

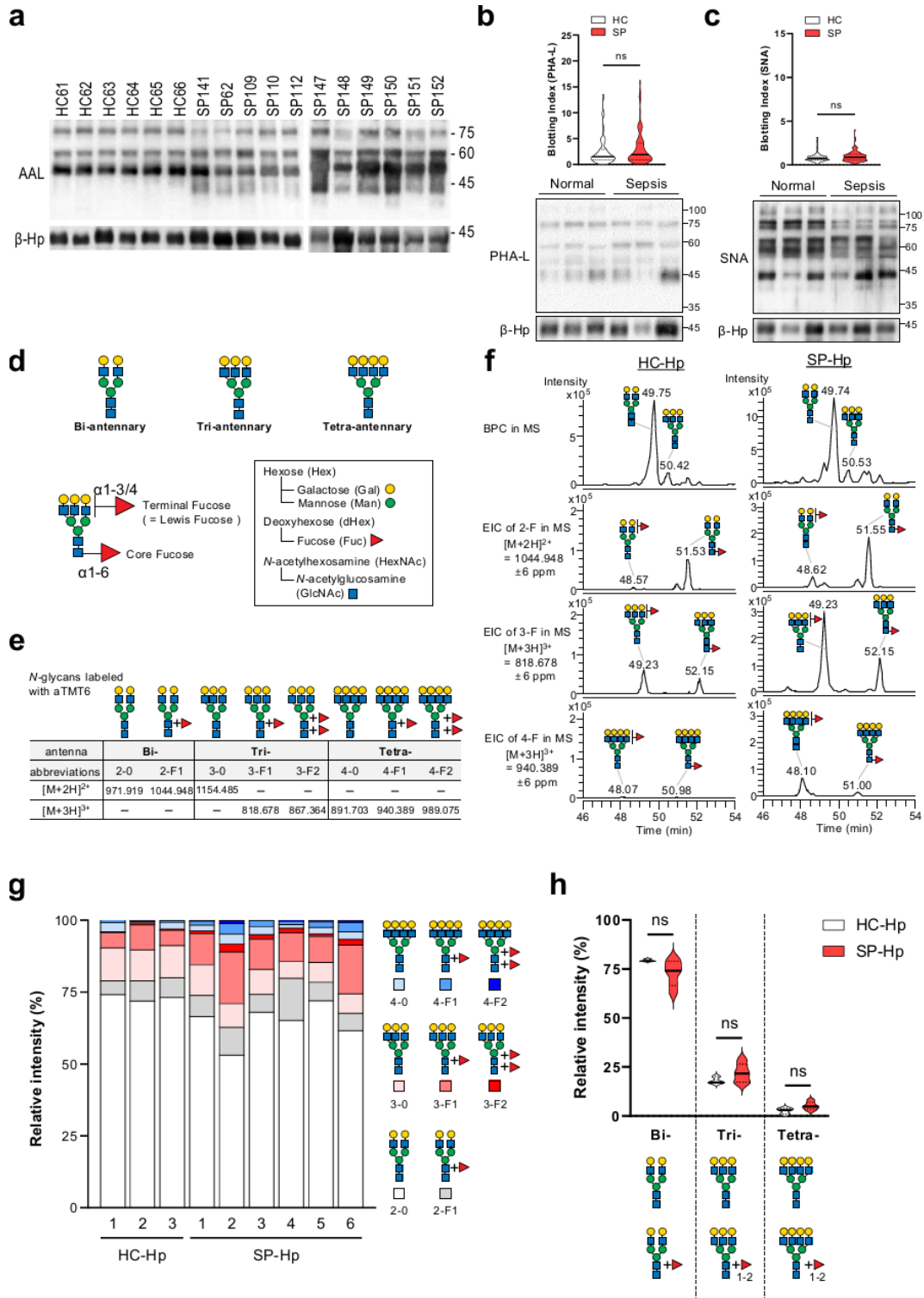
Supplementary Information

Fucosylated haptoglobin drives inflammation via Mincle in sepsis: an observational study

Taylor Roh^{1,2,3#}, Sungeun Ju^{4#}, So Young Park^{5#*}, Yeonghwan Ahn⁴, Jiyun Chung⁴, Miyako Nakano⁶, Gyoungah Ryu^{1,2}, Young Jae Kim^{1,2,3}, Geumseo Kim^{1,2,3}, Hyewon Choi⁴, Sung-Gwon Lee⁷, In Soo Kim^{2,8}, Song-I Lee⁹, Chaeuk Chung¹⁰, Takashi Shimizu^{11,12}, Eiji Miyoshi¹³, Sung-Soo Jung¹⁴, Chungoo Park¹⁵, Sho Yamasaki^{11,12,16}, Seung-Yeol Park^{4*} and Eun-Kyeong Jo^{1,2*}

