iScience, Volume *27*

Supplemental information

Recognition of yeast β-glucan particles triggers immunometabolic signaling required for trained immunity

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SUPPLEMENTAL INFORMATION LEGENDS

Figure S1. Macrophage responses to restimulation in β-glucan trained macrophages, *Related to Figure 1*. **A-C)** Training assays in hMDM trained with BGP or dWGP (10 μg/mL) or tolerized with LPS (10 ng/mL) and restimulated with LPS (10 ng/mL) for the indicated times (0-24h) and TNF production measured (A) or CXCL8 at 3h, TNF at 6h and IL-10 production 24h post-stimulation measured and expressed relative to untrained cells (B), or restimulated with LPS (100 ng/mL), pam3CSK4 (100 μg/mL) or HKCA (10^6cells/mL) and TNF production measured after 6h (C). **D-F)** Training assays in mBMDM trained with BGP or dWGP (100 μg/mL) or tolerized with LPS (10 ng/mL) and restimulated with LPS (10 ng/mL) for the indicated times (0-24h) and TNF production measured (D) or IL6, TNF and IL-10 production 24h post-stimulation measured and expressed relative to untrained cells (E), or restimulated with LPS (100 ng/mL), p(I:C) (5 μg/mL), Zymosan (ZYM) or pam3CSK4 (PAM, 100 μg/mL) and TNF production measured after 24h (F). **G)** hMDM derived from dWGP-trained (10 μg/mL) or LPS-tolerised (10 ng/mL) monocytes were treated with irradiated Mycobacterium tuberculosis H37Rv (iMtb, 500 μg/mL) for 24h and production of the indicated cytokines measured. **H-I)** hMDM derived from dWGP-trained monocytes (10 μg/mL) were infected with Mtb H37Ra (MOI 5 bacteria/cell) and intracellular bacterial survival measured by CFU analysis at the indicated times post-infection (H) or TNF production measured in culture supernatants (I). **J)** Training Assay in dWGP-trained mBMDM restimulated with LPS (10 ng/mL) or pam3CSK4 (100 μg/mL) for 6h. Intracellular cytokine staining for TNF was performed after brefeldin-A treatment (left panel) or ELISA on supernatants derived from cells without brefeldin-A. All data is mean ± sd for n=3 (A-B, D-F, G, J-K), n=4 (C) and n=5 (H-I) or n=2 (J) independent experiments. $^*P<0.05$ or indicated $P\geq0.05$ determined using multiple comparisons testing following one or two-way ANOVA.

Figure S2. Canonical Dectin-1 signalling does not distinguish β-glucan-induced trained immunity, *Related to Figure 2*. **B)** NFκB-linked SEAP activity in hDectin1a or hDectin-1b-HEK293 reporter cells incubated with the indicated β-glucans (1-100 μg/mL) or LPS (1-100 ng/mL) or left unstimulated (-) for 6h. **C)** Correlation of NFκB-linked SEAP in hDectin-1b-HEK293 reporter cells (Fig. 2A) with Monocyte Training in hMDM (Fig. 1A) for 100 μg/mL β-glucans treatment. **D)** Training Assay in hMDM incubated with solubilized WGP (sWGP, 100 μg/mL) for 1h prior to training with dWGP (1,10 & 100 ug/mL) for 24h. Mature macrophages were restimulated with LPS (10ng/mL, 6h) and TNF production measured. **E)** NFκB-linked SEAP activity in hDectin-1b-HEK293 reporter cells incubated with laminarin or sWGP at the indicated concentrations for 1h prior to treatment with BGP or dWGP at 100 μg/mL for 6h. **F)** hMDM Training Assay from monocytes pre-treated with laminarin or sWGP at the indicated concentrations for 1h prior to training with BGP or dWGP at 100 μg/mL for 24h. Mature macrophages were restimulated with LPS (10 ng/mL, 6h) and TNF production measured. **G)** NFκB-linked SEAP activity hDectin-1b-HEK293 reporter cells incubated with the indicated concentrations of piceatannol (PIC) for 1h prior to treatment with BGP or dWGP at 100 μg/mL for 6h. **H-I)** Production of the indicated cytokine in mBMDM after treatment with the indicated β-glucans (1-100 μg/mL) or LPS (1-100 ng/mL) or left unstimulated (-) for 24h and measured by ELISA. **J)** hMDM Training Assay using supernatants from WGP-trained monocytes (10 μg/mL, 24h) transferred to untrained monocytes (S/N Txt), left for 5-days. Mature macrophages were restimulated with LPS (10 ng/mL, 6h) and TNF production measured. **K)** NFκB-linked SEAP activity in RAW-Blue cells treated with β-glucans or left unstimulated (-) for 6h (E) **L)** TNF production from monocytes incubated with cytochalasin-D (Cyt-D, 10 μM, 1h) and subsequently treated with the indicated b-glucan (10 μg/mL) for 24h. **M)** Training Assay in dWGP-trained mBMDM pre-treated with Cyt-D (10 μM, 1h) and TNF (6h) and IL10 (24h) production measured after LPS restimulation. All data is mean ± sd for n=2 (E-G, L-M), n=3 (A-B, D, H-K) and n=4 (C) independent experiments. $^{*/\#}P<0.05$, ns or indicated $P\geq0.05$

determined using multiple comparisons testing following one or two-way ANOVA or Students t-test (B).

