

1                   **Helminth-derived metabolites induce tolerogenic functional, metabolic, and**  
2                   **transcriptional signatures in dendritic cells that attenuate experimental colitis**  
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18

19      **Summary Sentence:** Metabolites produced by *Heligmosomoides polygyrus* induce metabolic and  
20      transcriptional changes in DCs consistent with tolDCs, and adoptive transfer of these DCs  
21      attenuated DSS-induced intestinal inflammation.  
22

23 **ABSTRACT**

24 Inflammatory bowel diseases (IBD) are chronic inflammatory diseases in which abdominal pain,  
25 bloody diarrhea, weight loss, and fatigue collectively result in diminished quality of patient life.  
26 The disappearance of intestinal helminth infections in Western societies is associated with an  
27 increased prevalence of IBD and other immune-mediated inflammatory diseases. Evidence  
28 indicates that helminths induce tolerogenic dendritic cells (tolDCs), which promote intestinal  
29 tolerance and attenuate intestinal inflammation characteristic of IBD, but the exact mechanism is  
30 unclear. Helminth-derived excretory-secretory (HES) products including macromolecules,  
31 proteins, and polysaccharides have been shown to modulate the antigen presenting function of DCs  
32 with down-stream effects on effector CD4<sup>+</sup> T cells. Previous studies indicate that DCs in helminth-  
33 infected animals induce tolerance to unrelated antigens and DCs exposed to HES display  
34 phenotypic and functional features of tolDCs. Here, we identify that nonpolar metabolites (HnpM)  
35 produced by a helminth, the murine gastrointestinal nematode *Heligmosomoides polygyrus bakeri*  
36 (Hpb), induce tolDCs as evidenced by decreased LPS-induced TNF and increased IL-10 secretion  
37 and reduced expression of MHC-II, CD86, and CD40. Furthermore, these DCs inhibited OVA-  
38 specific CD4<sup>+</sup> T cell proliferation and induced CD4<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells. Adoptive transfer  
39 of HnpM-induced tolDCs attenuated DSS-induced intestinal inflammation characteristic of IBD.  
40 Mechanistically, HnpM induced metabolic and transcriptional signatures in BMDCs consistent  
41 with tolDCs. Collectively, our findings provide groundwork for further investigation into novel  
42 mechanisms regulating DC tolerance and the role of helminth secreted metabolites in attenuating  
43 intestinal inflammation associated with IBD.

44

## 45 INTRODUCTION

46 Inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis (UC) are  
47 characterized by symptoms of watery or bloody diarrhea, abdominal pain, fatigue, weight loss, and  
48 significantly diminished patient quality-of-life[1, 2]. In genetically-prone individuals,  
49 environmental factors contribute to dysregulated intestinal tolerance, leading to immune responses  
50 to foreign antigens that are usually innocuous[3]. This dysregulated tolerance results in  
51 exaggerated CD4<sup>+</sup> Th<sub>1</sub> and Th<sub>17</sub> cell responses[4-8], and, in some cases, atypical Th<sub>2</sub> responses[9,  
52 10].

53 Evidence indicate that a broad range of helminth species induce intestinal tolerance and inhibit  
54 intestinal inflammation by modulating CD4<sup>+</sup> Th cell responses. In particular, lamina propria (LP)  
55 T cells from wild-type (WT) C57Bl/6 (B6) mice infected with *Heligmosomoides polygyrus bakeri*  
56 (Hpb), a murine gastrointestinal nematode, secrete high levels of the regulatory cytokines IL-10  
57 and TGF-β[11]. Furthermore, T cells from the mesenteric lymph nodes (MLN) of Hpb-infected  
58 WT B6 mice alleviate established intestinal inflammation when transferred into IL-10<sup>-/-</sup>  
59 recipients[12]. Interestingly, Hpb infection mitigates the Th<sub>1</sub>/Th<sub>17</sub>-driven intestinal inflammation  
60 observed in IL-10<sup>-/-</sup> mice[12, 13]. Infections with various helminth species including Hpb also  
61 protect mice from chemically induced intestinal inflammation[13-18].

62 CD4<sup>+</sup> Th cell responses are shaped by antigen presenting cells (APCs) especially dendritic  
63 cells (DCs), which also promote immune tolerance by influencing CD4<sup>+</sup> T cell responses via  
64 inducing T cell depletion, T cell anergy, and regulatory T cells (T<sub>regs</sub>)[19, 20]. A potential  
65 mechanism by which helminths indirectly shape CD4<sup>+</sup> Th cell responses is by their ability to  
66 modulate DC function. Importantly, monocyte-derived DCs from patients with helminth  
67 infections[21] and DCs from mice with experimental helminth infections induce tolerance to

68 unrelated antigens[22]. Moreover, helminths release molecules that shift DCs toward a tolerogenic  
69 DC (tolDC) phenotype, which modulate CD4<sup>+</sup> Th cell polarization and inhibit experimental  
70 intestinal inflammation[23-25]. Helminth-derived products induce distinct DC phenotypes in  
71 terms of co-stimulatory molecule expression, cytokine production, and signaling pathways[25-27].  
72 Thus, it is critical to identify molecules in the helminth secretome and characterize their  
73 immunomodulatory effects, especially their ability to induce novel tolerogenic pathways. Previous  
74 studies have identified important phenotypic and functional changes in DCs and macrophages in  
75 response to secretome components, including macromolecules, proteins, and polysaccharides, of  
76 various helminths. Here, we investigated the responses of DCs to metabolites in the excretory-  
77 secretory products of Hpb adult worms by characterizing the functional, metabolic, and  
78 transcriptional signatures of DCs treated with Hpb-derived metabolites. We also determined the  
79 effects of these DCs on CD4<sup>+</sup> Th cell responses *in vitro* and their therapeutic effects *in vivo* on  
80 experimental colitis in mice induced by administration of DSS, a widely used method to induce  
81 intestinal inflammation.

82

## 83 **RESULTS & DISCUSSION**

### 84 **Hpb-derived non-polar metabolites (HnpM) induce a tolerogenic DC phenotype**

85 Engagement of TLR4 expressed on DCs by bacterial lipopolysaccharide (LPS) initiates a  
86 complex signaling cascade and the release of proinflammatory cytokines including TNF, IL-6, IL-  
87 8, and IL-1 $\beta$ [28]. To investigate if helminth-derived metabolites induce tolDCs, we isolated polar  
88 (HpM) and nonpolar (HnpM) metabolites from Hpb culture supernatants using chromatography.  
89 We first questioned if HpM and HnpM metabolites induce a tolerogenic phenotype in DCs by  
90 determining changes in proinflammatory and immunoregulatory cytokine secretion. Bone

91 marrow-derived DCs (BMDC) were prepared from B6 mice and cultured in the presence of HnpM  
92 or HpM for 20 h and stimulated subsequently with 100 ng/ml LPS for 4 h (**Fig. 1a**). Mock purified  
93 polar and non-polar fractions from media without Hpb were used as negative controls. As  
94 expected, LPS induced BMDC to secrete high levels of TNF (**Fig. 1b**). Remarkably, TNF  
95 production was significantly reduced when BMDCs were incubated with HnpM compared to LPS  
96 alone, the HpM fraction, or polar/nonpolar controls. BMDCs treated with HnpM and stimulated  
97 with LPS had significantly enhanced IL-10 production compared to LPS alone; there were no  
98 changes in IL-10 production by BMDCs pre-treated with HpM or polar/nonpolar controls (**Fig.**  
99 **1b**). It should be noted that in the absence of LPS stimulation, neither HpM nor HnpM altered  
100 BMDC secretion of TNF or IL-10 (data not shown). The TNF/IL-10 ratio was more than 2-fold  
101 higher in HnpM-treated compared to control BMDCs after LPS stimulation (**Fig. 1c**). Together,  
102 these data suggest that HnpM present in HES produced by Hpb induced a cytokine profile in  
103 BMDCs characteristic of TolDCs. There are conflicting reports regarding the ability of helminth  
104 products to modulate TLR-dependent pathways in DCs. For instance, *Schistosoma mansoni*  
105 soluble egg antigen has been shown to suppress MyD88-dependent and independent pathways  
106 leading to IL-12p40 production induced by engaging TLR3, TLR4, or TLR9[29]. We have shown  
107 previously that unfractionated HES from Hpb suppresses IL-12p70 as well as IL-10 secretion by  
108 BMDCs following TLR4 or TLR9 ligation[23]. Thus, it is likely that different helminth species  
109 secrete various molecules that have diverse effects on DC cytokine secretion and other functions.

110 In addition to cytokines produced by antigen presenting cells (APC) including DCs, activation  
111 of CD4<sup>+</sup> T cells as fully licensed effector cells requires T cell receptor (TCR) signaling initiated  
112 by binding to cognate peptide-MHC complexes expressed by DCs and co-stimulatory molecule  
113 expression. TolDCs are known to express low levels of MHC complexes and co-stimulatory

114 molecules[30]. BMDCs pre-treated for 20 h with HnpM had significantly reduced expression of  
115 MHC-II and the co-stimulatory molecules CD86 and CD40 (**Fig. 1d, 1e**). Moreover, CD11c<sup>+</sup> DCs  
116 isolated from the spleens of mice treated i.p. with HnpM once daily for 3 days also had reduced  
117 expression of MHC-II, CD86, and CD40, an observation that corroborates our *in vitro* findings in  
118 BMDCs treated with HnpM (**Fig. 1f, 1g**).

119 To functionally assess the tolerogenic capacity of DCs treated *in vitro* with HnpM, we  
120 investigated if these DCs inhibited antigen-specific CD4<sup>+</sup> T cell proliferation using the OT-II  
121 transgenic mouse model. HnpM-treated DCs were pulsed with OVA or the OT-II peptide 323-339  
122 for 6 h and co-cultured with purified CFSE-labelled OT-II CD4<sup>+</sup> cells for 72 h. HnpM-treated DCs  
123 significantly suppressed OVA-specific CD4<sup>+</sup> T cell proliferation (**Fig. 1h-1j**). In addition, CD4<sup>+</sup> T  
124 cells co-cultured with HnpM-treated DCs displayed increased polarization towards Treg cells that  
125 expressed Foxp3 (**Fig. 1k, 1l**).