¹Department of Microbiology, College of Medicine, Chungnam National University, Daejeon, Republic of Korea. ²Department of Medical Science, College of Medicine, Chungnam National University, Daejeon, Republic of Korea. ³Brain Korea 21 FOUR Project for Medical Science, College of Medicine, Chungnam National University, Daejeon, Republic of Korea. ⁴Department of Life Sciences, Pohang University of Science and Technology (POSTECH), Pohang, Republic of Korea. ⁵Division of Pulmonary, Allergy and Critical Care Medicine, Kangdong Sacred Heart Hospital, Seoul, Republic of Korea. ⁶Graduate School of Integrated Sciences for Life, Hiroshima University, Higashi-Hiroshima, Hiroshima, Japan. ⁷Section of Genetics and Physiology, Laboratory of Molecular and Cellular Biology, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institutes of Health (NIH), Bethesda, MD, USA. ⁸Department of Pharmacology, College of Medicine, Chungnam National University, Daejeon, Republic of Korea. ⁹Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, Chungnam National University Hospital, Daejeon, Republic of Korea. ¹⁰Division of Pulmonology and Critical Care Medicine, Department of Internal Medicine, College of Medicine, Chungnam National University, Daejeon, Republic of Korea. ¹¹Department of Molecular Immunology, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, Japan. ¹²Laboratory of Molecular Immunology, Immunology Frontier Research Center (IFReC), Osaka University, Suita, Osaka, Japan. ¹³Department of Molecular Biochemistry and Clinical Investigation, Osaka University Graduate School of Medicine, Osaka University, Suita, Osaka, Japan. ¹⁴Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, College of Medicine, Chungnam National University, Daejeon, Republic of Korea. ¹⁵School of Biological Sciences and Technology, Chonnam National University, Gwangju, Republic of Korea. ¹⁶Center for Infectious Disease Education and Research (CiDER), Osaka University, Suita, Osaka, Japan. #These authors contributed equally to this work *Corresponding authors e-mail: sy.park12@gmail.com; seungpark@postech.ac.kr; hayoungj@cnu.ac.kr.

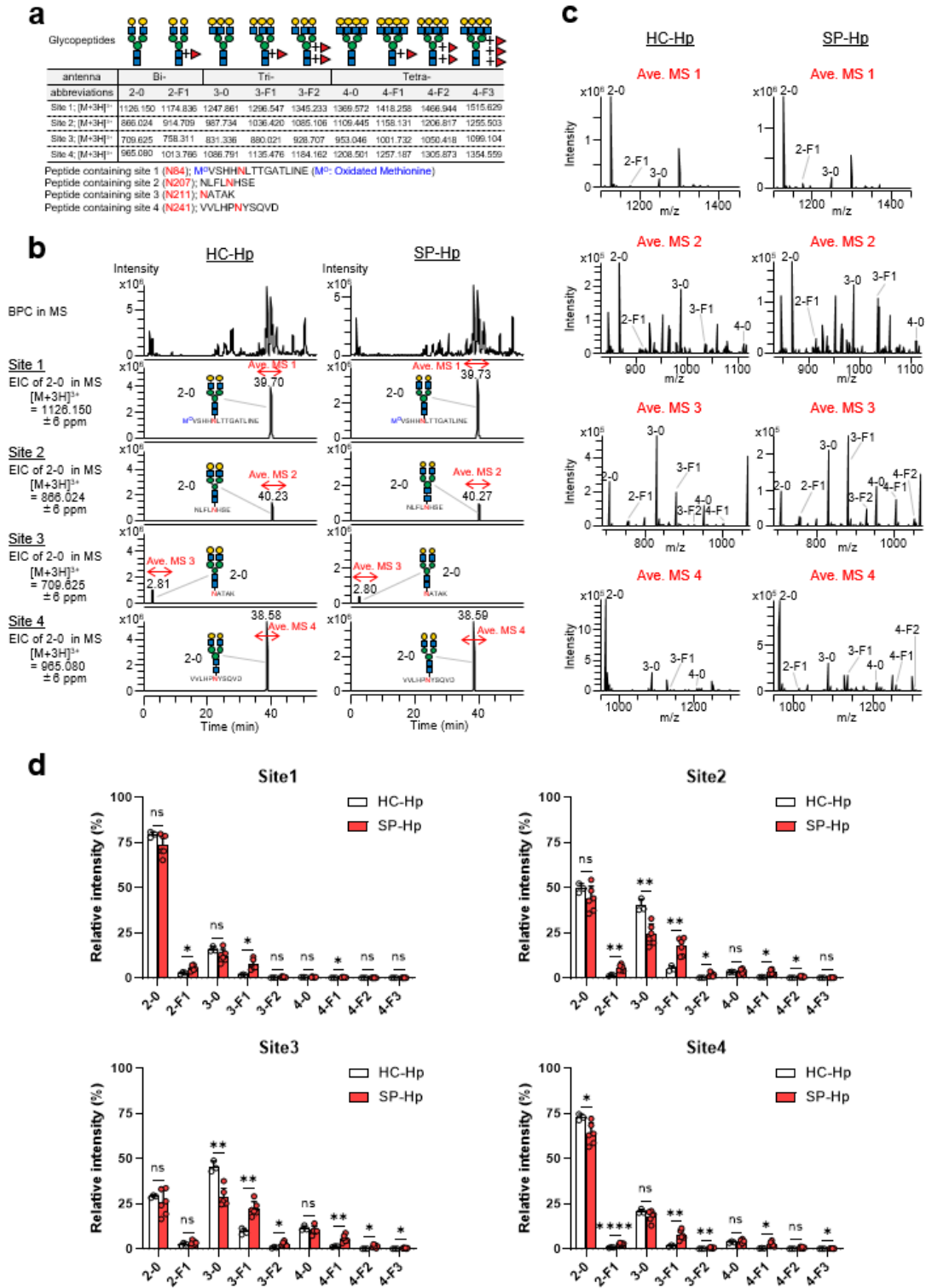
Supplementary Fig. 1



Supplementary Fig. 1. LC (GC)-ESI MS analysis for desialo-glycans of Hp. **a**, AAL