Figure S3. β-glucans drives long-term metabolic reprogramming for training, *Related to Figure 3*. **A)** Extracellular Lactate production in monocytes treated with the indicated β-glucans (1-100 μg/mL) or LPS (1-100 ng/mL) or left unstimulated (-) for 24-72h. **B)** dWGP training assay in mBMDM pre-treated with 2 deoxyglucose (2DG, 25 μM, 1h). IL-6 and IL-10 production were measured 24h after restimulation with LPS (10 ng/mL). **C-E)** Extracellular flux analysis for for mBMDM treated with the indicated β-glucans (100 μg/mL) or LPS (100 ng/mL) or left unstimulated (-) for 24-72h. Glycolytic capacity or maximal respiration rate was calculated and shown in C). Glycolytic traces based on extracellular acidification rates (ECAR) after inhibitor addition (OM; oligomycin and 2DG) shown in D). Respiration traces based on oxygen consumption rates (OCR) after inhibitor addition (OM, FCCP and Rot & AA; rotenone + antimycin-A) shown in E). **F-G)** Flow cytometry gating strategy for mitochondrial activity analysis on mBMDM (top plots) & representative plots for control stimuli (bottom, left -> right; untreated, LPS-stimulated, OM-treatment & FCCP-treatment) with fold changes in mitotracker green (MTG, mitochondrial mass) and tetramethylrhodamine methyl ester (TMRM, mitochondrial activity) signal shown in G). Data is mean ± sd for n=2 (A), n=3 (G) n=4 (B) or n=6 (C-E) independent experiments. $^{*/\#}$P<0.05 determined using multiple comparisons testing following one or two-way ANOVA.

Figure S4. mTOR independent remodelling of TCA during dWGP training, *Related to Figure 4*. **A)** Representative gating strategy used for intracellular staining of monocytes for Phospho-S6 (p-S6) activity. **B)** Representative flow cytometry histogram for p-S6 positivity in monocytes treated with dWGP or sWGP (10 μg/mL) for 2h or left untreated (-). **C)** p-S6 activity in human monocytes after stimulation with the indicated β-glucans (10 μg/mL) or LPS (10 ng/mL) or left unstimulated (-) for 1h. **D)** p-S6 staining in monocytes pre-treated with rapamycin (rapa, 10

nM, 1h) prior to stimulation with dWGP or sWGP (10 μg/mL) or LPS (100 ng/mL) for 2h. **E)** p-S6 staining in monocytes stimulated with dWGP (10 μg/mL) for the indicated times. **F)** dWGP Training Assay in mBMDM pre-treated with Rapa (10 nM). TNF production was measured between 3-24h post-LPS restimulation (10 ng/mL). Data is mean ± sd for n=3 (C-D,F) independent experiments or representative experiments. $^{*}P<0.05$ determined using multiple comparisons testing following one (C-D) or two-way (F) ANOVA.

Figure S5. Phagocytosis of particulate β-glucans, *Related to Figure 5*. **A)** Representative histogram plot of monocytes treated with dWGP (10 μg/mL, 2h) or left untreated (-) and subjected to staining for surface Dectin-1 expression detected by flow cytometry. **B)** Monocytes pre-treated with cytochalasin-D (Cyt-D, 10 μM) or vehicle treated for 1h prior stimulation with dWGP or BGP (100 μg/mL) for 15 min, were immediately fixed and stained for surface Dectin-1 expression measured by flow cytometry. **C)** Monocytes were stimulated with dWGP or BGP (100 μg/mL) for the indicated times and surface Dectin-1 expression detected by flow cytometry. **D)** hMDM Training Assay in monocytes pre-treated with bafilomycin-A1 (Baf-A1, 10 μM) 1h prior to treatment with dWGP (10 μg/mL) or LPS (100 ng/mL). Mature macrophages were restimulated with LPS (10 ng/mL, 6h) and TNF production measured. **E)** TNF production in monocytes pre-treated with PIC (between 4, 10 and 30 μM) 15min prior to stimulation with dWGP (10 μg/mL) for 24h. Data is mean ± sd for n=2 (D) or n=3 (B,E) independent experiments or representative experiments. $^{*}P<0.05$ determined using multiple comparisons testing following two-way ANOVA.

Figure S6. Phagocytosis of intact and pure yeast β-glucan particles drives trained immunity, *Related to Figure 6*. **A)** Schematic illustrating protocol to prepare fractions of solubilized WGP. **B)** NFκB-linked SEAP activity in hDectin1-HEK293 reporter cells incubated with dWGP, sWGP or defined fractions of sWGP F1-F4 (MW ranges indicated below, kDa) at 1, 10 and 100 μg/mL for 6h. **C)** Schematic illustrating protocol to conjugate solubilized WGP to polystyrene particles