126

### 127 **HnpM induce metabolic changes in DCs**

128 Since metabolic reprogramming of immune cells is closely intertwined with inflammatory and  
129 tolerogenic responses, we next sought to determine if a unique metabolic signature distinguishes  
130 HnpM-induced tolDCs. We performed untargeted metabolomics on supernatants harvested from  
131 BMDCs treated with PBS or HnpM for 4 and 20 h (**Fig 2a**). As the identity and chemical  
132 composition of most host metabolites are still undefined, our aim was to identify a comprehensive  
133 molecular weight profile of HnpM-treated DCs to pinpoint potentially new metabolite biomarkers.  
134 Using this unbiased approach, we observed that supernatants harvested from BMDCs treated with  
135 HnpM contained 1,992 unique metabolite peaks (**Sup. File**). To create a hierarchy of relevant  
136 metabolic changes, the peaks were selected using a 2-fold change cut-off against cell-free media

137 control samples, in which 227 peaks were identified (**Sup. File**). Data were analysed using  
138 MetaboAnalyst; interquartile range (IQR) filtering reduced the number of peaks to 225 in the  
139 supernatants of HnpM-treated DCs. Principal component analysis (PCA) indicated that BMDCs  
140 treated with HnpM for 4 h formed clusters distinct from control BMDCs (**Fig. 2c**). Curiously, DCs  
141 that were exposed to HnpM for 20 h clustered together independently of pre-treatment with HnpM.  
142 Moreover, a similar pattern was observed by heatmap analysis of peaks clustered by similarity  
143 (**Fig. S1a, S1b**).

144 As the metabolite analysis of DCs treated with HnpM for 20 h did not reveal meaningful  
145 differences, we pursued further analysis focusing on the 4 h group. Statistical analyses indicated a  
146 downregulated clustering of metabolites (**Fig. S1c**) as shown in the heatmap of the top 50 most  
147 enriched metabolites (**Fig. 2b**). Volcano plot analysis showed a total of 17 downregulated  
148 metabolites, as opposed to only one upregulated metabolite of the 225 total peaks analyzed (**Fig.**  
149 **S1d**). Functional analyses were performed to infer pathways related to the differentially enriched  
150 metabolites by using Mummichog enrichment and KEGG database. The results predicted a total  
151 of 29 pathways and 43 matched compounds (**Sup. File**). Scatter plot test of significant peaks  
152 revealed two differentially enriched pathways: sphingolipid metabolism and terpenoid backbone  
153 metabolism with the metabolite, C00418, a potential match to mevalonic acid (3,5-dihydroxy-3-  
154 methylvaleric acid), according to the KEGG compound database (**Fig. 2d**). Interestingly,  
155 sphingolipid metabolism has been associated with the resolution phase of inflammatory responses  
156 in human macrophages as shown by sharp increases in glycosphingolipids and cholesteryl esters  
157 during this phase[31]. Furthermore, sphingolipid metabolism is essential for DC activation and  
158 secretion of pro-inflammatory cytokines in response to stimulation by TLR ligands[32]. In  
159 particular, the mevalonate pathway has been implicated in DC maturation as evidenced by

160 increased expression of MHC class II and CD40 by DC and increased cytokine production[33].  
161 Together, these data support the conclusion that the metabolic profile induced in DCs by treatment  
162 with HnpM is consistent with HnpM interference in DC activation leading to polarization of DCs  
163 to a tolerogenic phenotype.

164

### 165 **HnpM induce a unique transcriptomic signature in DCs**

166 Previous studies have shown that immunogenic DCs and tolDCs express distinct  
167 transcriptional profiles[34, 35]. Using matched samples of DCs from the metabolomic study, we  
168 compared the transcriptional profiles of HnpM-treated and control DCs at 4 and 20 h using RNA  
169 sequencing (**Fig. 2a**). Differential expression analysis of HnpM-treated BMDCs against control  
170 samples showed that DCs treated with HnpM for 4 h did not present any statistically significant  
171 changes in gene expression (**Fig. S2a**). Conversely, DCs treated with HnpM for 20 h had 183  
172 significantly upregulated and 76 significantly downregulated genes (**Fig. 3a, 3b; Sup. File**). The  
173 differences in the metabolome at 4 h and changes in gene expression at 20 h suggest that HnpM  
174 initially induced a metabolic shift that does not require gene regulation, that is, the metabolic shift  
175 preceded HnpM-induced changes in gene expression. Indeed, previous studies indicate that  
176 metabolism may regulate gene expression. DNA and histones are modified by different  
177 modulators, such as histone-modifying enzymes, chromatin-remodelling complexes, and  
178 transcription regulators. The enzymatic activities of these modifiers are regulated by metabolism  
179 via production of metabolites and metabolic enzymes[36]. Thus, the changes in metabolites and  
180 gene expression that we observed in HnpM-treated BMDCs may be important signatures of tolDCs  
181 induced by metabolites produced by Hpb and other helminths.



182 Gene ontology (GO) annotation and pathway enrichment ontology clusters with network  
183 layouts of representative terms of the upregulated genes revealed statistically enriched terms,  
184 showing significant features associated with immune responses, including exogenous antigen  
185 processing and presentation, cell signaling, and regulation of cytokine production as well as the  
186 adaptive immune response (**Fig. 3c; Sup. File**). The protein-protein interactions (PPI) and  
187 MCODE algorithms were applied to this network to identify neighborhoods where proteins are  
188 densely connected. This analysis strongly confirmed features related to exogenous antigen  
189 presentation by MHC-II (**Fig. S2b, S2c**). Enrichment ontology clusters and network layouts for  
190 the downregulated genes showed significance in genes related to cell proliferation, including  
191 mitotic cell cycle and cytokinesis (**Fig. 3d, Fig. S2d-S2g, and Sup. File**).

192 GSEA analysis revealed that the differentially expressed genes identified in BMDCs treated  
193 with HnpM had significant similarities with genes previously identified to be upregulated in  
194 dexamethasone-[37] and vitamin D3-induced[38] tolDCs (**Fig. 3e, Fig. S2h, S2i, Supp. File**). Of  
195 note, there were common genes in the barcode leading-edge genes (**Sup. File**). Upregulated genes  
196 identified in our transcriptomics and enrichment ontology clusters indicated these genes are  
197 involved in regulating the inflammatory response (**Fig. S2j**). Moreover, integrative analysis of  
198 transcriptional and metabolic signatures showed relevant and statistically significant pathways  
199 related to the regulation of immune responses, including antigen processing and presentation,  
200 differentiation of Th<sub>1</sub> and Th<sub>2</sub> cells, D-arginine and D-ornithine metabolism, and the PI3K-Akt  
201 signaling pathway (**Fig. 3f and Sup. File**). Previous studies showed that subsets of DCs have  
202 molecular signatures including those involved in immune system processes, cell differentiation,  
203 metabolism, and cell death which are associated with their activation status. For example, the  
204 expression of critical genes, such as macrophage galactose N-acetyl-galactosamine (*Mgl2*),

205 cadherin-1 (*Cdhl*), CD137 (*TNF-asf9*), and RhoB (*RhoB*) were observed to change markedly with  
206 DC maturation[39]. Of note, our GSEA analysis displayed remarkable similarity in HnpM-treated  
207 DCs to differentially expressed genes in tolDCs. Together, these findings may lead to the  
208 identification of a helminth-induced tolerogenic transcriptional signature in DCs and expand our  
209 understanding of the interactions between the helminth secretome and host immune tolerance.

210

## 211 **Adoptive transfer of HnpM-treated DCs alleviates intestinal inflammation in experimental** 212 **colitis**

213 Recently, progress in the development of pharmaceutical strategies to attenuate intestinal  
214 inflammation characteristic of IBD has been noteworthy including immunosuppressive therapies,  
215 such as anti-cytokine monoclonal antibodies, and antibiotic therapy to control microbiota species  
216 and abundance<sup>45,46</sup>. Importantly, there is no available treatment to date to rescue or induce  
217 tolerance to alleviate intestinal inflammation and modulate disease severity. As described above,  
218 TNF secretion by HnpM-treated BMDC was significantly decreased *in vitro* in response to LPS  
219 stimulation together with increased IL-10 production. We also observed that exposure of DCs *in*  
220 *vitro* or *in vivo* to HnpM resulted in reduced expression of MHC-II and co-stimulatory molecules  
221 by BMDCs and splenic DCs, respectively. Co-culture of HnpM-treated BMDCs dramatically  
222 reduced OVA-specific CD4<sup>+</sup> T cell proliferation and increased the frequency of Foxp3<sup>+</sup> Tregs.  
223 These observations prompted us to investigate the tolerogenic potential of HnpM-treated BMDCs  
224 in mice with DSS-induced intestinal inflammation. B6 mice administered 3% DSS in drinking  
225 water for 5 days[40] were injected i.p. with either 1 x 10<sup>6</sup> control BMDCs or HnpM-treated  
226 BMDCs. Mice adoptively transferred with HnpM-treated DCs displayed significant reductions in  
227 disease activity score (DAS), colon shortening, and weight loss (**Fig. 4a-4c**). Histopathological

228 analysis indicated decreased loss of intestinal parenchyma and significantly less tissue damage in  
229 the mid-distal colon (**Fig. 4d**). We have previously reported that adoptively transferred  
230 BMDCs[41] or macrophages[42] migrate to the spleen, MLN, and GI tract of recipient mice where  
231 they can interact with T cells. In previous work, we confirmed that adoptively transferring  
232 regulatory/tolerogenic APCs, such as macrophages, which migrate to secondary lymphoid organs  
233 and interact with T cells to alleviate intestinal inflammation[42]. Others demonstrated that  
234 adoptive transfer of tolDCs[43] effectively blocked dinitrobenzene sulfonic acid (DNBS)-induced  
235 intestinal inflammation in immunocompetent mice. In contrast, adoptive transfer of tolDCs was  
236 ineffective in Rag<sup>-/-</sup> mice with DNBS-induced colitis, indicating interaction between tolDCs and  
237 T cells is critical to alleviate intestinal inflammation characteristic of IBD.

238

## 239 CONCLUSION

240 Tolerogenic DCs (tolDCs) promote immune homeostasis and tolerance by influencing CD4<sup>+</sup>  
241 Th cell responses via various mechanisms including inducing Foxp3<sup>+</sup> Tregs[19-22]. Recent studies  
242 indicate that different functional classes of DCs are marked by distinct phenotypic, molecular and  
243 transcriptional profiles, which translate to a diversity of immunological functions among DC  
244 subsets. Collectively, our findings demonstrate that DC maturation and function are influenced by  
245 exposure to HnpM produced by adult stage Hpb. Treatment with HnpM *in vitro* or *in vivo* induced  
246 tolerogenic features in DCs including modulating their cytokine secretion in response to the TLR4  
247 agonist LPS and expression of MHC-II, CD86, and CD40 responses associated with inhibition of  
248 antigen-specific, effector CD4<sup>+</sup> T cell responses and induction of Foxp3<sup>+</sup> Tregs. Importantly,  
249 adoptive transfer of HnpM-induced tolDCs resulted in dramatic reduction in intestinal  
250 inflammation in DSS-induced colitis in mice. Mechanistically, HnpM induced metabolic and

251 transcriptional signatures in BMDCs consistent with those established previously for tolDCs[19,  
252 20].

