

# Biochemical analysis of leukocytes after *in-vitro* and *in-vivo* activation with bacterial and fungal pathogens using Raman spectroscopy

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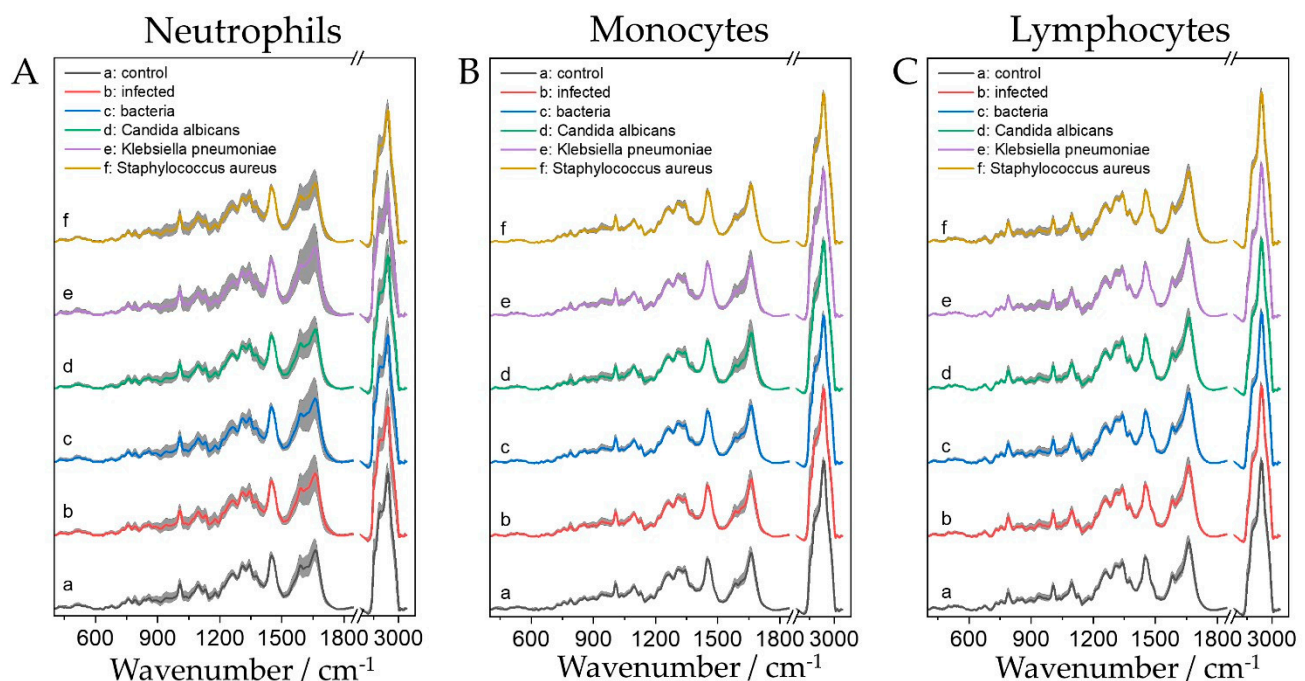
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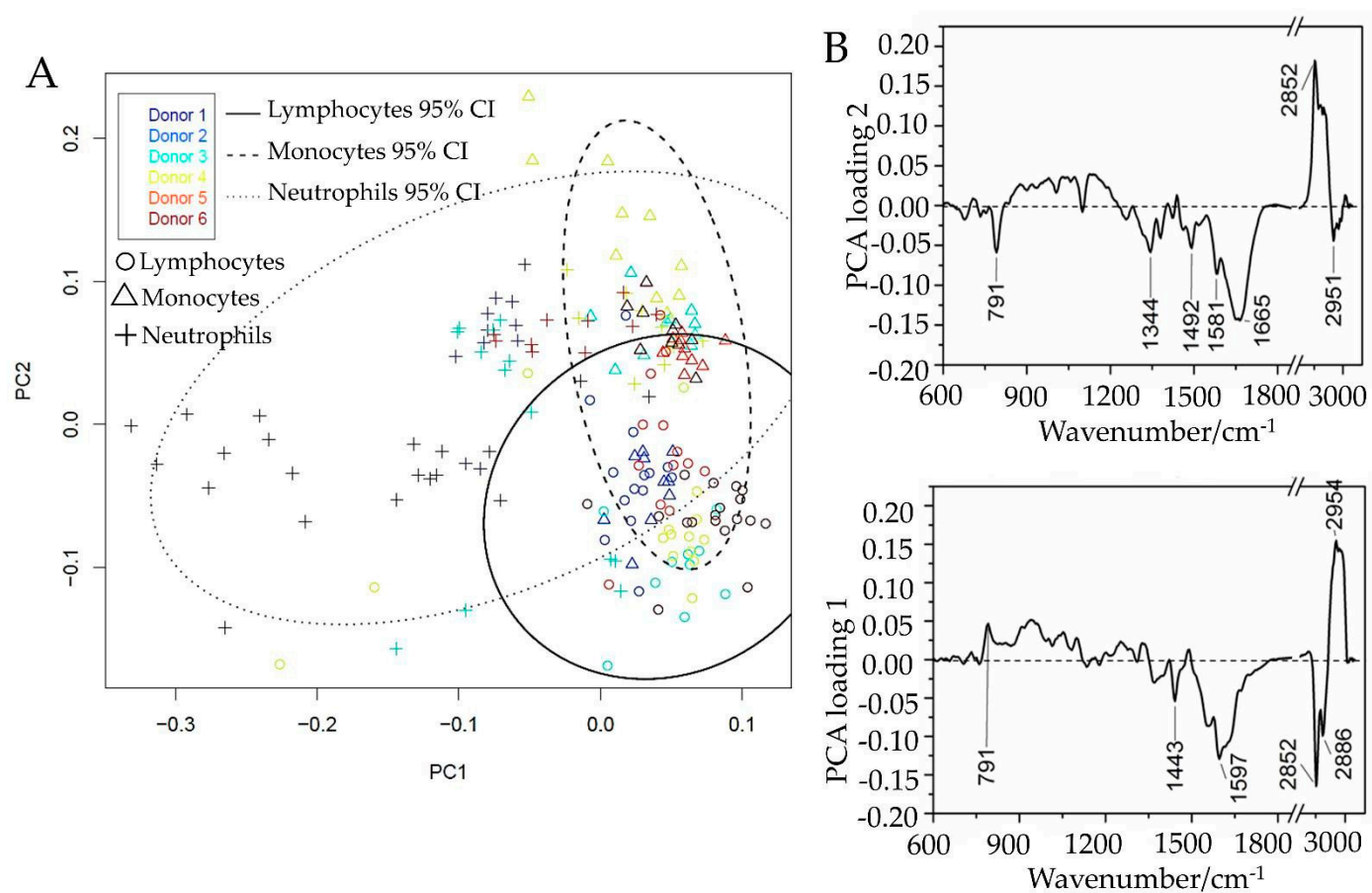