(PS). **D)** Sugar content of sWGP preparations after filtration following incubated with 2 μg aminated polystyrene particles (AM-PS) in the presence or absence of conjugating agent 1,1' Carbonyldi-imidazole (CDI). **E)** Lactate production in human monocytes after treatment with 200 μg/mL aminated polystyrene particles (PS) alone or conjugated with sWGP (PS-sWGP), the equivalent amount of sWGP roughly corresponding to 200 μg/ml of sWGP-PS (1 μg/ml) or dWGP (100 μg/ml) or left untreated (-) for 72h. **F)** Schematic illustrating protocol to prepare single particle "dispersible" WGP (dWGP) from aggregated "unsonicated" WGP (uWGP) via sonication. Light micrographs represent monocyte cultures with each preparation. **G)** NFκB-linked SEAP activity inhDectin1-HEK293 reporter cells treated with heat-killed *Candida albicans* (HKCA) at 10^4, 10^5 & 10^6 cells/mL, Zymosan (ZYM), dispersible WGP (dWGP), unsonicated WGP (uWGP) or solubilized WGP β-glucan (sWGP) all at 1,10 & 100 μg/mL, or LPS (1,10 and 100 ng/mL) for 6h. **H)** Extracellular Lactate production in monocytes treated with the indicated WGPs (1-10 μg/mL) or left unstimulated (-) sampled between 24-72h post-treatment. **I)** NFκB-linked SEAP activity in hDectin1a, hTLR2 or hTLR4-HEK293 reporter cells treated with the indicated β-glucans (1-10 μg/mL), LPS (10 ng/mL), PAM3CSK4 (PAM, 100 μg/mL) or left unstimulated (-) for 6h. **J-K)** Training Assay in hMDM (I) or mBMDM (J) trained with Zymosan-depleted (ZYM-d, 10/100 μg/mL). Mature macrophages were restimulated with LPS (10 ng/mL) and TNF production measured relative to untrained cells (-) at the indicated times. Data is mean ± sd for n=5 (B) or n=3 (D-E, G-K) independent experiments. $^{*/\#}P < 0.05$, ns $P \geq 0.05$ determined using multiple comparisons testing following two-way ANOVA.

Figure S7. dWGP delivery drives myeloid bone marrow reprogramming, *Related to Figure 7*. C57/BL6-J mice were injected intraperitoneally with dWGP (0.2 & 0.4 mg/mouse), BGP (1 mg/mouse) or vehicle PBS (A-B) or 2mg WGP IP or via oral gavage (C). After 7-days, bone-marrow (BM) was isolated, quantified, stained and analysed by flow cytometry for the indicated populations; c-Lin-1^-, c-Kit^+, $Sca1^+$ (LKS), Long-Term Hematopoietic Stem Cells (LT-HSC), Short-Term HSCs (ST-HSC), Multipotent Progenitors 1-3 (MPP) or CD150-/CD48- cells. **A)** %

of total LKS cells in bone-marrow cells. **B)** % of LKS subsets in total bone-marrow cells (BM). **C)** TNF production in total splenocytes prepared from mice treated with 0.2 mg dWGP as indicated and stimulated with LPS (10 ng/mL) for 6h. Data is mean ± sd for n=4 (A-C) mice per group. *P < 0.05 determined using multiple comparisons testing following one (A) or two-way (B-C) ANOVA.

Figure S8. dWGP-containing diets reprogramme bone-marrow macrophage responses, *Related to Figure 8*. **A-C)** Mature C57/BL6-J mice were fed control (0 WGP diet) for 2-weeks prior to initiation of feeding diets supplemented with increasing concentrations of dWGP (0, 0.003%, 0.025%, 0.050% per kg chow). Mice were sacrificed 1, 3 or 4-weeks post-dWGP feeding and bone-marrow (BM) examined by flow cytometry. Data is the is relative percentage for the indicated subset in the total LKS population and is mean± sd for n=6-8 mice per group. **C)** Volcano plot of differentially expressed genes in resting BMDM derived from mice fed a control (0 WGP) diet or mice fed 0.025% dWGP for 1-week (n=2 mice per group). *P<0.05 determined using multiple comparisons testing following Mixed Effect Model testing (A-C) or multiple Students t-tests (D). Log_{10} P value thresholds for RNA-sequencing are indicated on plots.

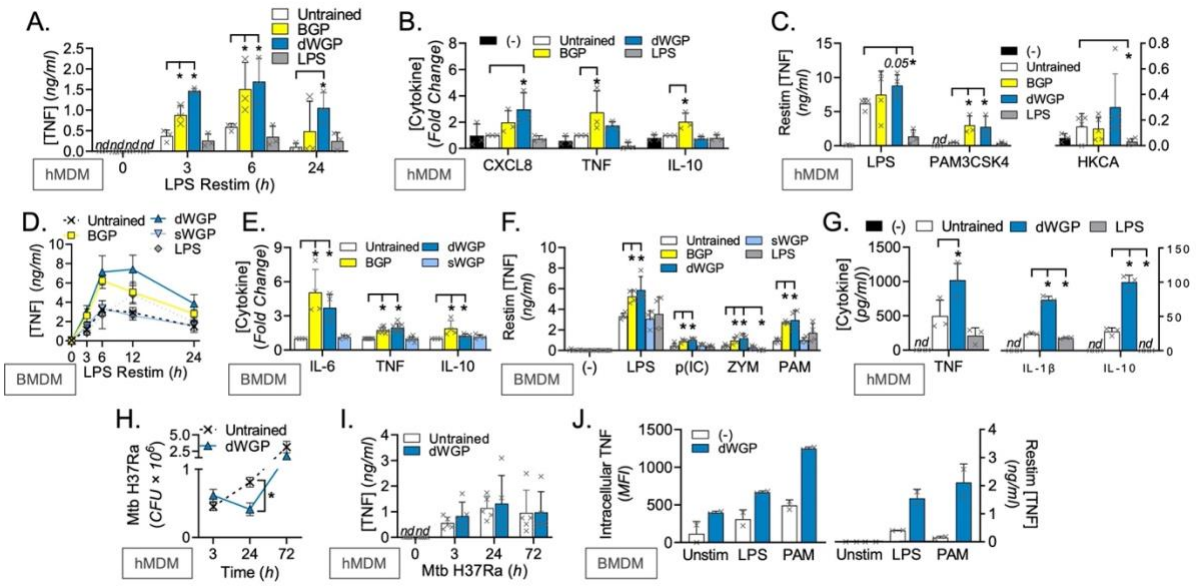
A.
Untrained
BGP
dWGP
LPS
[TNF] (ng/ml)
hMDM
LPS Restim (h)
B.
(-)
Untrained
BGP
dWGP
LPS
[Cytokine] (Fold Change)
CXCL8
TNF
IL-10
hMDM
C.
Restim [TNF] (ng/ml)
0.05
LPS
PAM3CSK4
HKCA
hMDM
D.
Untrained
BGP
dWGP
sWGP
LPS
[TNF] (ng/ml)
LPS Restim (h)
BMDM
E.
Untrained
BGP
dWGP
sWGP
[Cytokine] (Fold Change)
IL-6
TNF
IL-10
BMDM
F.
Untrained
BGP
dWGP
sWGP
LPS
Restim [TNF] (ng/ml)
(-)
LPS
p(IC)
ZYM
PAM
BMDM
G.
(-)
Untrained
dWGP
LPS
[Cytokine] (pg/ml))
TNF
IL-1β
IL-10
hMDM
H.
Untrained
dWGP
Mtb H37Ra (CFU × 10^6)
Time (h)
hMDM
I.
Untrained
dWGP
[TNF] (ng/ml)
Mtb H37Ra (h)
hMDM
J.
(-)
dWGP
Intracellular TNF (MFI)
Restim [TNF] (ng/ml)
Unstim
LPS
PAM
BMDM