blotting of the sera from sepsis patients (n = 11) and healthy donors (n = 6), followed by reblotting with anti-Hp antibody. The membrane was reblotting for β -Hp using anti-Hp antibody. **b**, PHA-L blotting of the sera from sepsis patients and healthy donors, followed by reblotting with anti-Hp antibody. **c**, SNA blotting in the sera of sepsis patients and healthy donors, followed by reblotting with anti-Hp antibody. **d**, Antennary branched structures of N-glycans and the types of linkage of fucose. **e**, Structures, abbreviations, theoretical mass for desialo-glycans labeled with aTMT detected in this study. N-glycan labeled with aTMT are calculated as doubly or triply charged ions. **f**, Base peak chromatogram (BPC) and eight extracted ion chromatogram (EIC) of haptoglobin samples (these are representative data for HC-Hp (HC2) and SP-Hp (SP32)). The EICs at m/z 971.919, 1154.485, 891.703, 1044.948, 818.678, 940.389, 867.364 and 989.075 suggest non-, mono- and di-fucosylated bi-, tri- and tetra-antennary N-glycans labeled with aTMT obtained from analyses of haptoglobin samples. **g**, 100% stacked bar graph indicates relative abundances (%) of N-glycans labeled with aTMT after setting the total peak intensities of all detected N-glycans in each EIC to 100%. **h**, Relative abundances (%) of bi-, tri- and tetra-antennary N-glycans labeled with aTMT obtained from analyses of haptoglobin samples.

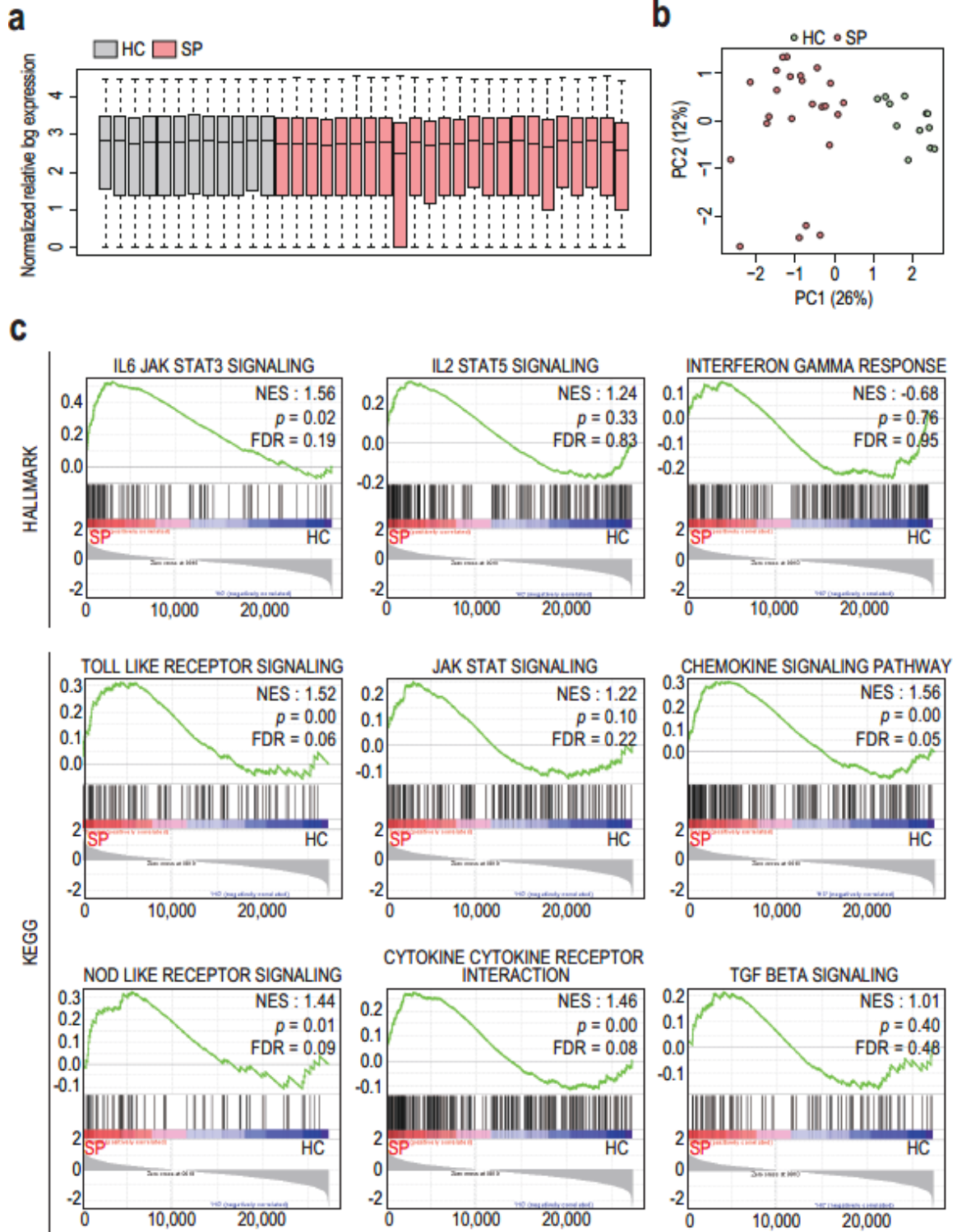
Supplementary Fig. 2



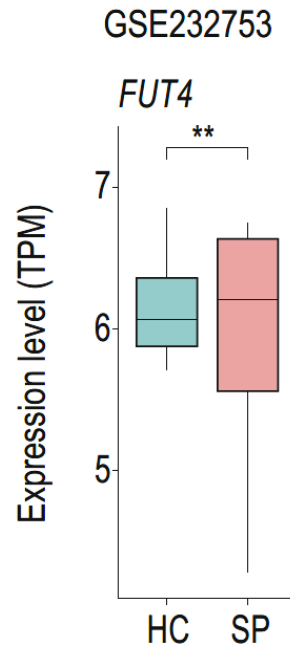
Supplementary Fig. 2. LC (ODS)-ESI MS analysis for desialo-glycopeptides of Hp. **a**,