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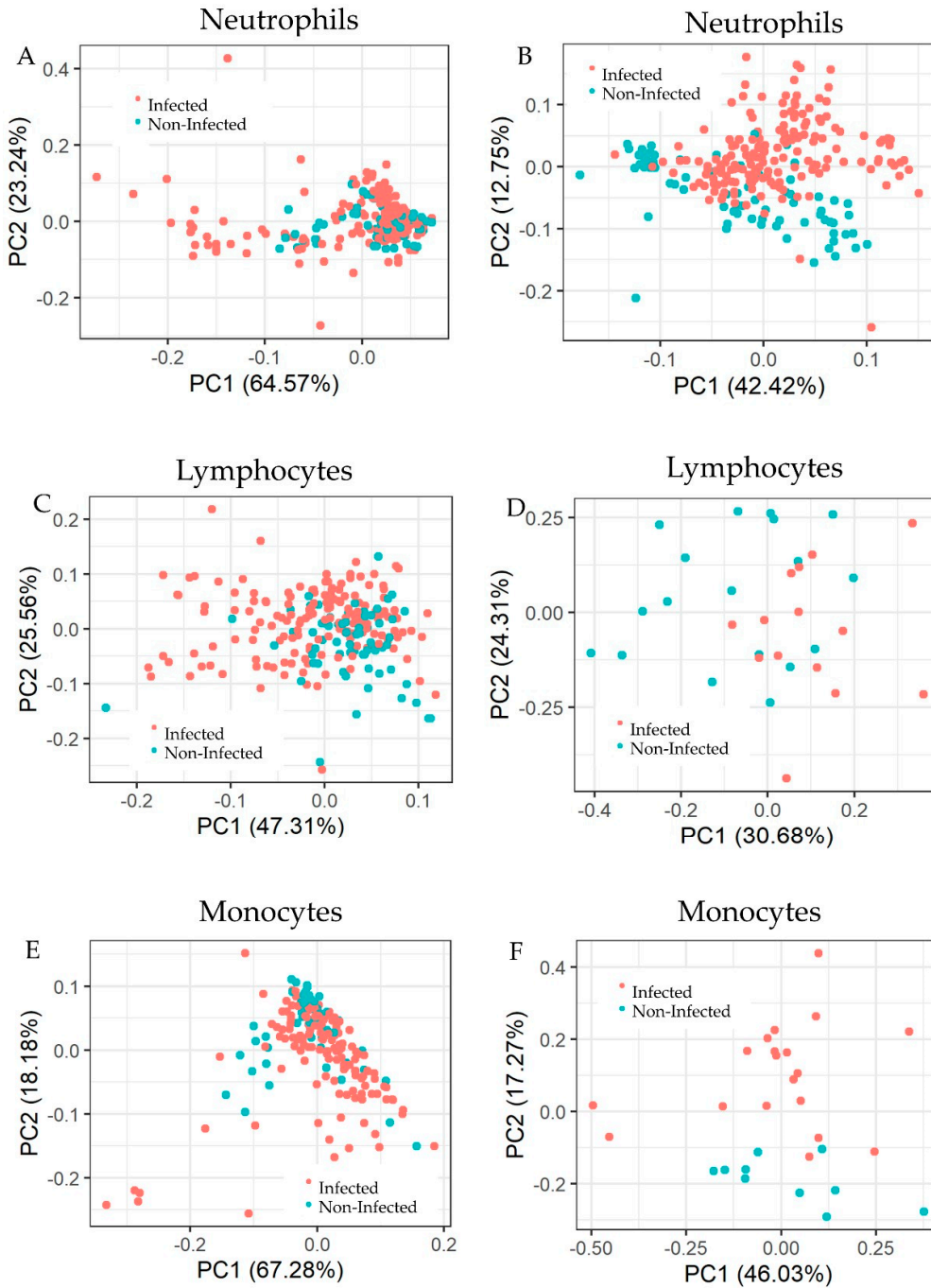
**Supplemental Figure S1.** Mean Raman spectra of A) neutrophils, B) monocytes, and C) lymphocytes. Mean spectra were computed from 6 donors (neutrophils) or 5 donors (monocytes and lymphocytes). The number of used cells can be calculated with the help of Table S5. Raman spectra are shifted along the y-axis for clarity. From bottom to top: a) non-infected, b) infected with pathogens, c) infected with bacteria, d) infected with fungi (*C. albicans*), e) infected with *K. pneumoniae*, f) infected with *S. aureus*.