Figure S1

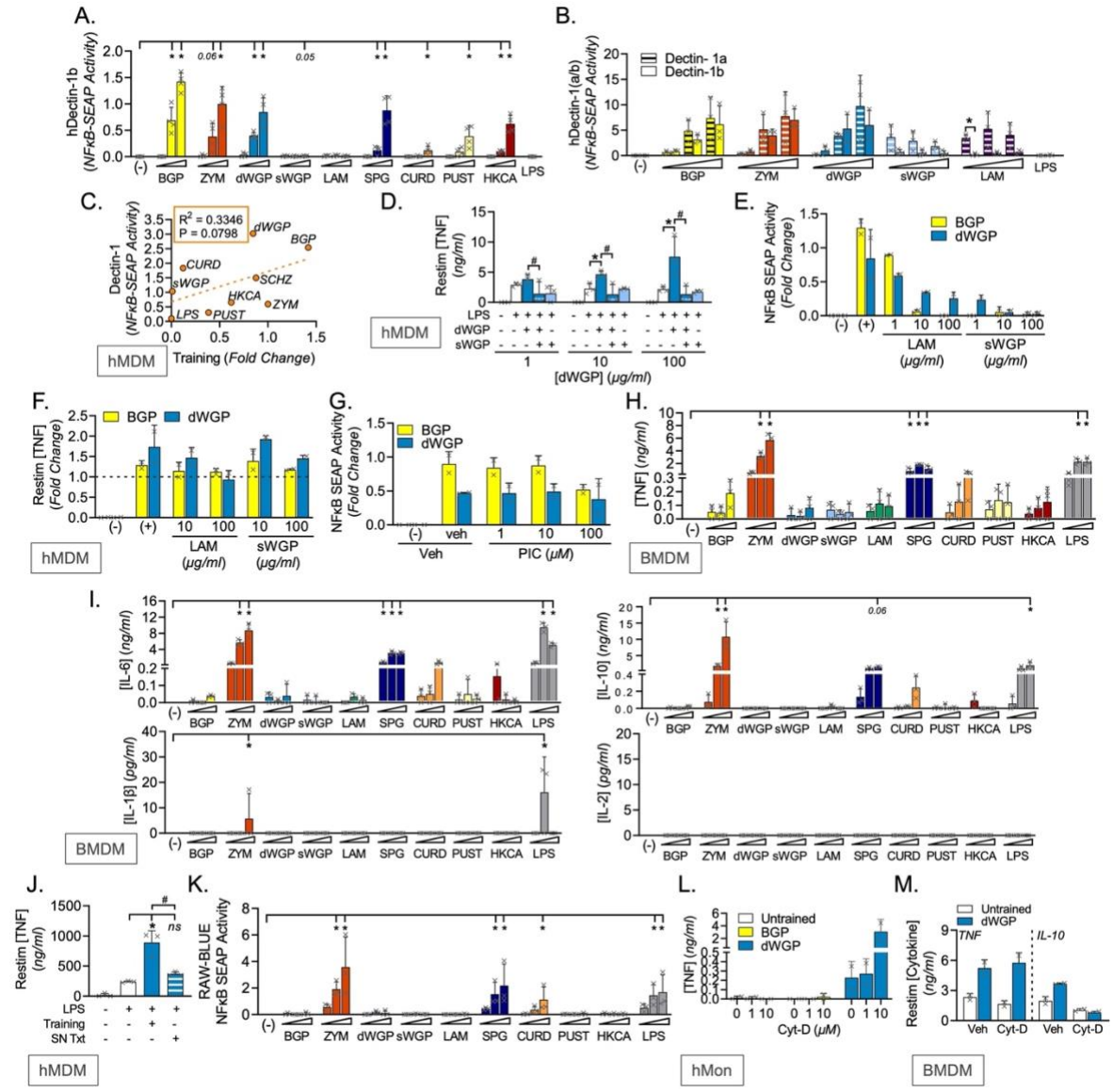
A.
hDectin-1b (NFκB-SEAP Activity)
(-) BGP ZYM dWGP sWGP LAM SPG CURD PUST HKCA LPS
B.
hDectin-1(a/b) (NFκB-SEAP Activity)
Dectin- 1a
Dectin-1b
C.
R² = 0.3346
P = 0.0798
Dectin-1 (NFκB-SEAP Activity)
Training (Fold Change)
hMDM
D.
Restim [TNF] (ng/ml)
LPS
dWGP
sWGP
[dWGP] (μg/ml)
hMDM
E.
NFκB SEAP Activity (Fold Change)
BGP
dWGP
LAM (μg/ml)
sWGP (μg/ml)
F.
Restim [TNF] (Fold Change)
hMDM
G.
NFκB SEAP Activity (Fold Change)
Veh
PIC (μM)
H.
[TNF] (ng/ml)
BMDM
I.
[IL-6] (ng/ml)
[IL-10] (ng/ml)
[IL-1β] (pg/ml)
[IL-2] (pg/ml)
BMDM
J.
Restim [TNF] (ng/ml)
LPS
Training
SN Txt
hMDM
K.
RAW-BLUE NFκB SEAP Activity
L.
[TNF] (ng/ml)
Untrained
BGP
dWGP
Cyt-D (μM)
hMon
M.
Restim [Cytokine] (ng/ml)
TNF
IL-10
Veh
Cyt-D
BMDM