253 It is likely that metabolites and other molecules released by helminths, which promote tolDCs,  
254 have evolved as an immune evasion mechanism to avoid expulsion by the host and establishment  
255 of a chronic infection characteristic of these parasites. Thus, our findings provide groundwork for  
256 further investigation into novel mechanisms regulating DC tolerance and the role of helminth  
257 secreted metabolites in attenuating intestinal inflammation associated with IBD. Future studies  
258 may uncover novel pathways to induce tolDCs by harnessing information from the list of  
259 metabolites and genes regulated up or down by HnpM presented here, leading to innovative  
260 therapies to treat IBD and other immune-mediated, inflammatory diseases.

261

## 262 **AUTHOR CONTRIBUTIONS**

263 Conceptualization, N.L.M., A.N.M., A.J., M.M.S., F.L.; Data Curation, N.L.M., A.N.M., T.S.,  
264 G.F., I.L.K., I.C., A.J., F.L.; Data Analysis, N.L.M., A.N.M., T.S., G.F., I.L.K., I.C., A.J., F.L.;  
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266 N.L.M., A.N.M., E.S., A.G.M., T.S., G.F., I.L.K., I.C., A.J., M.M.S., F.L.; Project Administration,  
267 F.L.; Resources, I.L.K., M.M.S., F.L.; Software, I.C.; Supervision, M.M.S, F.L.; Validation, F.L.;  
268 Visualization, N.L.M., A.N.M.; Writing - Original Draft, N.L.M., A.N.M.; Writing - Review &  
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277 F.L.

## 278 MATERIALS & METHODS

279 **Mice** Male BALB/c mice, 8-10 weeks old, and female C57Bl/6 (B6) mice, 6-8 weeks old, obtained  
280 from Charles River Laboratories (St. Constant, QC, Canada) and B6 OT-II/Rag<sup>-/-</sup> mice, 6-8 weeks  
281 old, were housed under specific pathogen-free conditions at McGill University. All experiments  
282 were approved and conducted in accordance with the guidelines of the Canadian Council on  
283 Animal Care.

284 ***H. polygyrus bakeri* (Hpb)-Derived Metabolites** Helminth-derived excretory/secretory (HES)  
285 products were obtained as previously described[44]. BALB/c mice were infected with 400-500  
286 infective larvae (L3) of Hpb for 21 days. Adult worms were collected from the small intestine and  
287 cultured in RPMI 1640 medium containing 2% w/v glucose, 80 µg/ml gentamicin, 100 U/ml  
288 penicillin/streptomycin, and 20 µg/ml polymyxin B for 40 h at 37°C, 5% CO<sub>2</sub>. Supernatant was  
289 collected, centrifuged at 8000 × g for 10 min at 4°C, and passed through a 0.2 µm filter.  
290 Metabolites were separated from proteins and exosomes using a 3 kDa MWCO centrifugation unit  
291 (UFC910024, Millipore) and centrifuged at 4000 × g for 40 min at 4°C. Hpb-derived metabolites  
292 were further separated into polar (HpM) and nonpolar (HnpM) fractions by chromatography using  
293 a C<sub>18</sub> column. The flow-through containing polar metabolites from the C<sub>18</sub> column was further  
294 subjected to a universal polymeric reversed-phase sorbent containing column (WAT106202,  
295 Waters), and the polar metabolites were eluted using acetonitrile at 100% v/v. HpM and HnpM  
296 stock fractions were concentrated using a speed vacuum concentrator, resuspended in ultrapure  
297 water and maintained at -20°C.

298 **Dendritic Cells** Bone marrow-derived dendritic cells (BMDCs) and splenic dendritic cells (DCs)  
299 were obtained from B6 mice. Bone marrow cell suspensions were cultured and differentiated in  
300 10 cm petri dishes in complete RPMI 1640 medium supplemented with 10% fetal bovine serum

301 (FBS), 1% 100X penicillin/streptomycin solution, 1% 100 mM sodium pyruvate, and 20 ng/ml  
302 GM-CSF (StemCell Technologies) for 8 days at 37°C, 5% CO<sub>2</sub>. On days 3 and 6, BMDCs were  
303 given an additional 10 ml and 5 ml, respectively, of complete medium containing 20 and 40 ng/ml  
304 GM-CSF. Floating cells were collected and used for all experiments; flow cytometry confirmed  
305 the purity of BMDCs as >90% CD11c<sup>+</sup>. Aliquots of BMDCs (1 x 10<sup>5</sup>) were seeded into 96-well  
306 plates, treated with 50 µg/ml HnpM, 50 µg/ml HpM, or 50 µg/ml polar and nonpolar control mock  
307 extractions in complete RPMI 1640 containing 20 ng/ml GM-CSF and cultured at 37°C, 5% CO<sub>2</sub>  
308 for 20 h, washed, and stimulated with LPS (100 ng/ml; Millipore-Sigma) for 4 h. For splenic DCs,  
309 spleens were minced, passed through a 40 µm cell strainer to obtain single cell suspensions, and  
310 red blood cells lysed using ACK lysis buffer (ThermoFisher).

311 **Cytokine ELISAs** Supernatants were collected from BMDCS for TNF (R&D Technologies) or  
312 IL-10 (R&D Technologies) quantification. ELISAs were performed according to manufacturer's  
313 instructions.

314 **Immunophenotyping** Cultured BMDCs or splenic DCs were treated with Fc block, and labeled  
315 with the following antibodies from BioLegend for 30 min at 4°C: anti-CD45-PeCy7, -CD11c-  
316 FITC), -MHC-II (I-A/I-E)-BV421, -CD40-PE, and -CD86-PerCP mAbs. To quantify CD4<sup>+</sup>Foxp3<sup>+</sup>  
317 cells, spleen cells were stained with anti-CD4-FITC mAb (eBioscience), fixed, and permeabilized  
318 using the Foxp3 staining kit (Invitrogen), and stained with anti-Foxp3-Alexa Fluor 488 mAb  
319 (eBioscience) for 30 min at 4°C. Cells were acquired in Attune Nxt flow cytometer (ThermoFisher  
320 Scientific) and analyzed using FlowJo software v.10.2 (Tree Star).

321 **OVA-specific CD4<sup>+</sup> T cell proliferation** To evaluate OVA-specific CD4<sup>+</sup> T cell proliferation,  
322 spleens were harvested from naïve OT-II TCR-transgenic mice, and single cell suspensions were  
323 prepared as described above. CD4<sup>+</sup> T cells were isolated using the EasySep Mouse CD4<sup>+</sup> T cell

324 Isolation Kit (StemCell Technologies) according to the manufacturer's instructions. BMDCs were  
325 either untreated or treated with 50 µg/ml HnpM for 24 h prior to co-culture with OT-II CD4<sup>+</sup> T  
326 cells. During the last 6 h of culture, BMDCs were pulsed with 250 µg/ml OVA (Sigma) or 10  
327 µg/ml OVA peptide 323-339 (Sigma) at 37°C, 5% CO<sub>2</sub>, prior to co-culture for 72 h with purified,  
328 CFSE-labelled (eBioscience) OT-II CD4<sup>+</sup> T cells at a final T cell:BMDC ratio of 2:1. Cells were  
329 collected and labelled with anti-CD4-FITC and anti-CD44-APC mAbs (BD Biosciences). Flow  
330 cytometry was used to determine CFSE dilution in gated CD4<sup>+</sup>CD44<sup>+</sup> cells (LSR Fortessa; BD  
331 Biosciences); data were analyzed using FlowJo software.

332 **DSS Treatment** Female B6 mice at 6-8 weeks old were provided with 3% w/v DSS (MP  
333 Biomedicals) in drinking water *ad libitum* for 5 days. On day 5, drinking water was changed to  
334 normal drinking water and mice were injected i.p. with 1 x 10<sup>6</sup> vehicle control (PBS) DCs, naïve  
335 DCs, or HnpM-treated DCs. Mice were weighed daily. On day 8, necropsies were performed and  
336 colon lengths, percent weight changes and disease activity scores (DAS) based on a scale of 0-5  
337 were determined as previously described[45].

338 **Histology** Mid-distal colon sections were fixed in 10% neutral buffered formalin, embedded in  
339 paraffin wax, and sectioned to 4 µm thickness. Sections were de-paraffinized in two changes of  
340 xylene and rehydrating through graded ethanol solutions (100%, 95%, 70% EtOH) and distilled  
341 H<sub>2</sub>O. Sections were stained in Gills II hematoxylin (Leica Biosystems) for 5 minutes and rinsed in  
342 warm tap water for 5 minutes and counter-stained in eosin (Leica Biosystems) for 1 min followed  
343 by 1 min in distilled H<sub>2</sub>O. Slides were dehydrated through reverse graded ethanol solutions (70%,  
344 95%, 100% EtOH) and cleared in two changes of xylene before mounting overnight with Permount  
345 (Fisher Scientific). Brightfield microscopy on mid-distal histological sections was performed  
346 using a 100X objective on an EVOS XL Cell Imaging System (ThermoFisher Scientific) in which

347 5 mid-distal colon sections per mouse per group were blindly assessed using a validated 12-point  
348 scoring system[45].

349 **Metabolomics and Transcriptomics** As shown in **Fig. 2a**, matched BMDCs prepared and treated  
350 with HnpM for 4 and 20 h were used for metabolomics and transcriptomics. For metabolomics,  
351 supernatants and cell-free media controls were collected and incubated with ice-cold HPLC-grade  
352 methanol for 30 min on ice, centrifuged at  $10,000 \times g$  for 10 min at  $4^{\circ}\text{C}$ , and stored at  $-80^{\circ}\text{C}$  until  
353 analysis. For transcriptomics, RNA from HnpM-treated and control BMDCs was extracted with  
354 TRIzol (Life Technologies) and chloroform and precipitated with isopropanol and extracted using  
355 PureLink RNA Micro Scale Kit (ThermoFisher).

356 **Metabolomic profiling** The profiling of nonpolar metabolites was performed at the UVic-Genome  
357 BC Proteomics Centre by LC-MS/MS analysis of the deproteinated conditioned media by injecting  
358 3 mL of sample onto a Dionex UHPLC system equipped with an Agilent Eclipse  $\text{C}_{18}$  ( $2.1 \times 15$   
359 mm, 1.8 mm) column incubated at  $45^{\circ}\text{C}$ . Metabolites were resolved with a 30 min linear running  
360 0-80% using the buffer system 0.05% formic acid and 0.05% formic acid in acetonitrile at a flow  
361 rate of 300 mL/min. The column effluent was introduced by electrospray ionization onto a Velos  
362 LTQ Orbitrap Analyzer (Thermo Scientific) using a spray voltage of 3.6 kV, a source heater  
363 temperature of  $350^{\circ}\text{C}$ , and a sheath gas flow of 40 l/min. Survey scans were performed using the  
364 Orbitrap mass spectrometer and the 10 most intense ions were selected for fragmentation using a  
365 30-40 V-stepped collision-induced dissociation energy. Fragmentation products were analyzed in  
366 the linear ion trap mass spectrometer. Fragmentation was used to perform an online search using  
367 the XCMS database (<https://xcmsonline.scripps.edu>) to identify possible metabolites.