**Supplemental Figure S2.** Principal component analysis of non-activated leukocytes subpopulations using Raman spectra. (A) PCA score plot showing the distribution of leukocyte subpopulations (lymphocytes, monocytes, and 28 neutrophils) with the ellipses indicating 0.95 confidence interval for each cell population and (B) PC loadings 1 and 2 29 obtained from the PCA analysis show the contributing Raman bands.

## Healty donors

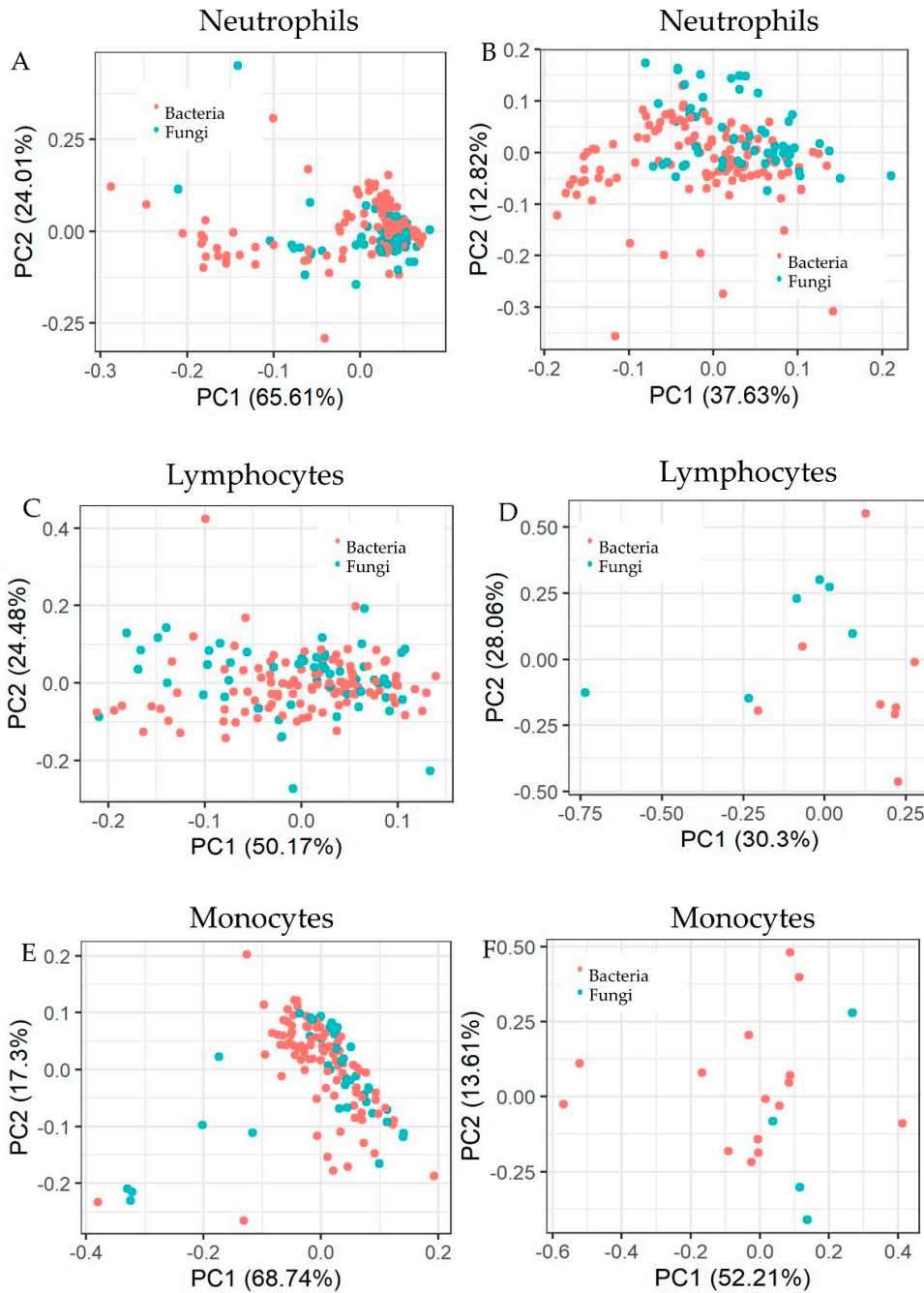
## Patients



**Figure S3.** PCA score plots of Raman data to separate infected and non-infected cells. Score plots are shown for different leukocyte subpopulations obtained from healthy donor (left column) and patient (right column) samples. The colors represent infected (pink) and non-infected (cyan) cells. For neutrophils from donor samples, the range of PC scores for infected cells is larger than for the non-infected cells, which points to higher heterogeneity of the infected samples. The differentiation between the infected and non-infected cells is more prominent for patient samples. The separation between infected and non-infected cells appears among patient's lymphocytes visible already in the first PC.