Figure S2

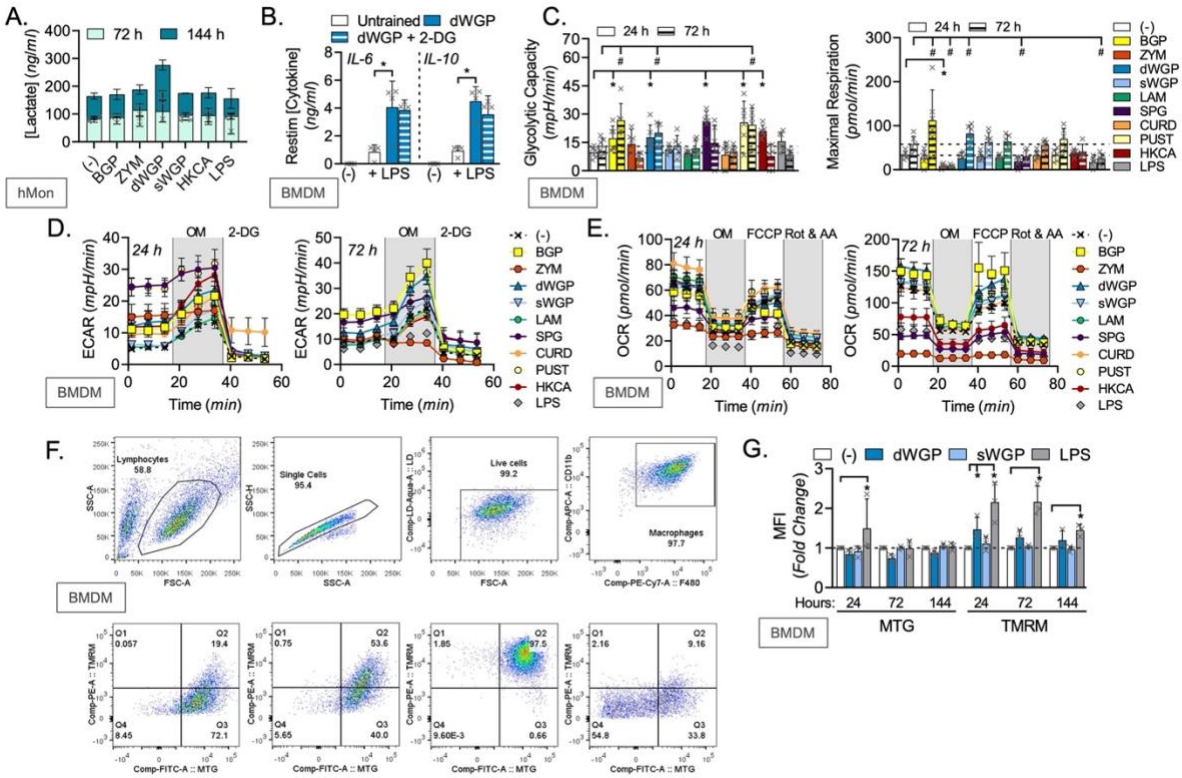
A.
[Lactate] (ng/ml)
72 h
144 h
(-)
BGP
ZYM
dWGP
sWGP
HKCA
LPS
hMon
B.
Untrained
dWGP
dWGP + 2-DG
Restim [Cytokine] (ng/ml)
IL-6
IL-10
(-)
+ LPS
BMDM
C.
24 h
72 h
Glycolytic Capacity (mpH/min)
Maximal Respiration (pmol/min)
BMDM
D.
ECAR (mpH/min)
OM
2-DG
Time (min)
E.
OCR (pmol/min)
FCCP
Rot & AA
F.
Lymphocytes
58.8
Single Cells
95.4
Live cells
99.2
Macrophages
97.7
G.
MFI (Fold Change)
Hours:
MTG
TMRM

