: **p*<0.05; ***p*<0.01 (One-way ANOVA with Bonferroni's post tests and Tukey's multiple comparisons test).

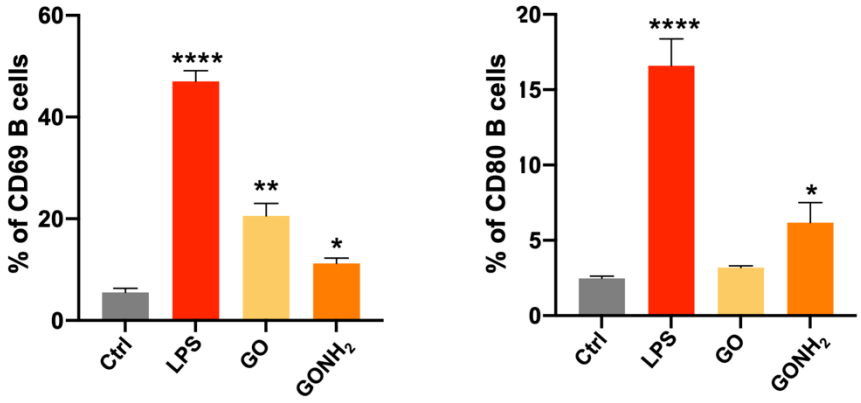

Figure S8. LPS strongly induces CD69 and CD80 secretion in B cell. CD69 and CD80 analyzed as described for figure 2 after treatment with GO or $GONH_2$ at fixed concentration (50 µg/mL) or LPS (2µg/mL) or left untreated (Ctrl). All experiments were performed in triplicate and shown as means ±SD. Statistical differences: **p*<0.05; ***p*<0.01; *p*<0.0001**** (One-way ANOVA with Bonferroni's post tests and Tukey's multiple comparisons test).

Figure S9. Enriched pathways in GO using RMDB. Genes used for this analysis are differentially expressed genes between GO and controls (p value < 0.005 and FDR < 0.1). Pathways are clustered according to similarity.