368 Following peak profiling, 2-fold change enriched peaks (compared to control samples, i.e.,  
369 samples with media only) were selected to perform statistical and functional interpretation using



370 MetaboAnalyst 5.0 (<https://www.metaboanalyst.ca/>)[46]. Data were analyzed using interquartile  
371 range (IQR) and normalized by doing a  $\log_{10}$  transformation. Principal component (PC) analysis,  
372 volcano plot (2-fold change threshold, 0.1 p-value threshold, and equal group variance), and  
373 heatmap (with Euclidean distance measure and Ward clustering algorithm with clustered group  
374 samples) were performed. Peak annotations were further analyzed using Mummichog algorithm  
375 and KEGG database for functional interpretation.

### 376 **RNA sequencing**

377 RNA sequencing of poly-A enriched RNAs was performed at Centre d'expertise et de services  
378 Génome Québec, as previously described[47]. Briefly, libraries were sequenced by Illumina RNA-  
379 Seq Libraries on two experimental replicates for each experiment. Reads alignment and gene ID  
380 mapping were performed using Bowtie2 and HTSeq, respectively, against the Genome Reference  
381 Consortium Mouse Build 38 patch release 6 (GRCm38.p6) database. Read counts were obtained  
382 with featureCounts from package subread and differential gene expression was obtained using  
383 EdgeR with a quasi-likelihood negative binomial generalized log-linear model likelihood ratio test.  
384 One count per million (CPM) minimum per gene, or five counts per gene in the smallest library,  
385 and counts represented by at least two replicate libraries were required for a gene to be considered  
386 for analysis. Genes were considered differentially expressed if the fold changes were  $\geq 2$  and  $p \leq$   
387 0.05. Further GSEA tests were performed using the fgsea and gage packages in R. Gene sets were  
388 constructed with genes differentially expressed up and down. Gene sets were analyzed using  
389 published data on tolerogenic and inflammatory DCs (**Supp. File**)[39, 48, 49]. Gene sets are  
390 represented as a barcode plot using the Limma package in R.

391 **Statistical analysis** Statistical analyses of metabolomic and transcriptomic data are described in  
392 each corresponding method sections. All other data are presented as boxplots indicating the median

393 (center line), upper and lower box bounds (IQR = first and third quartiles), and whiskers (minimum  
394 and maximum values), with individual data points superimposed onto the boxplot. Normality of  
395 datasets were tested using the Kolmogorov-Smirnoff test. Data that were normally distributed were  
396 analyzed by one-way ANOVA with appropriate post-hoc tests. Unpaired data were compared  
397 using two-tailed unpaired Student's *t* test. Statistical analysis was performed using GraphPad  
398 Prism v.9.3.1, and significance was assumed for  $p < 0.05$ .

399 **FIGURE LEGENDS**

400 **Figure 1. Helminth-derived nonpolar metabolites (HnpM) induce a tolerogenic phenotype in**  
401 **DCs**

402 **(a)** Experimental design workflow. Created in BioRender.

403 **(b)** TNF and IL-10 secreted protein quantified by ELISA from BMDCs either untreated controls  
404 or treated with 50 µg/ml helminth-derived polar metabolites (HpM), 50 µg/ml HnpM, or 50  
405 µg/ml of mock-extracted polar/nonpolar media controls for 20 h, and then stimulated with 100  
406 ng/ml LPS for 4 h. Data are represented as boxplots and analyzed by one-way ANOVA with  
407 Holm-Sidak multiple comparisons: \*\*\*\* $p < 0.0001$ , \*\*\* $p < 0.001$ ,  $n = 4$  biological replicates for  
408 BMDCs from 4 individual mice per group.

409 **(c)** Ratio of secreted TNF/IL-10 based on data presented in Figure 1a. Data are represented as  
410 boxplots and analyzed by unpaired t test:  $p = 0.0022$ ,  $n = 4$  individual mice per group.

411 **(d)** Gating strategy used to assess CD45/CD11c<sup>+</sup> BMDC populations.

412 **(e)** FACS analysis of MHC-II, CD86, and CD40 expression assessed by BMDCs were treated  
413 with vehicle control (PBS) or 50 µg/ml HnpM for 20 h. Data are represented as boxplots and  
414 analyzed by unpaired t test:  $p = 0.0447$ ,  $p = 0.0391$ ,  $p = 0.0007$ , for MHC-II, CD86 and CD40,  
415 respectively.  $n = 4$  biological replicates for BMDCs from 4 individual mice/group that were  
416 isolated, differentiated, and treated as described.

417 **(f)** Gating strategy used to assess splenic CD45/CD11c<sup>+</sup> DC populations.

418 **(g)** Splenic DC surface expression of MHC-II, CD86, and CD40 assessed by flow cytometry. Mice  
419 were injected i.p. with 25 µg HnpM or PBS as a control per day for 3 days and splenic DCs  
420 were isolated for flow cytometry. Data are represented as boxplots and analyzed by unpaired t  
421 test:  $p = 0.0260$ ,  $p = 0.0194$ ,  $p = 0.0066$ , for MHC-II, CD86 and CD40, respectively.  $n =$ biological  
422 replicates of splenic DCs from 5 individual mice per group.

423 **(h)** Gating strategy used to assess the proliferation of CD4<sup>+</sup>CD44<sup>+</sup> T cells co-cultured with either  
424 control (PBS) or HnpM-treated BMDCs for 72 h. Plots indicate the CFSE<sup>+</sup> cells in gated  
425 CD4<sup>+</sup>CD44<sup>+</sup>T cells.

426 **(i)** Data presented as mean fluorescence intensity of CSFE dye dilution in gated CD4<sup>+</sup>CD44<sup>+</sup> T  
427 cells and the percentage of cells undergoing > 3 cell divisions.

428 **(j)** Histogram of the percentage of CD4<sup>+</sup>CD44<sup>+</sup> T cells undergoing > 3 cell divisions. Data are  
429 represented as bar graphs with experimental replicate data points and analyzed by paired t test:

430 p=0.0001 (top), p=0.0429 (bottom). n=3 biological replicates for CD4<sup>+</sup>CD44<sup>+</sup> T cells obtained  
431 from 3 individual OT-II mice.

432 **(k)** Gating strategy used to assess Foxp3<sup>+</sup> populations in gated CD4<sup>+</sup> T cells co-cultured with  
433 control or HnpM-treated BMDCs.

434 **(l)** CD4<sup>+</sup> T cell expression of Foxp3 assessed by flow cytometry. BMDCs were treated with either  
435 vehicle control (PBS) or 50 µg/ml HnpM for 20 h, and co-cultured for 48 h with OT-II CD4<sup>+</sup>  
436 T cells isolated from spleen by negative selection using magnetic beads. Data are represented  
437 as boxplots and analyzed by unpaired t test: p= 0.0077, n=6 biological replicates of BMDCs  
438 from 4 individual mice per group.

439

440 **Figure 2. Helminth-derived nonpolar metabolites (HnpM) induce metabolic changes in**  
441 **BMDCs.**

442 **(a)** Experimental design workflow schematic of matched metabolomics and transcriptomics  
443 experiments. BMDCs were isolated and differentiated with 20 ng/ml GM-CSF and treated with  
444 HnpM or vehicle control (PBS) for 4 and 20 h. The supernatants were collected for untargeted  
445 metabolomics analysis and mRNA was extracted for RNA sequencing. n=biological replicates  
446 from 3 individual mice per group of BMDCs. Graphic created in BioRender.

447 **(b)** Heatmap of top 50 statistically enriched peak intensities at 4 h. Hierarchical clustering of  
448 metabolite concentrations with a higher enrichment (red) and lower enrichment (blue) were  
449 determined by Euclidean distance measuring and Ward clustering algorithms.

450 **(c)** Principal component (PC) analysis was performed using the prcomp package. The calculation  
451 was based on singular value decomposition and the Rscript chemometrics. The 2-D score plots  
452 between selected PCs are shown and the respective variances are provided.

453 **(d)** Scatter plot of inferred pathways with potential matched compounds. The peak annotations  
454 were performed using the Mummichog algorithm and the KEGG database was used for  
455 functional interpretation.

456

457 **Figure 3. Helminth-derived nonpolar metabolites (HnpM) induce a unique transcriptomic**  
458 **signature in BMDCs.**

459 Data for BMDCs treated with HnpM or vehicle control (PBS) for 20 h are presented. RNA was  
460 extracted as described in Materials and Methods for RNA sequencing by Illumina RNA-seq. Reads

461 alignment and gene ID mapping were performed using Bowtie2 and HTSeq, respectively, against  
462 the Genome Reference Consortium Mouse Build 38 patch release 6 (GRCm38.p6) database.

463 **(a)** Venn diagram and **(b)** volcano plot for differentially expressed genes at 20 h. Read counts  
464 were obtained with Get\_ReadCount.py and differential gene expression was obtained using  
465 EdgeR with a likelihood ratio test. Genes were considered differentially expressed if the fold  
466 change  $\geq 2$  and  $p \leq 0.05$ .

467 **(c)** GO annotation and pathway enrichment. All statistically enriched terms were identified based  
468 on the default choices under accumulative hypergeometric p-values and enrichment factors  
469 were calculated and used for filtering. Remaining significant terms were then hierarchically  
470 clustered into a tree based on Kappa-statistical similarities among their gene memberships.  
471 Then 0.3 kappa score was applied as the threshold to cast the tree into term clusters.

472 **(d)** Protein-protein interaction (PPI) network among the input genes. A subset of representative  
473 terms from the full cluster was converted into a network layout and each term is represented  
474 by a circular node, where the size is proportional to the number of input genes falling under  
475 that term with similar color representing cluster identity. Terms with a similarity score  $> 0.3$   
476 are linked by an edge and the thickness of the edge represents the similarity score.

477 **(e)** GSEA analysis was performed with phenoTest package in R. Gene sets were constructed with  
478 genes that are differentially expressed up and down. Gene sets were analyzed using published  
479 data on tolerogenic and inflammatory DCs (**Supp. File**) Gene sets are represented using a  
480 barcode plot using the Limma package in R.

481 **(f)** Integrative analysis of transcriptional and metabolic signatures. Enrichment analysis by  
482 hypergeometric test and topology measured degree centrality. Combined queries integration  
483 method was performed, and the overviews indicate all matched pathways according to the p  
484 values from the pathway enrichment analysis and pathway impact values from the pathway  
485 topology analysis.