Figure S3

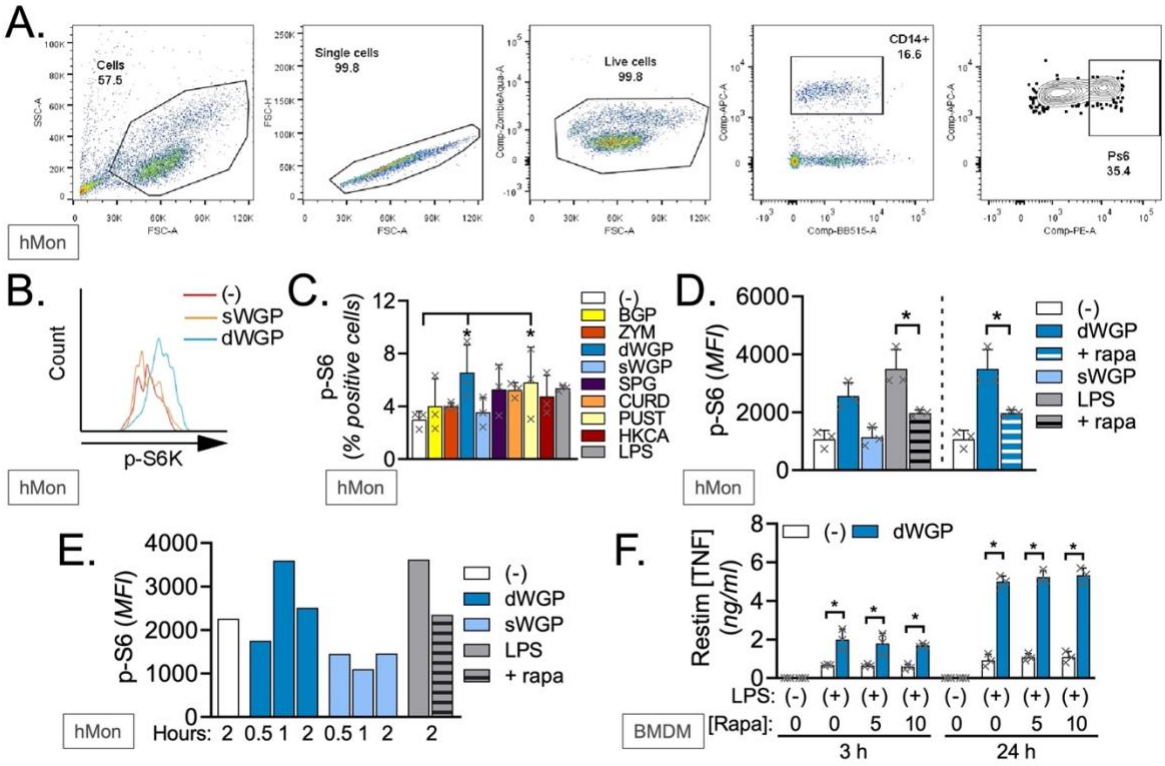
A.
Cells
57.5
Single cells
99.8
Live cells
99.8
CD14+
16.6
Ps6
35.4
hMon
B.
(-)
sWGP
dWGP
Count
p-S6K
hMon
C.
p-S6
(% positive cells)
(-)
BGP
ZYM
dWGP
sWGP
SPG
CURD
PUST
HKCA
LPS
hMon
D.
p-S6 (MFI)
(-)
dWGP
+ rapa
sWGP
LPS
+ rapa
hMon
E.
p-S6 (MFI)
(-)
dWGP
sWGP
LPS
+ rapa
Hours: 2 0.5 1 2 0.5 1 2 2
hMon
F.
Restim [TNF]
(ng/ml)
(-) dWGP
LPS: (-) (+) (+) (+) (-) (+) (+) (+)
[Rapa]: 0 0 5 10 0 0 5 10
3 h
24 h
BMDM

Figure S4

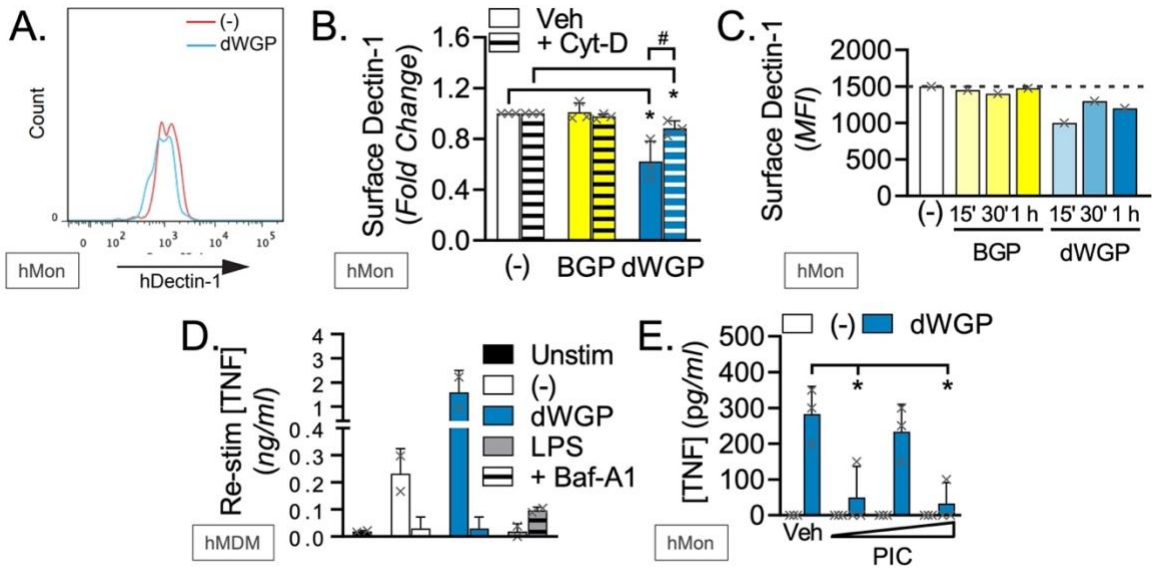
A.
(-)
dWGP
Count
hMon
hDectin-1
B.
Veh
+ Cyt-D
Surface Dectin-1
(Fold Change)
hMon
(-) BGP dWGP
C.
Surface Dectin-1
(MFI)
(-) 15' 30' 1 h 15' 30' 1 h
BGP
dWGP
hMon
D.
Re-stim [TNF]
(ng/ml)
Unstim
(-)
dWGP
LPS
+ Baf-A1
hMDM
E.
(-) dWGP
[TNF] (pg/ml)
Veh
PIC
hMon