Structures, abbreviations, theoretical mass for desialo-glycopeptides detected in this study. Desialo-glycopeptides which include sites 1-4 are calculated as triply charged ions. **b**, Base peak chromatogram (BPC) and four extracted ion chromatogram (EIC) of Hp samples (these are representative data for HC-Hp (HC2) and SP-Hp (SP32)). Four desialo-glycopeptide carrying non-fucosylated bi-antennary N-glycan (2-0) peaks were observed at 39.7 min (site 1: Asn184), 40.2 min (site 2: Asn207), 2.8 min (site 3: Asn211) and 38.6 min (site 4: Asn241). **c**, Average mass spectra (Ave. MS) around 39.7 min for site 1, 40.2 min for site 2, 2.8 min for site 3 and 38.6 min for site 4 of haptoglobin samples (these are representative data for HC-Hp (HC2) and SP-Hp (SP32)). **d**, Relative abundances (%) of N-glycans on glycopeptide at each site after setting the total peak intensities of all detected glycopeptides in each EIC to 100%. The annotations with two asterisks indicate $p < 0.05$, and those with one asterisk indicate $p < 0.01$ in this figure. The annotations with ns indicate not significant difference.

Supplementary Fig. 3

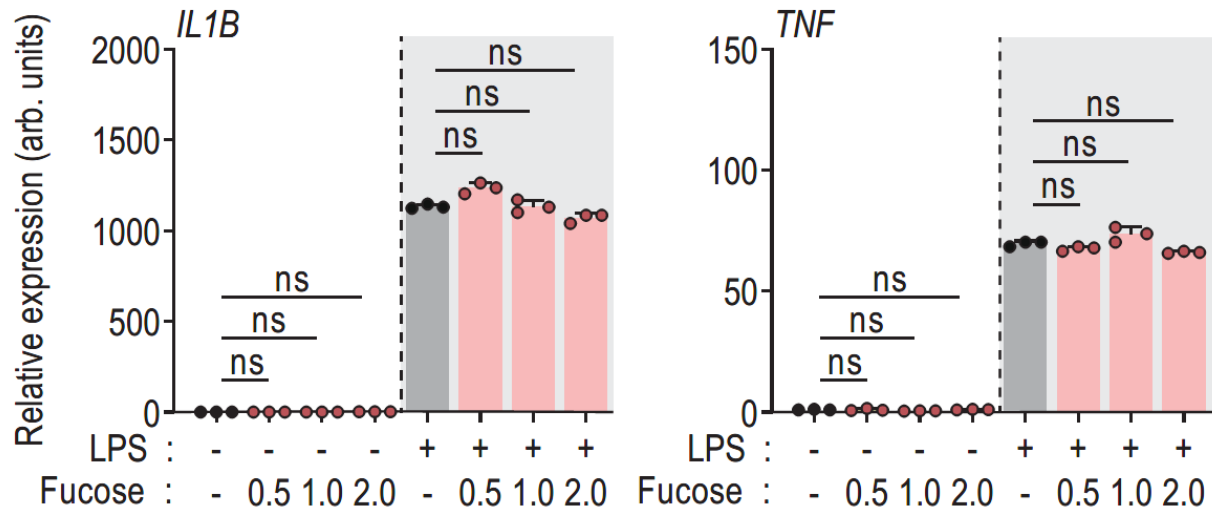


Supplementary Fig. 3. Bulk RNA-seq analysis reveals pro-inflammatory profiling in PBMCs of SPs (n = 24) compared to HCs (n = 12). **a**, Box plot showing normalized gene expressions for each sample. **b**, PCA plot depicting samples from SP and HC clustered separately. **c**, GSEA plot (HALLMARK and KEGG pathways) showing the inflammatory response-related pathways comparing PBMCs from SP and HC (BH-adjusted *p* values shown were computed from the GSEA test). FDR, False discovery rate; NES, normalized enrichment score.