## Healthy donors

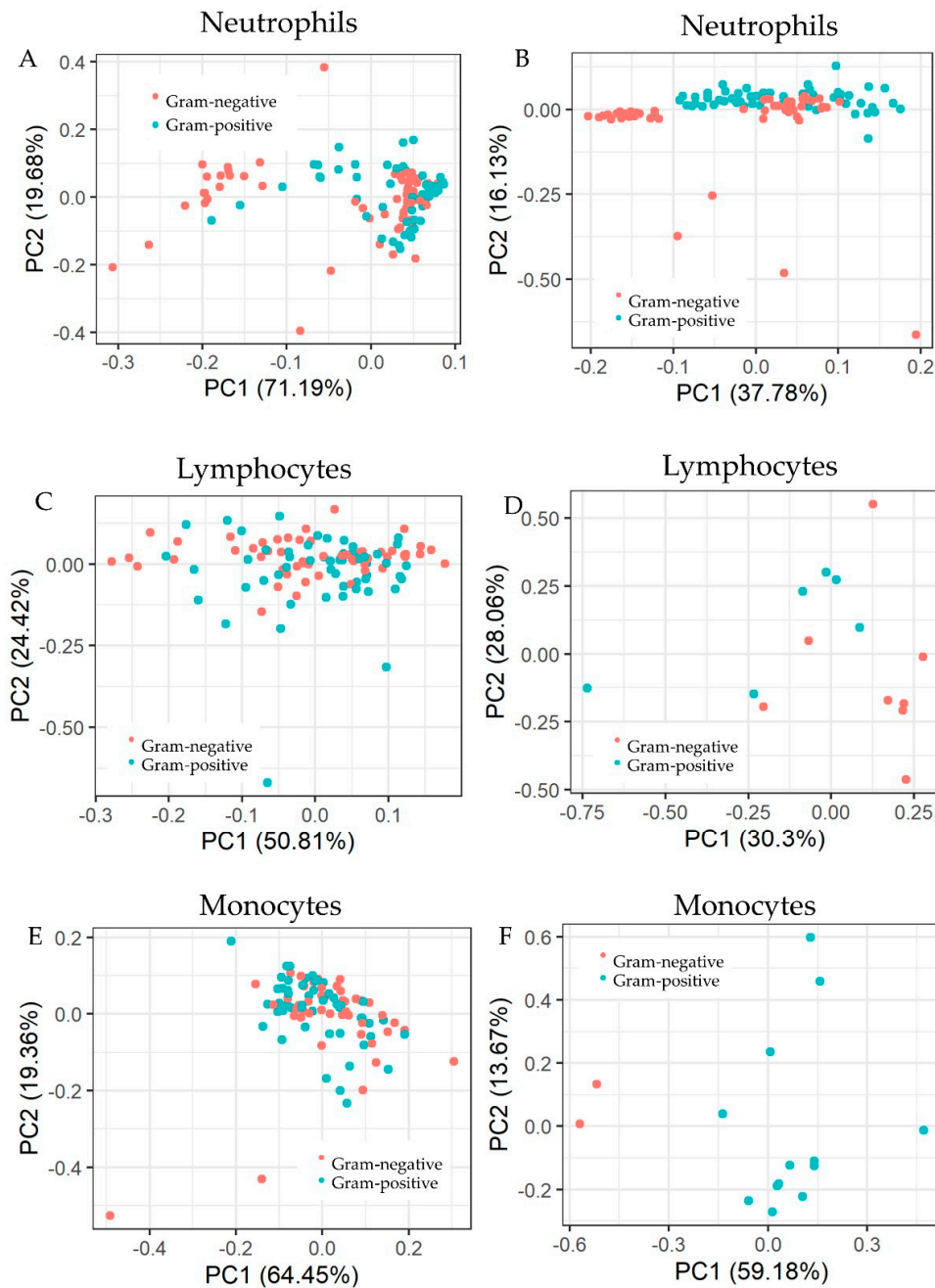
## Patients



**Figure S4.** PCA score plots of Raman spectra of cells infected with bacteria and fungi. Score plots are shown for different leukocyte subpopulations obtained from healthy donor (left column) and patient (right column) samples. The colors represent cells infected with bacteria (pink) and fungi (cyan). The differences between neutrophils infected with different pathogens is more prominent in patient samples, which is similar to Figure S3. Due to the large difference in number of lymphocytes and monocytes obtained from donor and patient samples, the scatterplots for these two cell subpopulations cannot be reliably compared between each other.

## Healthy donors

## Patients

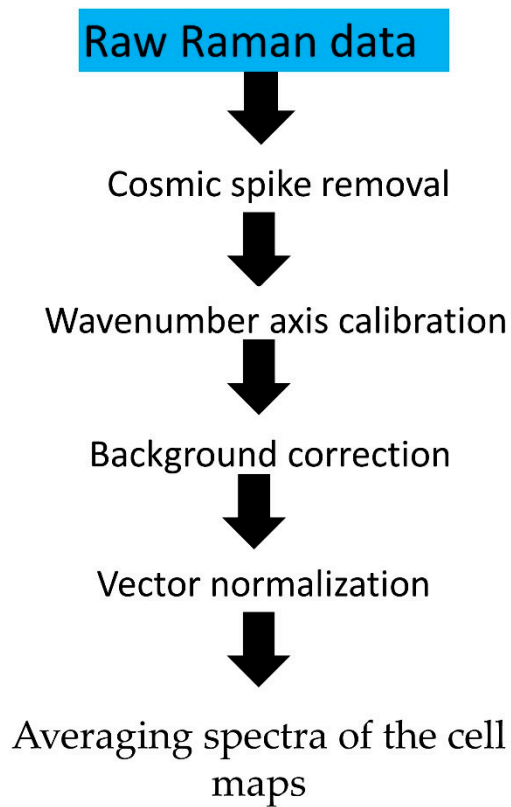


**Figure S5.** PCA score plots of Raman spectra of cells infected with bacteria. Score plots are shown for different leukocyte subpopulations obtained from donor (left column) and patient (right column) samples. The colors represent cells infected with gram-negative (pink) and gram-positive (cyan) bacteria. Both donor and patient samples infected with gram-negative bacteria show high heterogeneity for neutrophils along the first PC. Good separation of monocytes from the patient infected with gram-negative bacteria should be interpreted carefully, since only two monocytes from a single patient were analyzed in the gram-negative patient samples.