Figure S5

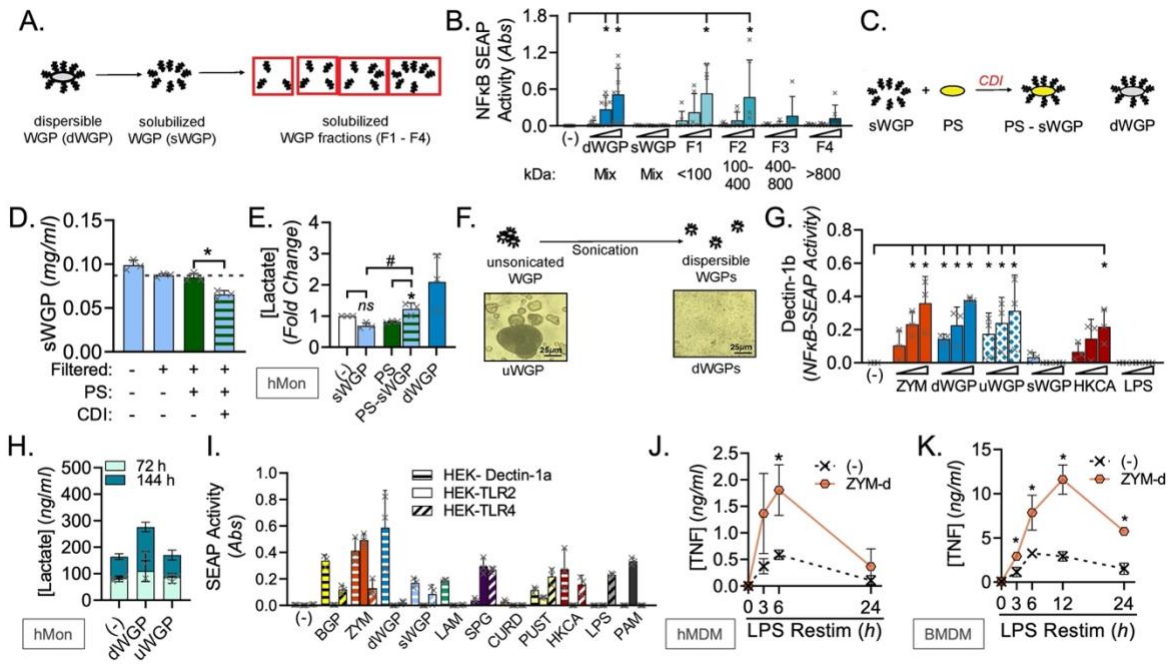
A.
dispersible WGP (dWGP)
solubilized WGP (sWGP)
solubilized WGP fractions (F1 - F4)
B.
NFκB SEAP Activity (Abs)
(-) dWGP sWGP F1 F2 F3 F4
kDa: Mix Mix <100 100-400 400-800 >800
C.
sWGP
PS
CDI
PS - sWGP
dWGP
D.
sWGP (mg/ml)
Filtered:
PS:
CDI:
E.
[Lactate] (Fold Change)
ns
hMon
(-) sWGP PS PS-sWGP dWGP
F.
unsonicated WGP
Sonication
dispersible WGPs
uWGP
dWGPs
G.
Dectin-1b (NFκB-SEAP Activity)
(-) ZYM dWGP uWGP sWGP HKCA LPS
H.
[Lactate] (ng/ml)
72 h
144 h
hMon
(-) dWGP uWGP
I.
SEAP Activity (Abs)
HEK- Dectin-1a
HEK-TLR2
HEK-TLR4
(-) BGP ZYM dWGP sWGP LAM SPG CURD PUST HKCA LPS PAM
J.
[TNF] (ng/ml)
(-)
ZYM-d
hMDM
LPS Restim (h)
K.
[TNF] (ng/ml)
(-)
ZYM-d
BMDM
LPS Restim (h)