Supplementary Fig. 4

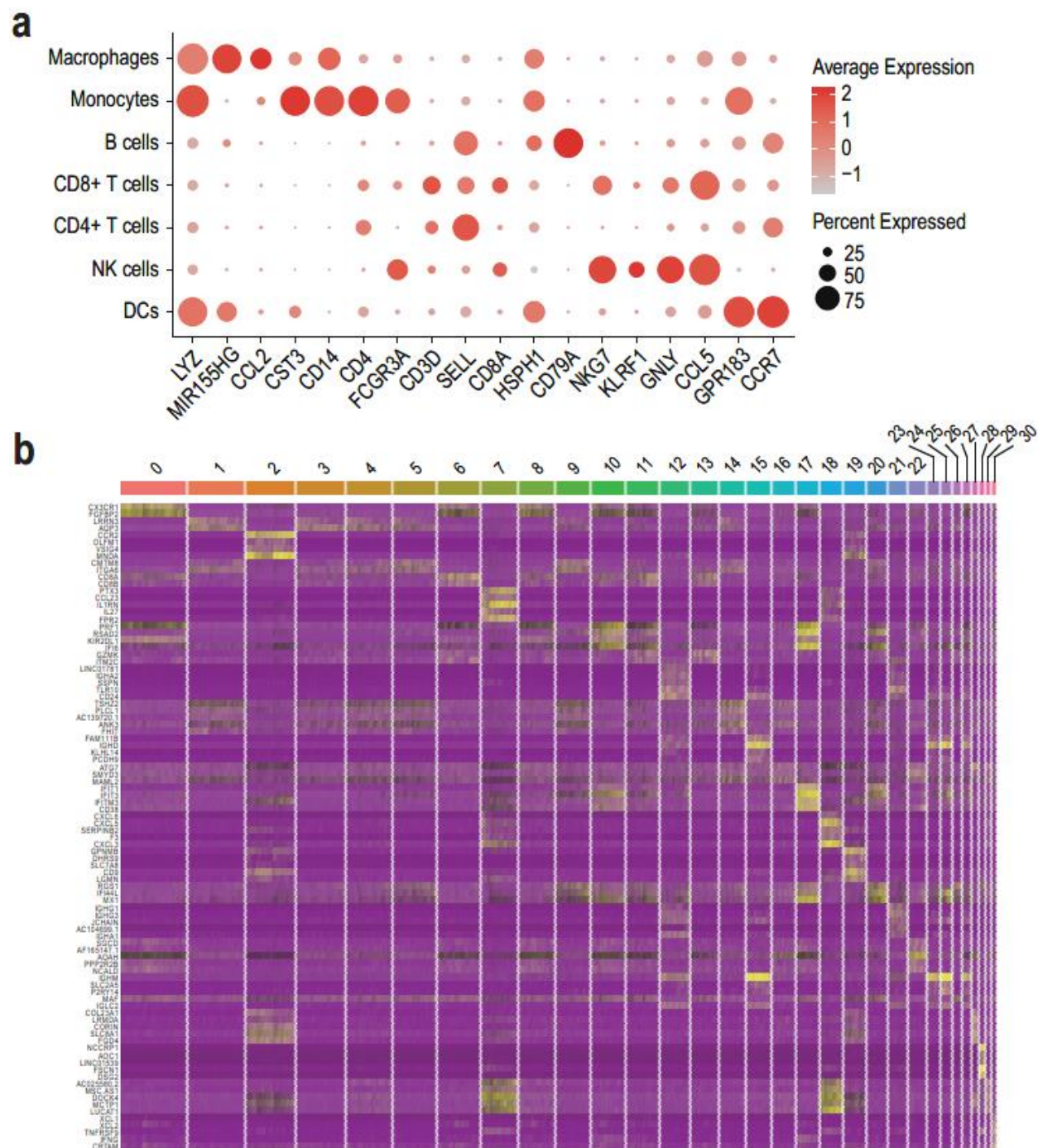
Supplementary Fig. 4. *FUT4* expression level is increased in SPs (n = 20) compared to HCs (n = 8). Bar plot displaying the expression level of fucosyltransferase-related genes in GSE232753 cohort. Statistical significance was calculated with a two-tailed *t*-test without adjustment (** $p < 0.01$).

Supplementary Fig. 5



Supplementary Fig. 5. Fucose alone does not induce inflammatory responses in human PBMCs. The relative expression of *IL1B* and *TNF* genes in PBMCs after treatment with L-Fucose (0.5, 1.0, and 2.0 mM) using qRT-PCR (technical replication = 3). Statistical significance was calculated with an unpaired *t*-test with Mann-Whitney test and presented as means $\pm$ SD. arb. units, arbitrary unit; ns, not significant.

Supplementary Fig. 6

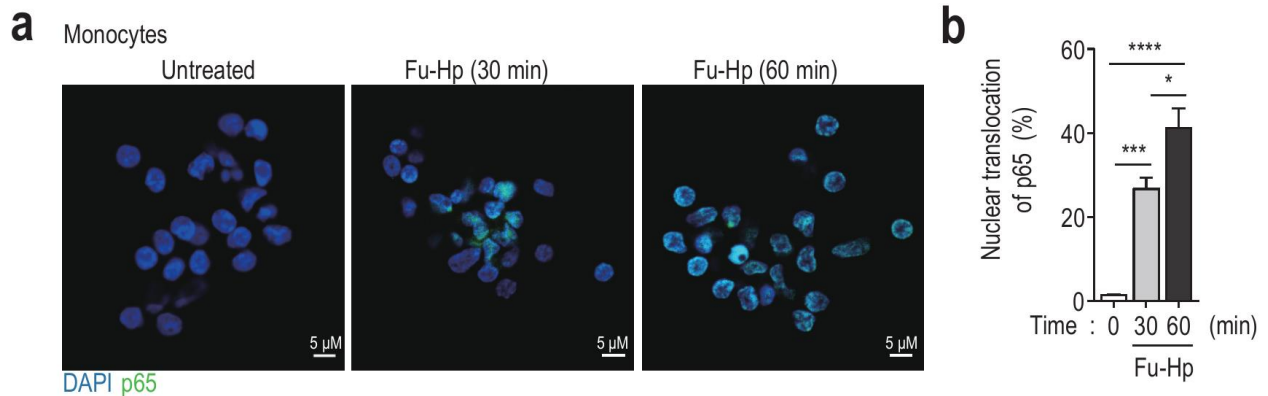


Supplementary Fig. 6. Analysis of scRNA-seq in Fu-Hp-treated human primary PBMCs (n = 3 for untreated; n = 3 for Fu-Hp-treated human primary PBMCs). **a**, Expression of selected markers for cell types. The dot size corresponds to the ratio of cells expressing

the gene in the cell type. The color scales correspond to the averaged expression levels.