486  
487 **Figure 4. DCs treated with HnpM alleviate DSS-induced intestinal inflammation.**

488 Intestinal inflammation was chemically-induced in B6 mice by administration of 3% DSS in  
489 drinking water for 5 days. On day 5, mice were injected i.p. with PBS as a control or  $1 \times 10^6$  naïve  
490 BMDCs or HnpM-treated BMDCs. Mice without DSS served as a negative control. Control mice

491 and mice adoptively transferred with BMDCs were sacrificed on day 8 post-DSS and necropsies  
492 were performed to determine the following:

493 **(a)** Disease activity scores (DAS) on a scale from 0-5 were determined based on body weight,  
494 colon length, health of the animal, rectal bleeding, and macroscopic appearance of the colon.

495 **(b)** Colon lengths in cm.

496 **(c)** Percent weight changes were determined on day 8 as a percentage compared to weight before  
497 DSS exposure.

498 **(d)** Histopathology scores (left) assessed in 5 sections per mouse per group of H&E-stained mid-  
499 distal colon sections (right).

500 Data in **Fig. 4a-d** are represented as boxplots and analyzed by one-way ANOVA with Holm-Sidak  
501 multiple comparisons: \*\*\*\* $p < 0.0001$ , \*\*\* $p < 0.001$ ,  $n = 4-5$  mice per group.

502

### 503 **COMPETING INTERESTS**

504 The authors declare no competing interests.

505

### 506 **DATA AVAILABILITY**

507 Source data associated with Figure and Supplemental Figure panels are provided with this paper  
508 as a Source Data file. Metabolite LC-MS data was deposited to Metabolomics Workbench under  
509 accession number ST002230. Transcriptomics data (RNAseq) was deposited to the NCBI  
510 Sequence Read Archive (SRA) under accession number PRJNA856720.