Figure S6

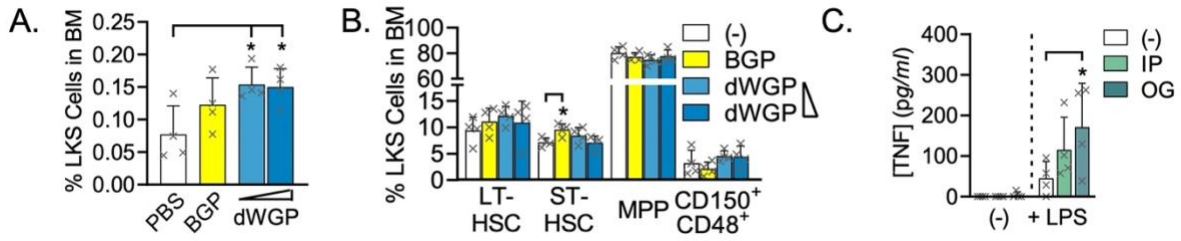
A.
% LKS Cells in BM
0.25
0.20
0.15
0.10
0.05
0.00
PBS
BGP
dWGP
B.
% LKS Cells in BM
100
80
60
15
10
5
0
(-)
BGP
dWGP
dWGP
LT-HSC
ST-HSC
MPP
CD150+ CD48+
C.
[TNF] (pg/ml)
400
300
200
100
0
(-)
IP
OG
(-)
+ LPS

Figure S7

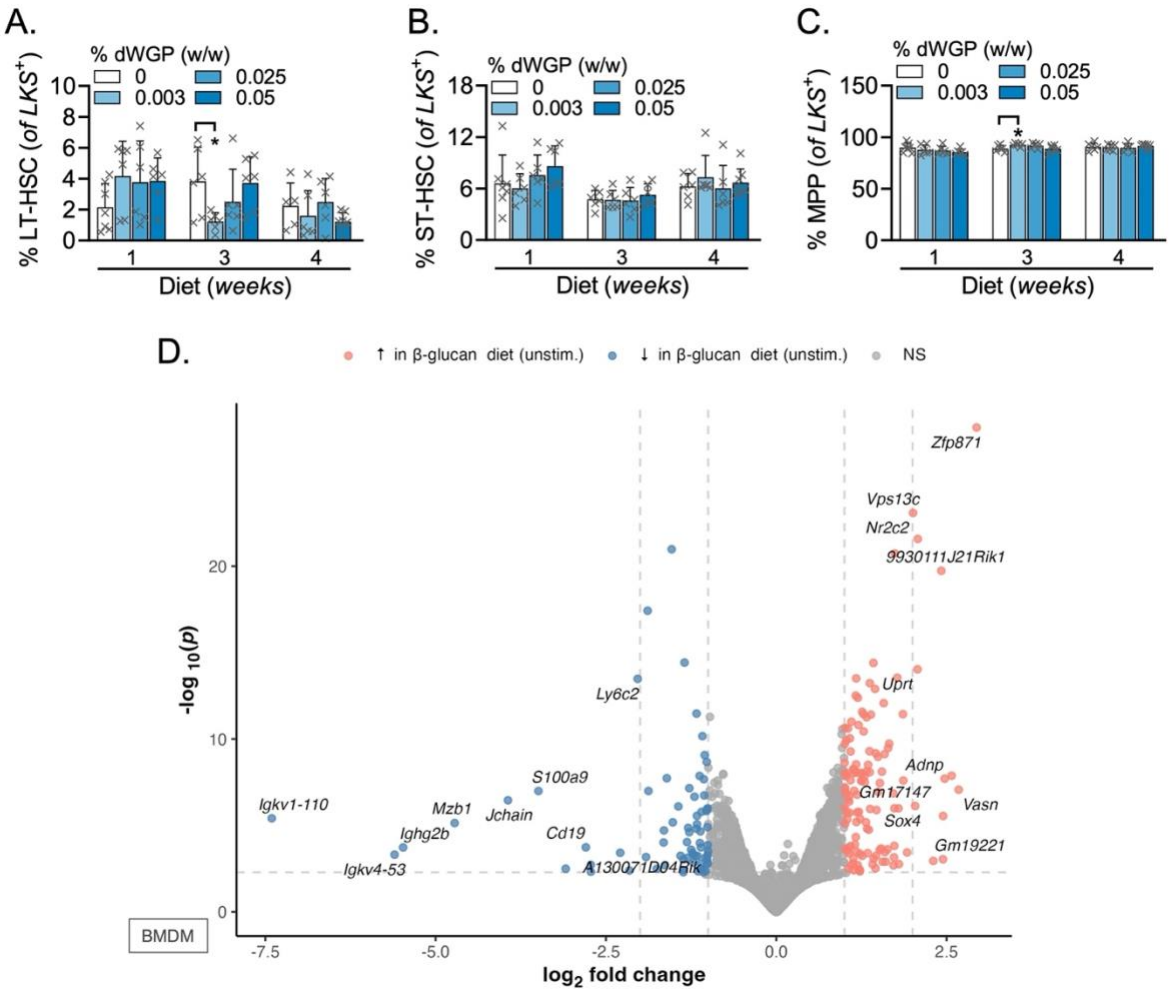
A.
% dWGP (w/w)
0
0.003
0.025
0.05
% LT-HSC (of LKS+)
Diet (weeks)
B.
% ST-HSC (of LKS+)
C.
% MPP (of LKS+)
D.
↑ in β-glucan diet (unstim.)
↓ in β-glucan diet (unstim.)
NS
Zfp871
Vps13c
Nr2c2
9930111J21Rik1
Uprt
Ly6c2
Adnp
Gm17147
Vasn
Sox4
Gm19221
S100a9
Jchain
Mzb1
Cd19
Ighg2b
Igkv1-110
Igkv4-53
A130071D04Rik
BMDM
-log 10(p)
log2 fold change

Figure S8