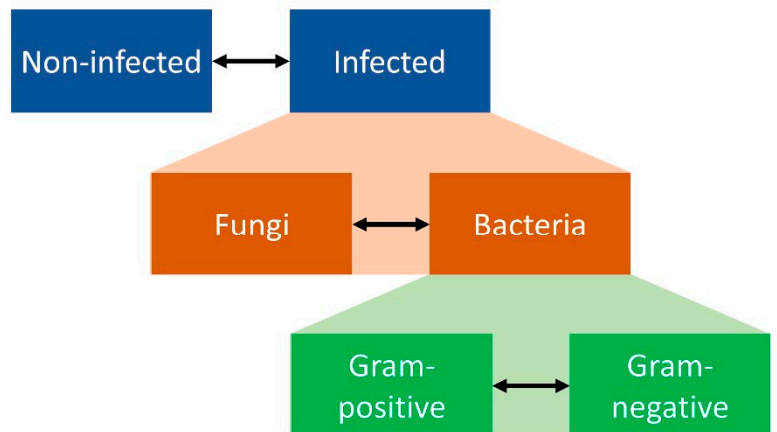


# Analysis

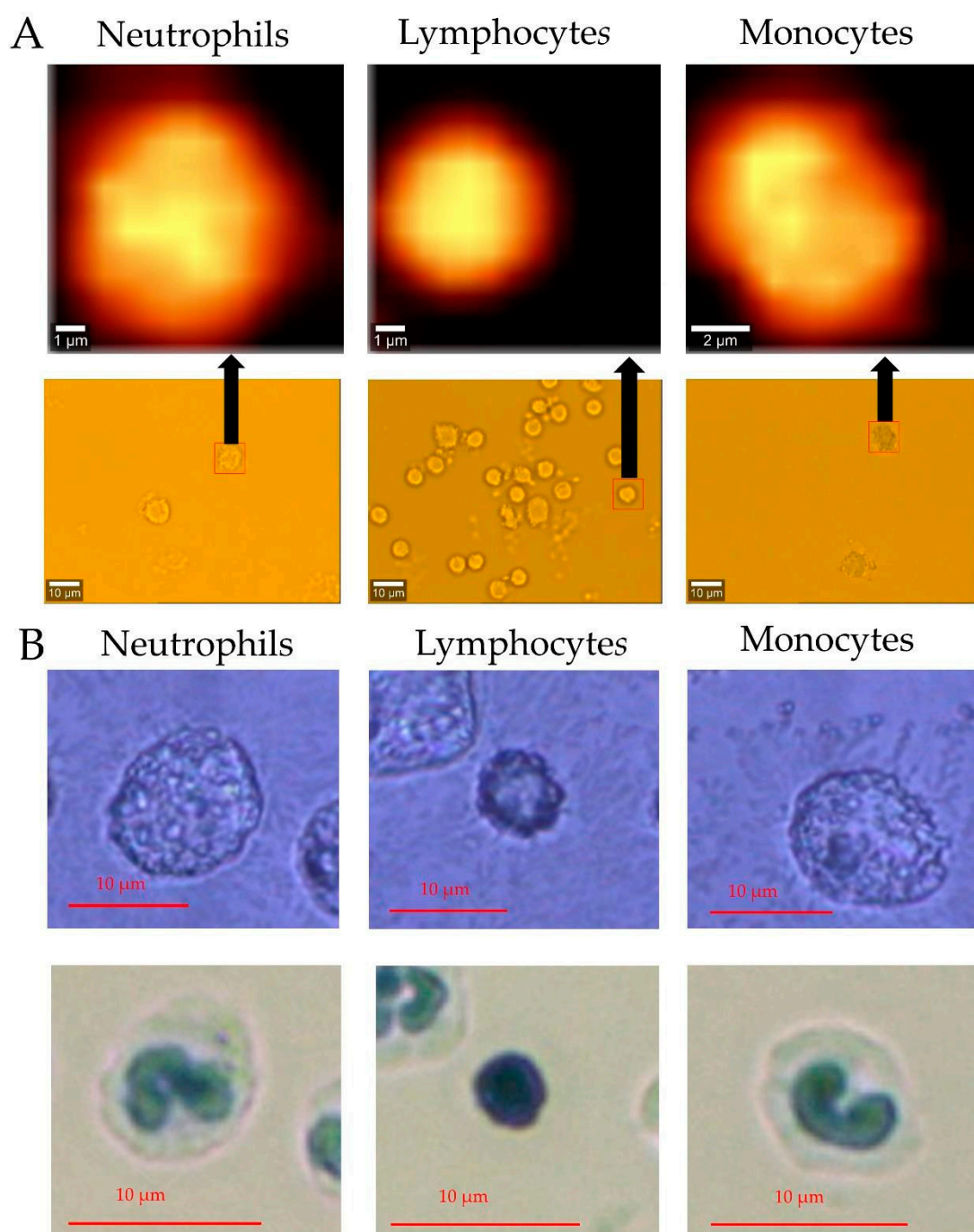
## Data Pre-processing



## Step by step decision tree



**Figure S6.** Schematics of the statistical analysis. **Left:** Data pre-processing work-flow. **Right:** Step-by-step decision tree to differentiate leukocytes (in different treatment groups or from different patients).



**Figure S7. A In-vitro model: Overview** white light images are depicted on the bottom. From those images, area scans were selected. Exemplarily, a false color Raman image representing overall Raman intensity of the CH-stretching band ( $2800\text{ cm}^{-1}$ -  $3000\text{ cm}^{-1}$ ) is shown for each subtype.

**B Leukocytes from patients:** In the top row white light images of leukocytes isolated from patients after spin-coating onto CaF<sub>2</sub> slides are shown. The cells were measured in dried condition to avoid detachment from the slide. After Raman measurements, leukocytes were stained using Kimura. The cells were relocated in the microscope and based on the nucleus shape the leukocytes sub-population were identified. Bottom row shows images of Kimura stained neutrophils, lymphocytes and monocytes from patients which are depicted in the top row.

**Table S1.** Interpretation of biochemical information from Raman spectra in non-infected and infected (*S. aureus*, *K. pneumoniae*, and *C. albicans*) lymphocytes and monocytes.

<b>Neutrophils</b>						