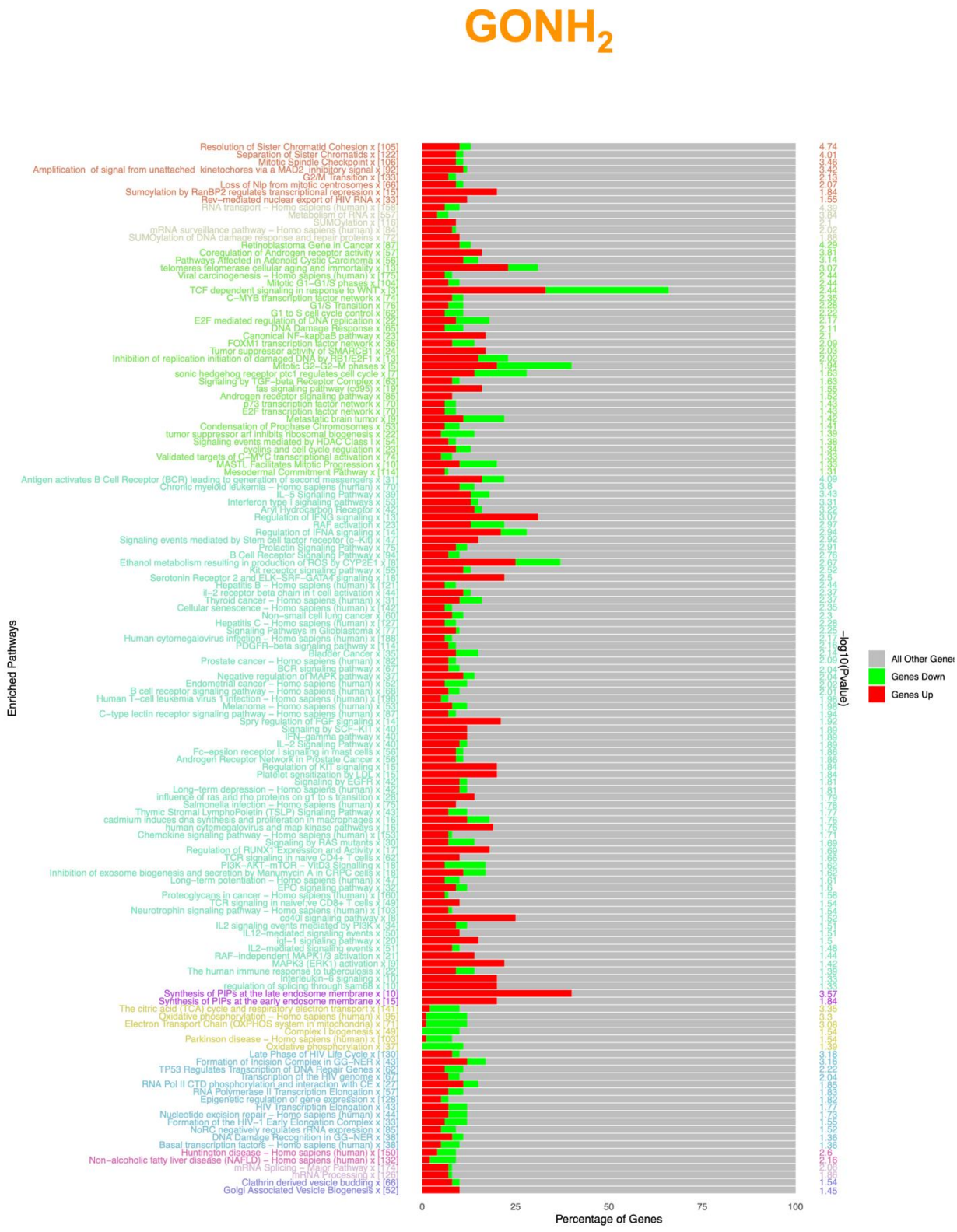

Figure S10. Enriched pathways in $GONH_2$ using RMDB. Genes used for this analysis are differentially expressed genes between $GONH_2$ and controls (p value < 0.005 and FDR < 0.1). Pathways are clustered according to similarity.

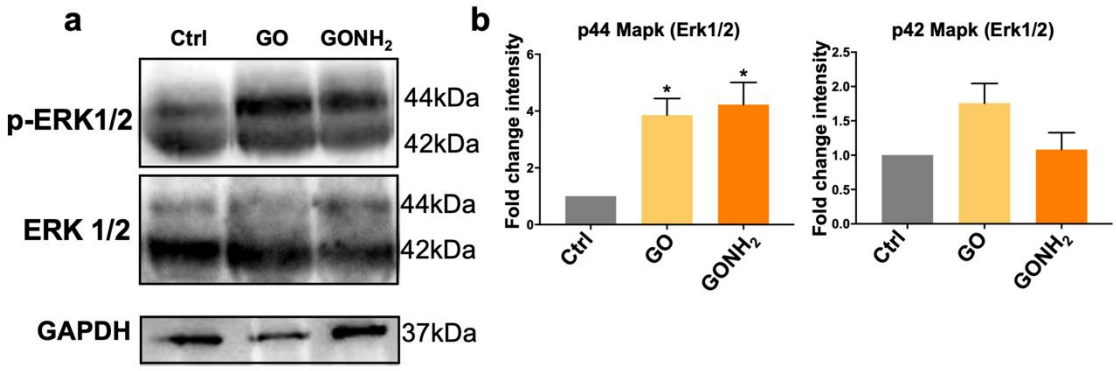

Figure S11. GO and $GONH_2$-induced Erk1/2 phosphorylation in B cells. Purified B cells from PBMCs were incubated with GO or $GONH_2$ at fixed concentration (50 μg/mL) for 24 h or left untreated. (**a**) Erk1/2 phosphorylation was assessed by western blot. p-ERK1/2 signal intensity was normalized based on GAPDH and total ERK1/2. (**b)** Histograms showing p44 and p42 band signal intensity fold change vs Ctrl. The results are presented as the mean ± SEM from three independent quantifications. Statistical differences: **p*<0.05 (One-way ANOVA and Bonferroni's post test and Tukey's multiple comparisons test).