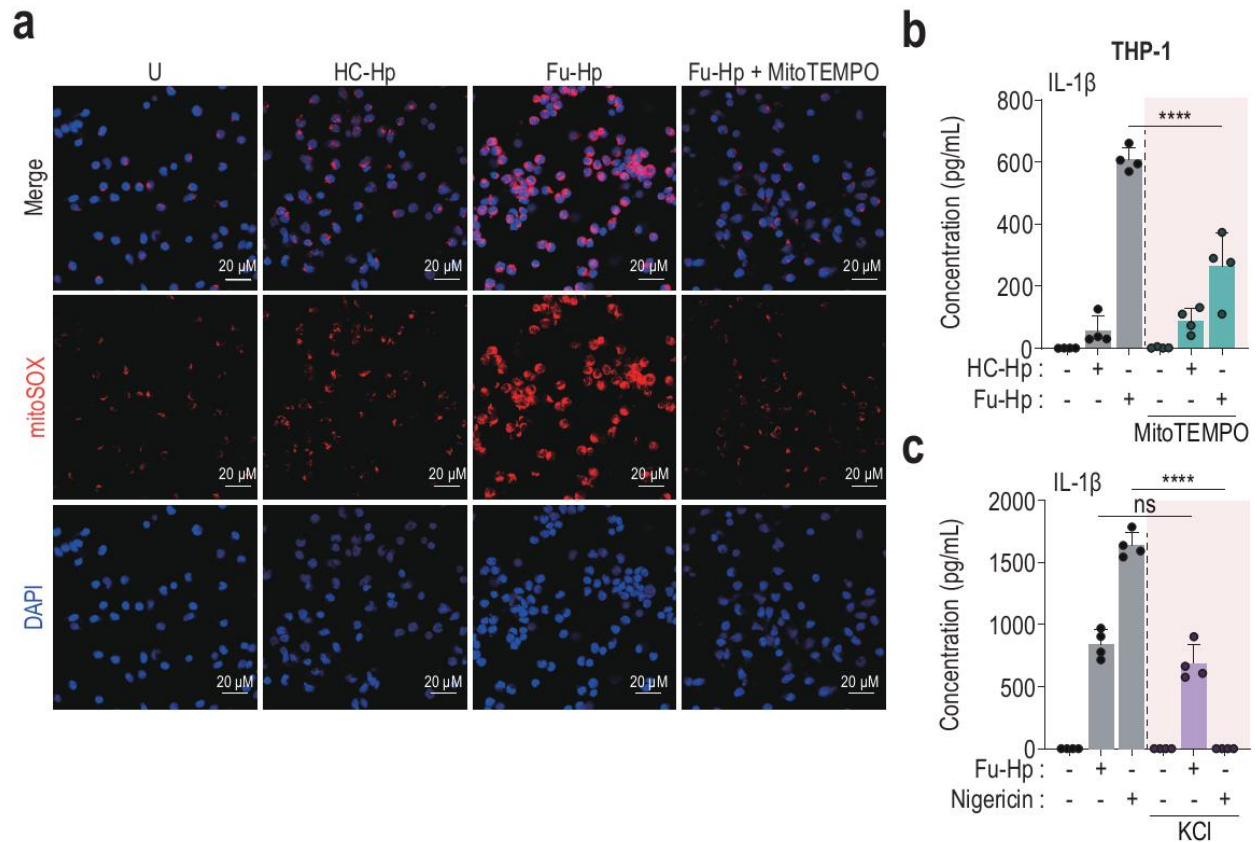
b, Heatmap depicting the expression of marker genes in the indicated clusters.

Supplementary Fig. 7



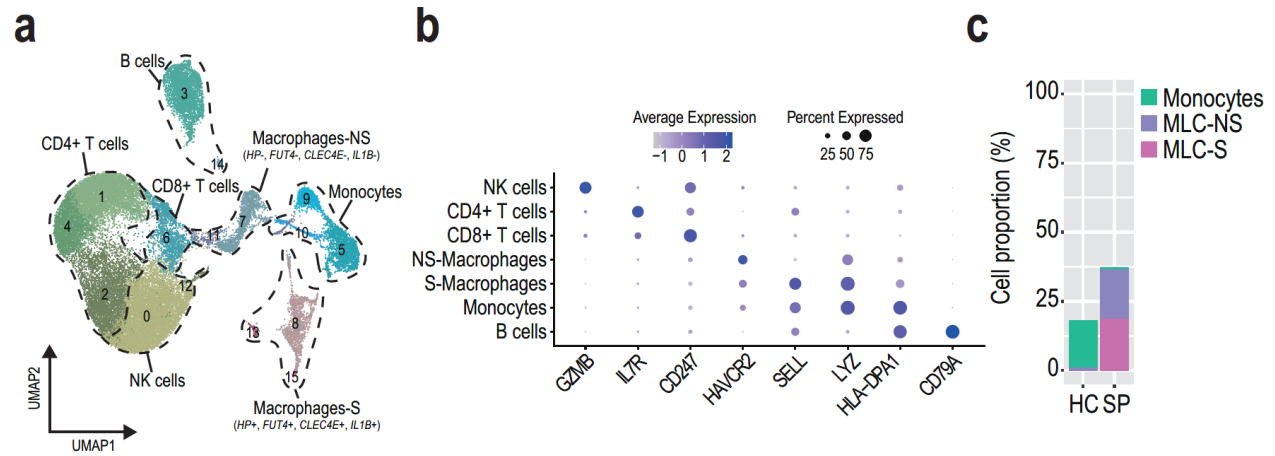
Supplementary Fig. 7. Fu-Hp promotes NF- κ B nuclear translocation in human primary monocytes ($n = 5$ for untreated; $n = 6$ for 30 min and 60 min treated). **a,b**, Representative immunofluorescence images (scale bar = $5\ \mu\text{m}$; **a**) and a quantitative analysis on the percentage of p65 (NF- κ B) nuclear translocation (**b**) in Fu-Hp-treated monocytes. Statistical significance was calculated with an ordinary one-way ANOVA with Bonferroni's multiple comparisons test ($*p < 0.05$; $***p < 0.001$; $****p < 0.0001$).