511

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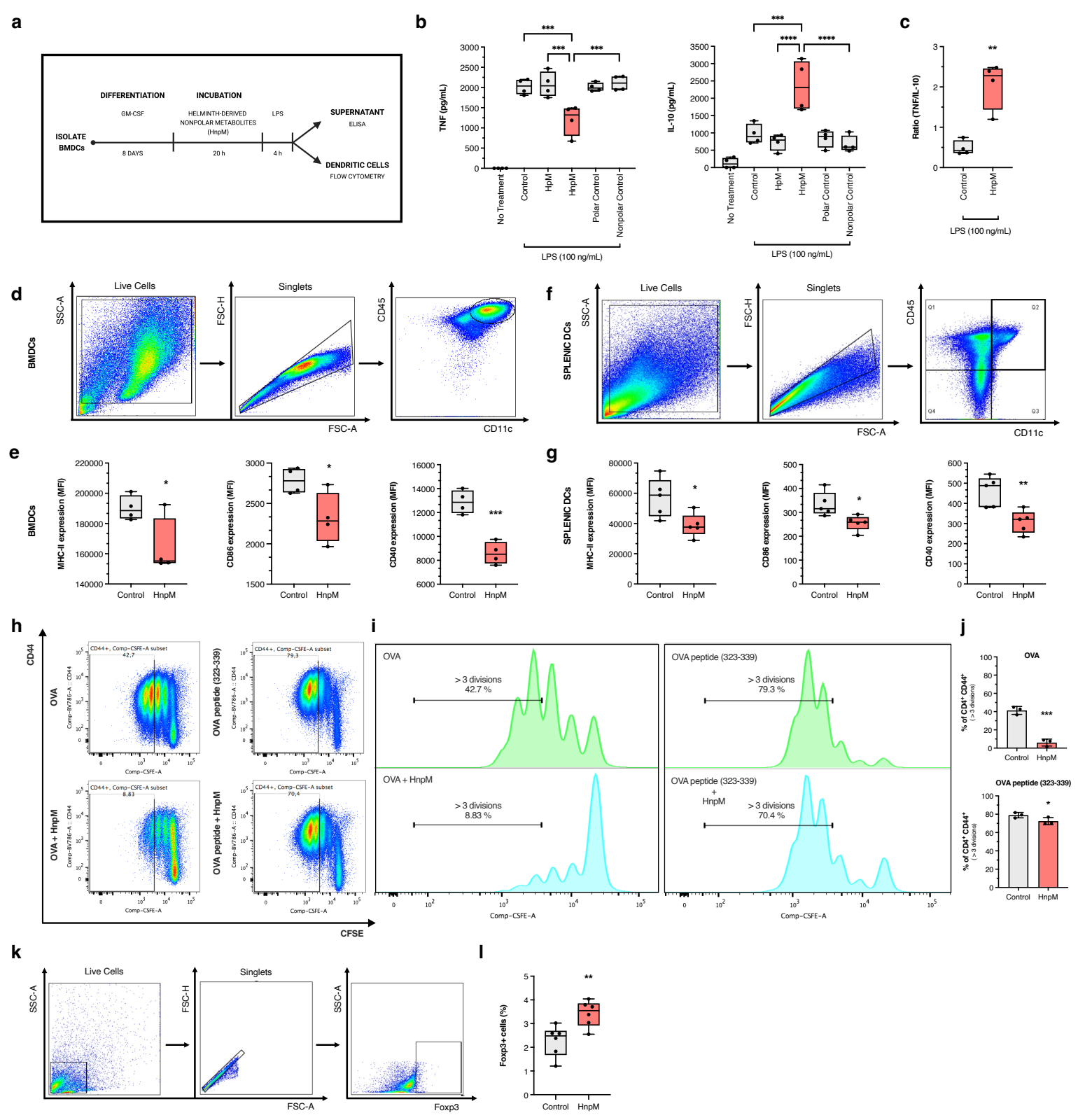
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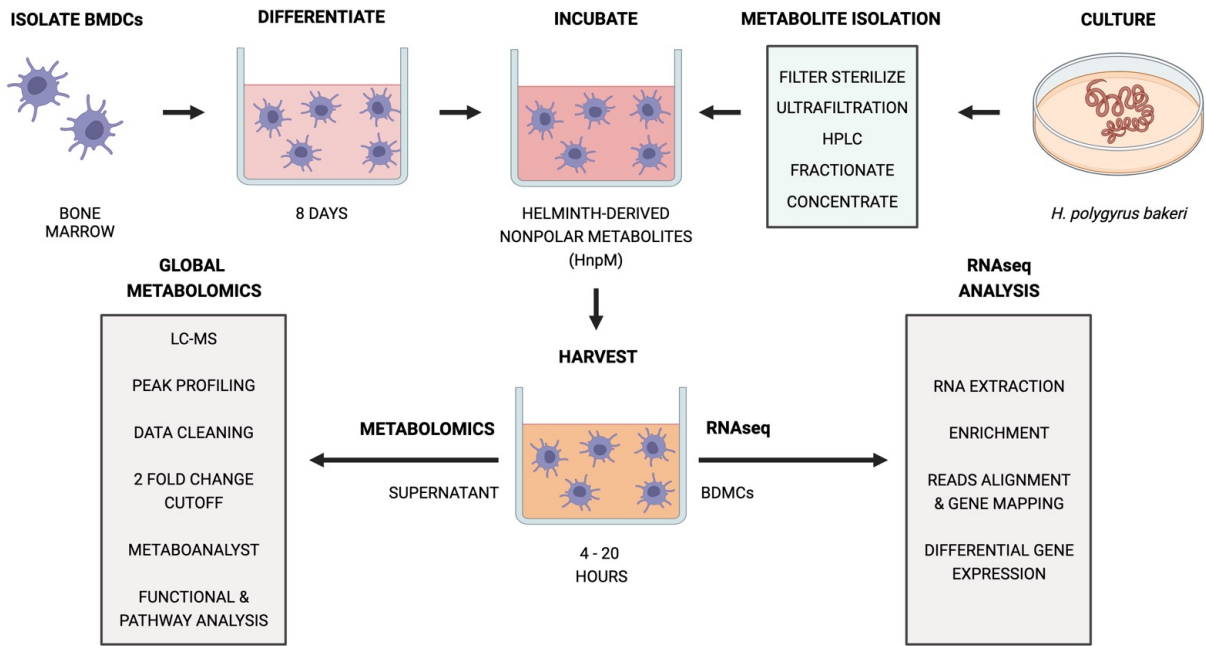
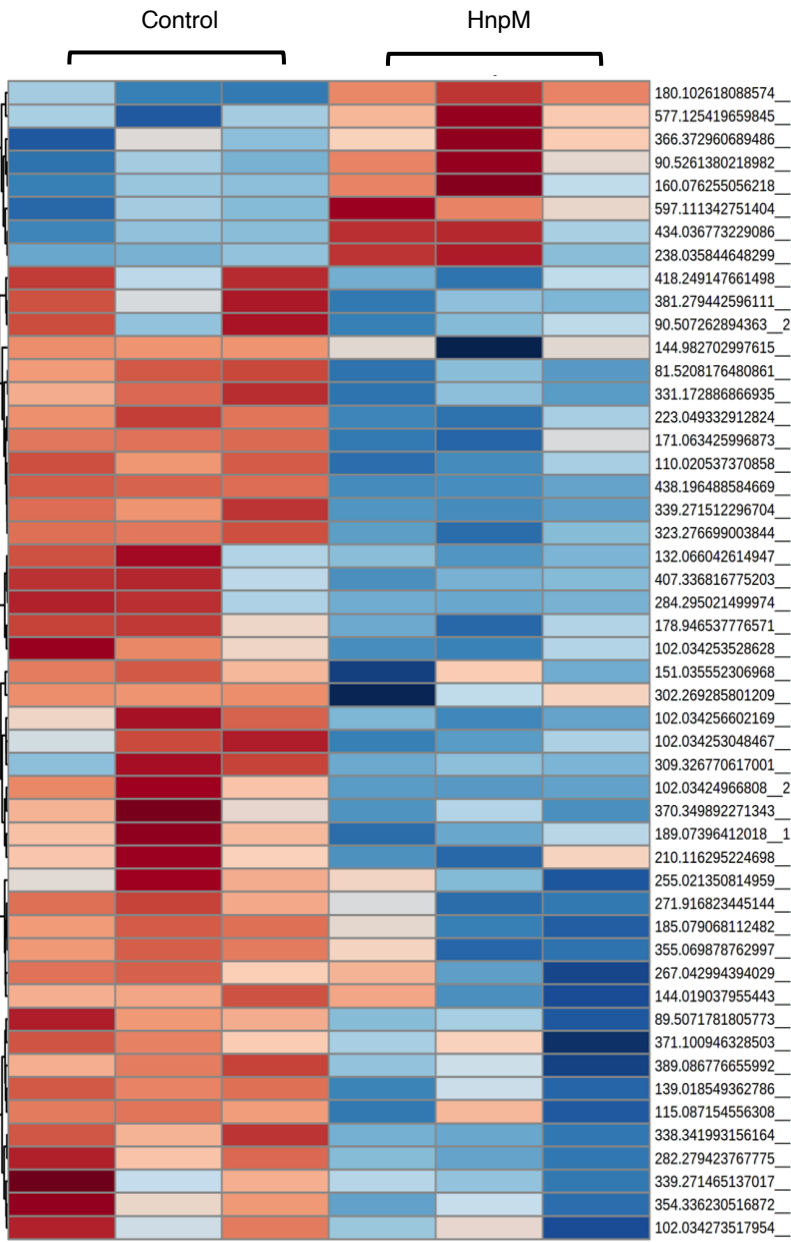
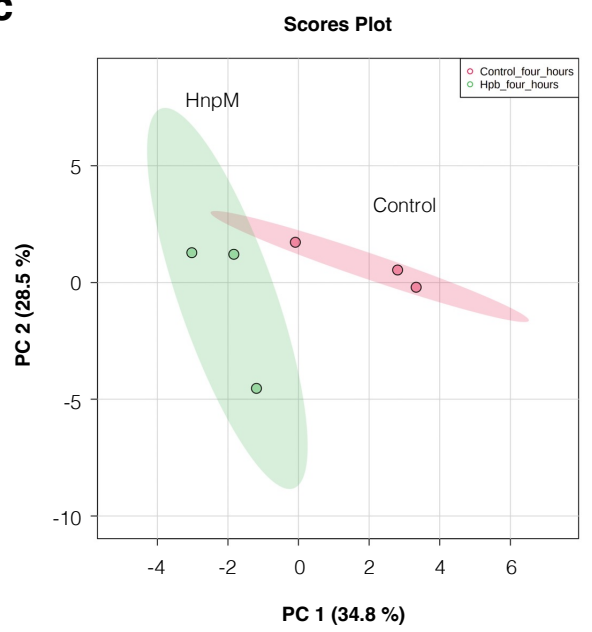
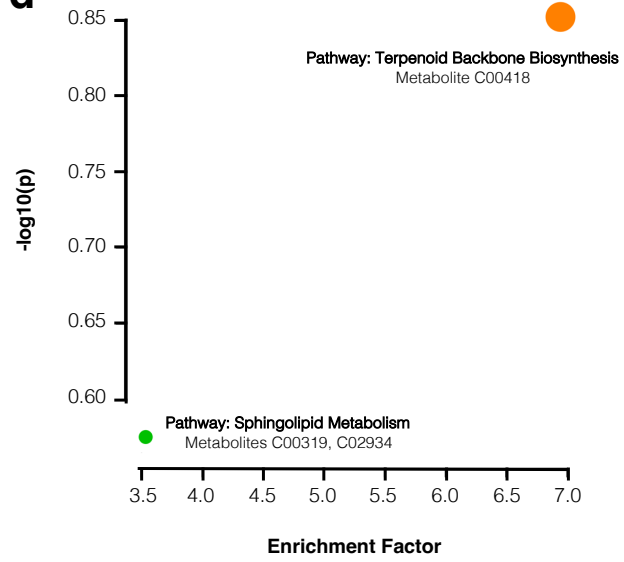
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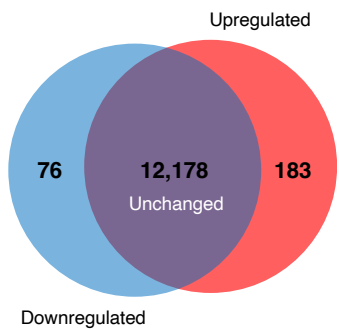
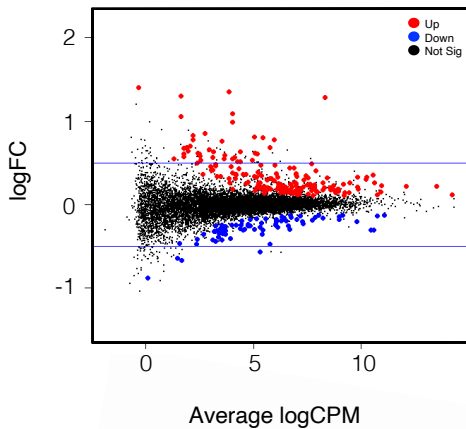
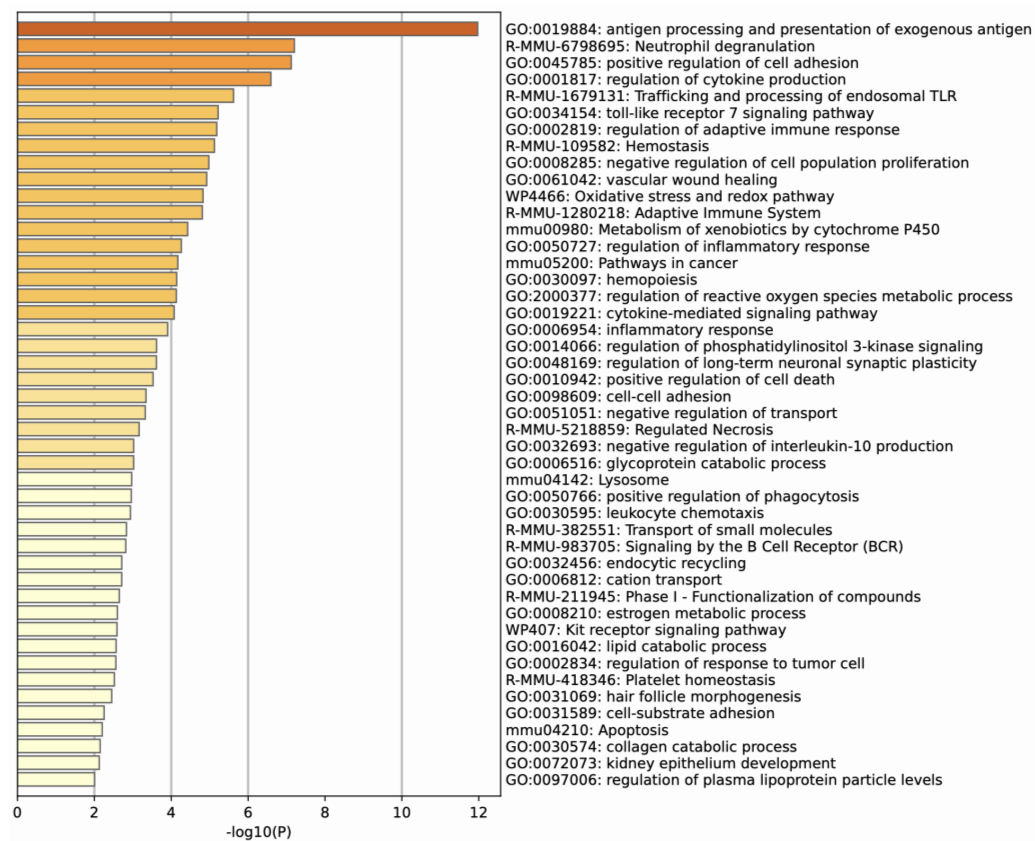
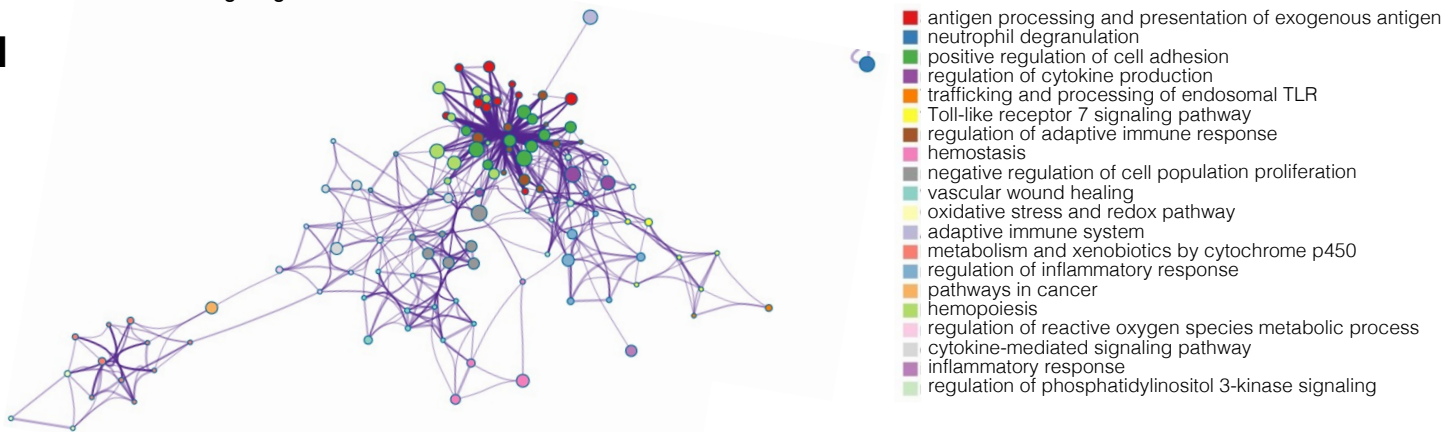
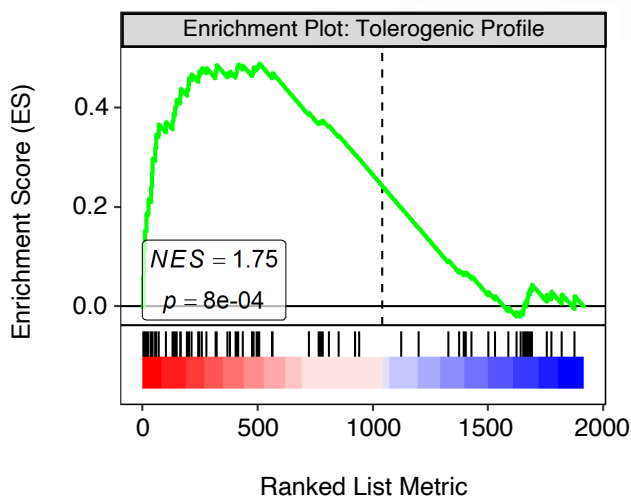
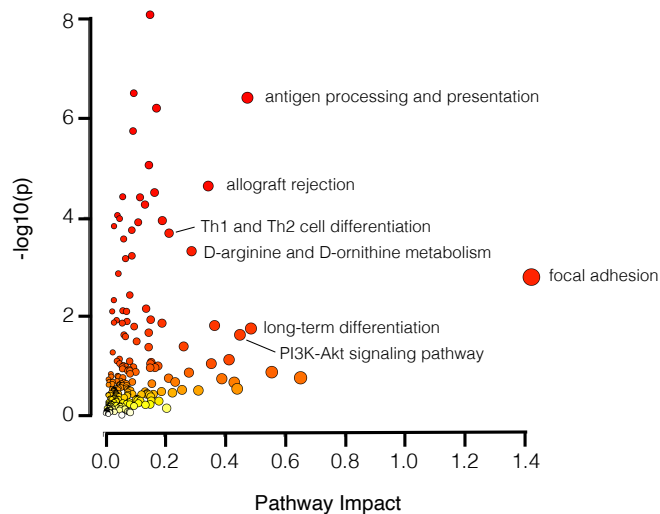
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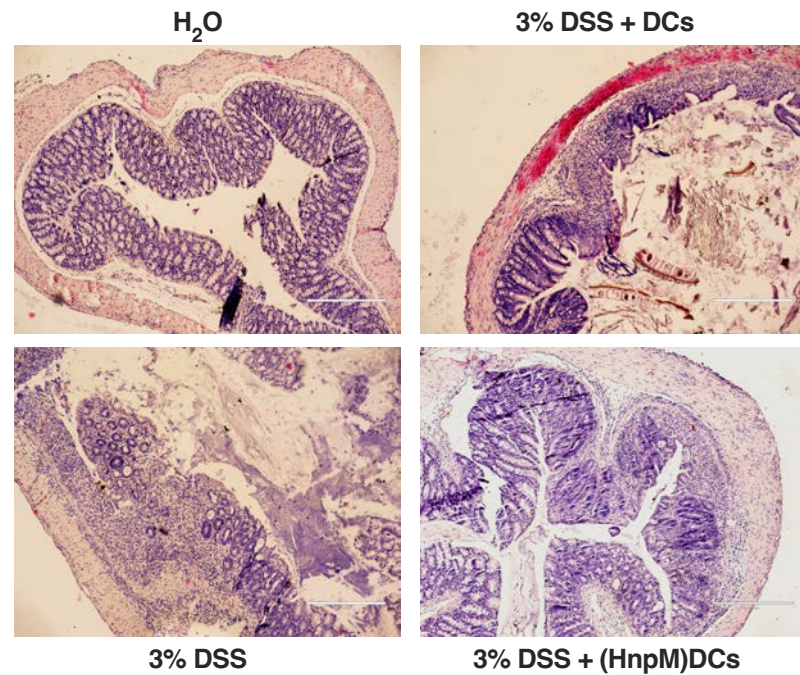
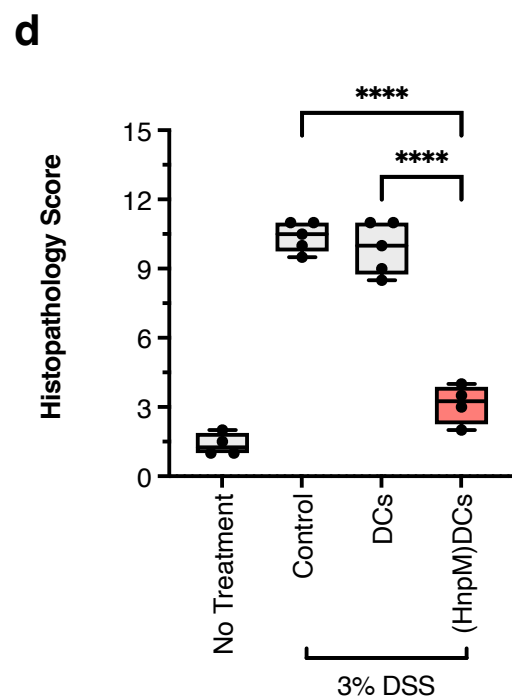
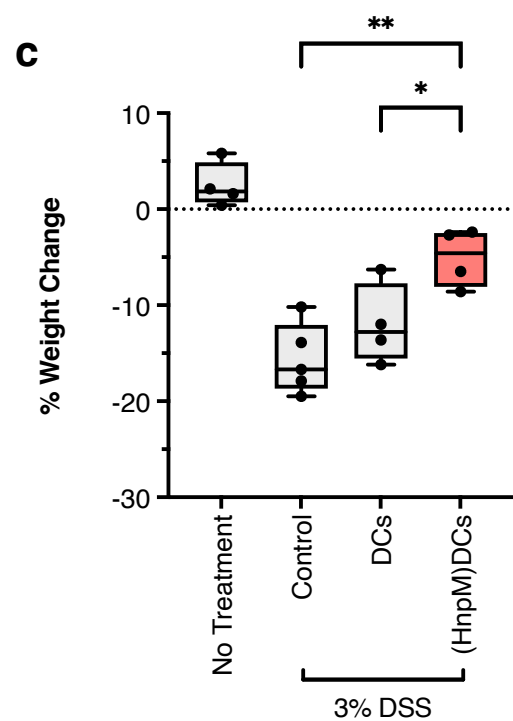
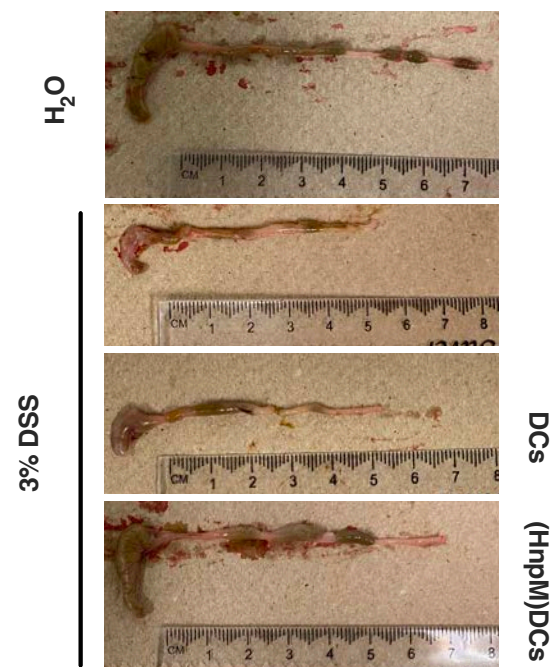
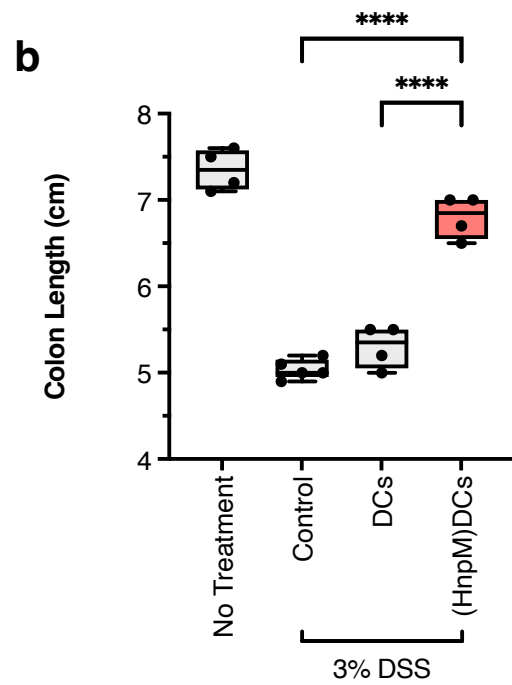
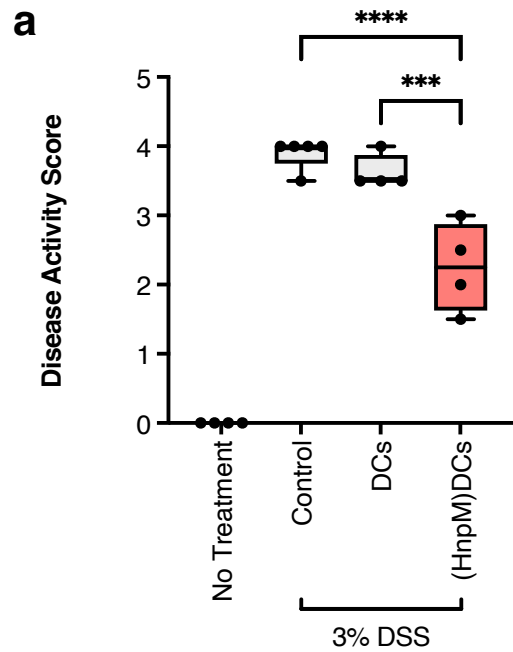