Supplementary Fig. 8



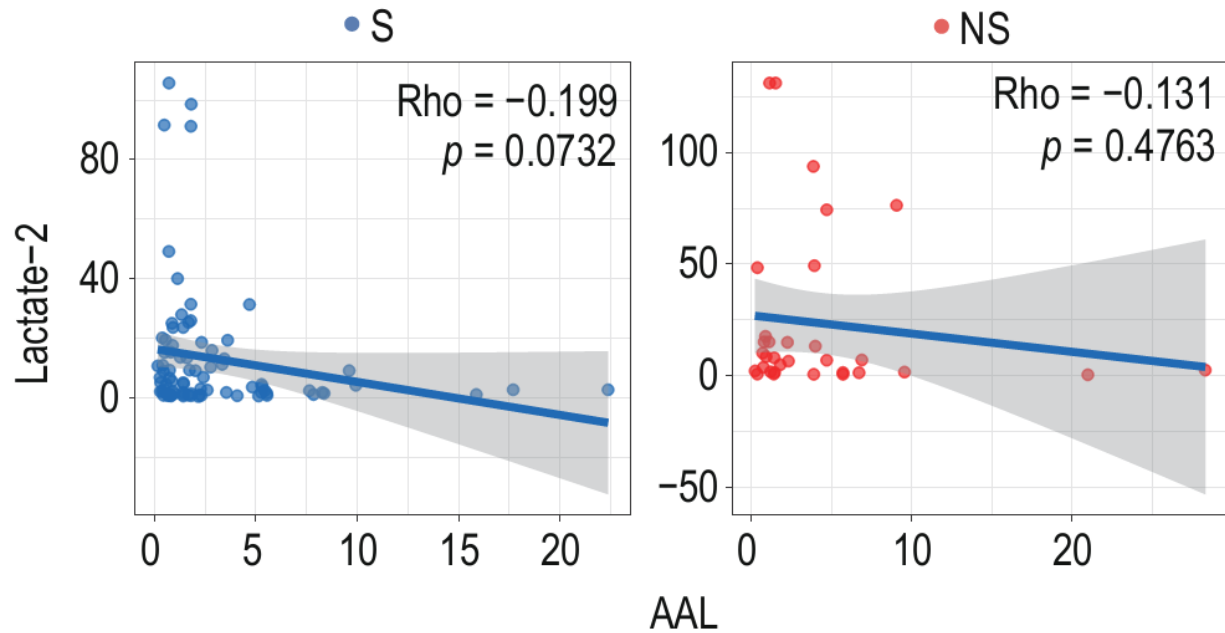
Supplementary Fig. 8. Fu-Hp-induced NLRP3 inflammasome activation depends on mtROS generation in human monocytes. **a**, Representative immunofluorescence images (scale bar = 20 μ m). Human primary monocytes were treated with Fu-Hp or HC-Hp in the presence or absence of MitoTEMPO. The mtROS and nuclei were stained with MitoSOX Red (red) and DAPI (blue), respectively. MT, MitoTEMPO. **b**, The concentrations of IL-1 β in the supernatants of THP-1 cells with Fu-Hp treatment after mitoTEMPO pre-treatment or not measured by ELISA (n = 4 per group). Statistical significance was calculated with an ordinary one-way ANOVA with Bonferroni's multiple comparisons test (**** p < 0.0001) and presented as means $\pm$ SEM. **c**, The concentrations of IL-1 β in the supernatants of THP-1 cells with Fu-Hp treatment after potassium chloride (KCl) pre-treatment or not measured by ELISA (n = 4 per group). Statistical significance was calculated with an ordinary one-way ANOVA with Bonferroni's multiple comparisons test (**** p < 0.0001) and presented as means $\pm$ SEM. ns, not significant.

Supplementary Fig. 9



Supplementary Fig. 9. Analysis of scRNA-seq in public sepsis cohort. **a**, Unsupervised UMAP analysis on PBMCs pooled from two HC and two SP (1 surviving patient and 1 non-surviving patient) subjects. Cell populations were mapped onto the UMAP landscape to visualize the distribution of cells across clusters. **b**, Expression of selected markers for cell types. The dot size corresponds to the ratio of cells expressing the gene in the cell type. The color scales correspond to the averaged expression levels. **c**, Bar plot of the proportion of indicated cell types shown in **a** and **b**, separated by groups. HC, Healthy control; SP, Sepsis patients; MLC-NS, Macrophage-like cell population from non-surviving patient; MLC-S, Macrophage-like cell population from surviving patient.

Supplementary Fig. 10



Supplementary Fig. 10. Correlation analysis of AAL and lactate levels in SPs. Correlation between the expression of the AAL level (x-axis) and the expression of either the level of lactate-2 (measurement within 6 hours following antibiotic administration) (y-axis) with the best linear fit plotted in our cohort ($n = 84$ for S; $n = 33$ for NS). Pearson's correlation coefficient and the p value are displayed in the top-right corner. The shaded area represents the 95% confidence interval. P values were calculated using a t -distribution with $[n-2]$ degrees of freedom. S, Surviving sepsis patients; NS, Non-surviving sepsis patients.

Supplementary Table 1

Supplementary Table 1. Characteristics of subjects for Hp purification.

	Sample group	
	HC	SP
Individuals	73	121
Sex, M/F (N, %)	37/36 (51/49%)	79/42 (65/35%)
Age (mean±SD, years)	61±6	72±11

Supplementary Table 2

Supplementary Table 2. Primers used in this study.

Species	Target	Primer sequence (5' - 3')
Human	<i>IL1B</i>	CGCCAGTGAAATGATGGCTTA TGCTGTAGTGGTGGTCGGA
	<i>IL6</i>	TTCTCCACAAGCGCCTTCG CTGAGATGCCGTCGAGGATG
	<i>TNF</i>	CATCCAACCTTCCCAAACGC CGAAGTGGTGGTCTTGTTGC
	<i>IL10</i>	GTTGCCTGGTCCTCCTGACTG CTCGAAGCATGTTAGGCAGGT
	<i>IL18</i>	CCACCACAAGGCGCTGTTCTACA AGGCGGAGGACTCGAACTCGTA
	<i>CCL2</i>	CCCCAGTCACCTGCTGTTAT TGGAATCCTGAACCCACTTC
	<i>CCL3</i>	GGCTCTCTGCAACCAGTTCTC GTTAGGAAGATGACACCGGGCT
	<i>CCL4</i>	GCTTCCTCGCAACTTTGTGGTAG GGTCATACACGTA CTCTGGAC
	<i>CXCL2</i>	CCGAAGTCATAGCCACACTC GGATTTGCCATTTTTTCAGCA
	<i>CXCL8</i>	GAGAGTGATTGAGAGTGGACCAC CACAACCCTCTGCACCCAGTTT
	<i>CXCL10</i>	TCCTGCAAGCCAATTTTGTCC TGTGGTCCATCCTTGGAAGC
	<i>CLEC4E</i>	TCTTATGGACTGTTGCTGGGA TGCGAAATGTCACAACACATCT
	<i>NLRP3</i>	GAGCACCAGCCAGAGTCTAA CAGCTTCTGGTTGCACAATCTC
Mouse	<i>Il1b</i>	TGACGGACCCCAAAGATGA AAAGACACAGGTAGCTGCCA
	<i>Il6</i>	ACAAAGCCAGAGTCCTTCAGA TGGTCCTTAGCCACTCCTTC
	<i>Tnf</i>	CCCACGTCGTAGCAAACCAC GCAGCCTTGTCCTTGAAGA
	<i>Il10</i>	GCTCTTGCACTACCAAAGCC CTGCTGATCCTCATGCCAGT
	<i>Ccl2</i>	TCTCTCTTCCTCCACCACCATG GCGTTAACTGCATCTGGCTGA
	<i>Ccl3</i>	CACCCTCTGTCACCTGCTCAA

Ccl4

ATGGCGCTGAGAAGACTTGGT
TCTGTGCAAACCTAACCCCG
GAGGGTCAGAGCCCATTGGT

Supplementary Table 3

Supplementary Table 3. Antibodies used in this study.

Antibody	Target size (kDa)	Company	Dilution
β-Actin (13E5) Rabbit mAb (HRP Conjugate)	45	Cell signaling Technology	1:2000
Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb #4060	60	Cell signaling Technology	1:1000
Phospho-NF-κB p65 (Ser536) (93H1) Rabbit mAb #3033	65	Cell signaling Technology	1:1000
Phospho-p38 MAPK (Thr180/Tyr182) (D3F9) XP® Rabbit mAb #4511	38	Cell signaling Technology	1:1000
Phospho-SAPK/JNK (Thr183/Tyr185) Antibody #9251	46, 54	Cell signaling Technology	1:1000
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody #9101	42, 44	Cell signaling Technology	1:1000
HA-Tag (C29F4) Rabbit mAb, Cell Signaling, #3724S	-	Cell signaling Technology	1:1000
CLEC-4E (B-7): sc-390806	35	Santa Cruz Biotechnology	1:1000
Caspase-1 Antibody, #2225	20 p20. 30 to 45 beta, delta, gamma. 50 alpha.	Cell signaling Technology	1:1000
IL-1β human Antibody CST#2022	31, 17	Cell signaling Technology	1:1000
ASC (N-15)-R Antibody rabbit SC-22514	22	Santa Cruz Biotechnology	1:1000
IL-1β Antibody (Mouse Specific) #8689	31	Cell signaling Technology	1:1000
IL-1β (3A6) Mouse mAb #12242	31, 17	Cell signaling Technology	1:1000
IL-1β (D3H1Z) Rabbit mAb #12507	31, 17	Cell signaling Technology	1:1000
Cryopyrin (N-14): sc-34411	110	Santa Cruz Biotechnology	1:1000
Anti-HP antibody produced in rabbit (H8636)	45	Sigma-Aldrich	1:2000
AAL lectin, B-1395	-	VectorLabs	1:1000
SNA lectin, B-1305	-	VectorLabs	1:1000
PHA-L lectin, B-1115	-	VectorLabs	1:500
IL-6 (10E5): sc-57315	21	Santa Cruz Biotechnology	1:1000
Anti-rabbit IgG, HRP-linked Antibody #7074	-	Cell signaling Technology	1:2000
Donkey anti-goat IgG-HRP SC-2020	-	Santa Cruz Biotechnology	1:2000
Anti-mouse IgG, HRP-linked Antibody #7076	-	Cell signaling Technology	1:2000