**a****b****c****d**

**a**

12,437 genes analyzed by RNAseq

**b****c****d****e****f**



## Supplementary Information

### **Helminth-derived metabolites induce tolerogenic transcriptional and metabolic signatures in dendritic cells in experimental intestinal inflammation**

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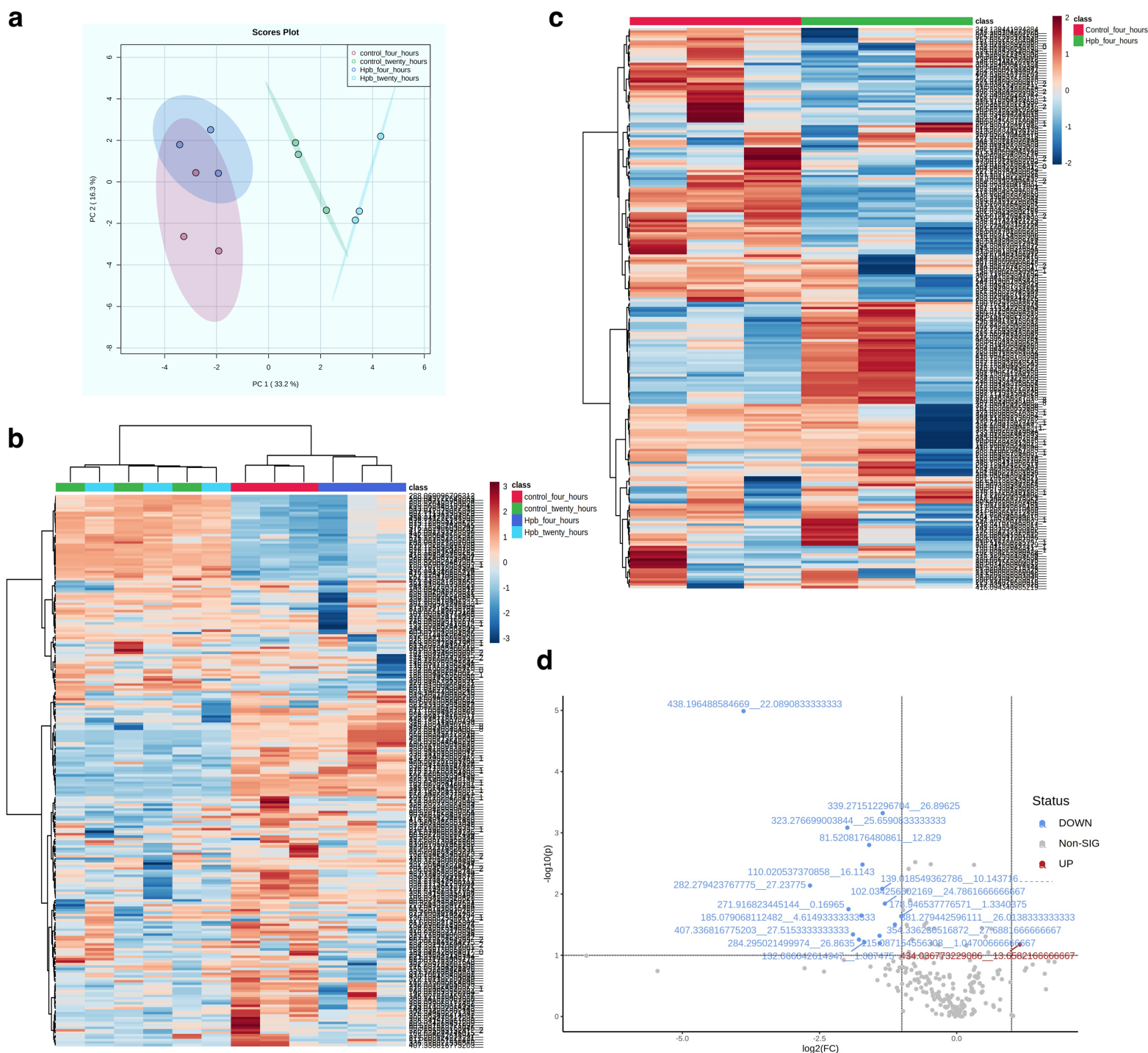
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## Supplementary Figure 1



**Supplementary Figure 1. Metabolomics and transcriptomics characterization of (HnpM)BMDCs at 4h and 20h.** BMDCs were isolated and differentiated with 20ng/ml of GM-CSF. Cells were incubated with helminth-derived nonpolar metabolites for 4 and 20 h. The supernatants were collected, and metabolomics analysis was performed.

**(a)** Principal component analysis in which analysis was performed using the procomp package. The calculation was based on singular value decomposition and the Rscript chemometrics.R is required. The 2-D scores plot between selected PCs is shown and the explained variances are given in brackets.

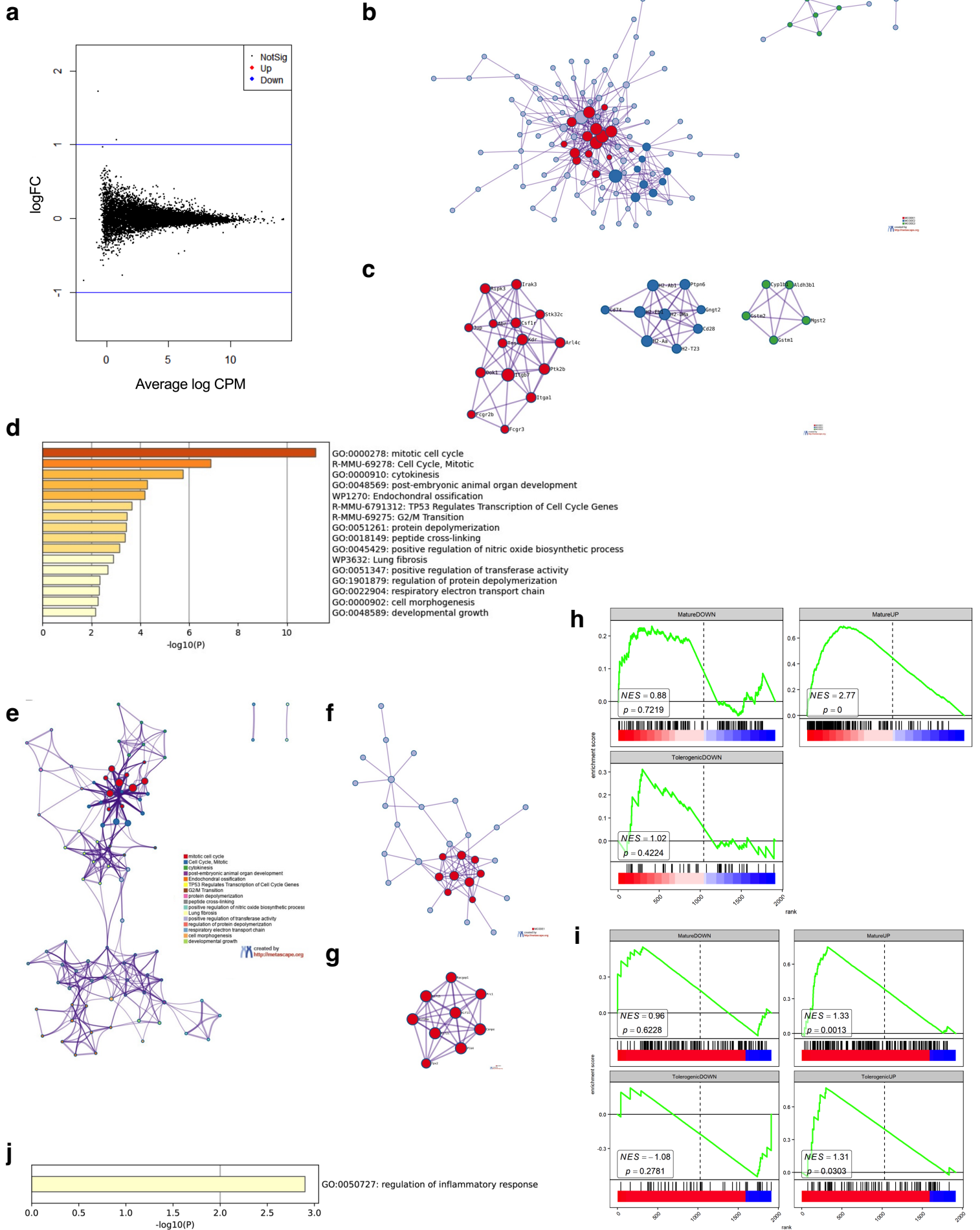
**(b)** Heatmap of all statistically enriched peak intensities. Red color represents metabolites with higher enrichment and blue for lower enrichment. The clustering was a result of distance measuring using Euclidean and clustering algorithm using ward.D.

**(c)** Heatmap of all statistically enriched peak intensities. Red color represents metabolites with higher enrichment and blue for lower enrichment. The clustering was a result of distance measuring using Euclidean and clustering algorithm using ward.D.

**(d)** Volcano plot. Important features selected by volcano plot with fold change threshold (x) 2 and t-tests threshold (y) 0.1. Both fold changes and p values were log transformed.



## Supplementary Figure 2



## Supplementary Figure 2. Helminth-derived nonpolar metabolites induce a transcriptional signature in BMDCs

BMDCs were incubated with HnpM for 4 and 20h. The cell pellets were collected, mRNA was extracted with Trizol, and sequenced by Illumina RNA-seq Libraries,  $n = 3-4$ . Reads alignment and gene ID mapping were performed using Bowtie2 and HTSeq, respectively, against the Genome Reference Consortium Mouse Build 38 patch release 6 (GRCm38.p6) database.

**(a)** Volcano plot for gene expression at 4h. Read counts were obtained with Get\_ReadCount.py and EdgeR with a likelihood test was performed to obtain differentially expressed genes. Genes were considered differentially expressed if the fold change  $\geq 2$  and  $p \leq 0.05$ .

**(b)** All protein-protein interactions among input genes were extracted from PPI data source and formed a PPI network.

**(c)** Network modules. Molecular complex detection (MCODE) algorithm was then applied to this network to identify neighborhoods where proteins are densely connected. MCODE1: GO:0046777|protein autophosphorylation|6.6;GO:0043408|regulation of MAPK cascadel-6.4;GO:0006468|protein phosphorylation|5.2; MCODE2: mmu05332|Graft-versus-host disease|13.3;mmu05330|Allograft rejection|13.3;GO:0019886|antigen processing and presentation of exogenous peptide antigen via MHC class III|13.2; MCODE3: mmu00980|Metabolism of xenobiotics by cytochrome P450|12.3;mmu00982|Drug metabolism - cytochrome P450|9.2;mmu05204|Chemical carcinogenesis - DNA adducts|8.9.

**(d)** Go annotation and pathway enrichment. All statistically enriched terms were identified based on the default choices under accumulative hypergeometric p-values and enrichment factors were calculated and used for filtering. Remaining significant terms were then hierarchically clustered into a tree based on Kappa-statistical similarities among their gene memberships. Then 0.3 kappa score was applied as the threshold to cast the tree into term clusters.

**(e)** Protein-protein interactions (PPIs) network among the input genes. A subset of representative terms from the full cluster was converted into a network layout and each term is represented by a circle node, where its size is proportional to the number of input genes fall under that term, and its color represent its cluster identity. Terms with a similarity score  $> 0.3$  are linked by an edge and the thickness of the edge represents the similarity score.

**(f)** All protein-protein interactions among input genes were extracted from PPI data source and formed a PPI network.

**(g)** Network modules. Molecular complex detection (MCODE) algorithm was then applied to this network to identify neighborhoods where proteins are densely connected. MCODE1: R-MMU-983189|Kinesin|13.8;R-MMU-6811434|COPI-dependent Golgi-to-ER retrograde traffic|12.3;GO:0002226|microtubule cytoskeleton organization|11.8.

**(h, i)** GSEA analysis, performed with phenoTest package in R. Gene sets were constructed with genes that are differentially expressed up and down. Gene sets were analyzed using published data on tolerogenic and inflammatory DCs (Supplementary File 5) Gene sets were represented using a barcode plot using the Limma package in R. **(h)** 20h and **(i)** 4h of incubation.

**(j)** Bar graph of enriched term across input gene lists. For each given gene list, pathway and process enrichment analysis has been carried out with the following ontology sources: GO Biological Processes, KEGG Pathway, Reactome Gene Sets, CORUM, TRRUST, PaGenBase and WikiPathways. All genes in the genome have been used as the enrichment background. Terms with a p-value  $< 0.01$ , a minimum count of 3, and an enrichment factor  $> 1.5$  was collected and grouped into clusters based on their membership similarities. More specifically, p-values are calculated based on the cumulative hypergeometric distribution<sup>2</sup>, and q-values are calculated using the Benjamini-Hochberg procedure to account for multiple testing. Kappa scores<sup>4</sup> are used as the similarity metric when performing hierarchical clustering on the enriched terms, and sub-trees with a similarity of  $> 0.3$  are considered a cluster. The most statistically significant term within a cluster is chosen to represent the cluster.