<i>In-vitro</i>		<i>In-vivo</i>				
<i>Non-infected</i>	<i>Infected</i>	<i>Non-infected</i>	<i>Infected</i>	<i>Raman assignments</i>	<i>Biomolecule</i>	<i>References</i>
1348				G(DNA/RNA)	Nucleic acid	(1)
1381						
	1438/43			CH <sub>2</sub> CH <sub>3</sub> deformation	All proteins, carbohydrates and lipids	(2)
1589				Cytosine and Guanine of DNA	Nucleic acid	(3)
	1597			Guanine/Adenine	Nucleic acid	(1)
	1633			Amide I (β-sheet structure)	Proteins	(3)
	2849/52		2846	CH <sub>2</sub> symmetric stretch	Lipids	(4)
2944/47		2948		CH stretching	Proteins	(4)
<b>Lymphocytes</b>						
<i>In-vitro</i>		<i>In-vivo</i>				
<i>Non-infected</i>	<i>Infected</i>	<i>Non-infected</i>	<i>Infected</i>	<i>Raman assignments</i>	<i>Biomolecule</i>	<i>References</i>
1074				PO <sub>2</sub> backbone C-N str	Nucleic acid	(1, 5)
			754	Thymine, Tryptophan	Nucleic acid	(1, 5)
	791			Phosphate backbone vibrations	Nucleic acid	(1, 5)
	1340/1344			G(DNA/RNA)	Nucleic acid	(1)
	1385					
1443		1436		CH <sub>2</sub> CH <sub>3</sub> deformation	All proteins, carbohydrates and lipids	(2)
	1492			C and G of DNA	Nucleic acid	(4)
	1581/1589			Cytosine and Guanine of DNA	Nucleic acid	(1)
		1600		Guanine/Adenine	Nucleic acid	(1)
	1649			Amide I	Proteins	(1, 5)
	1677/1681			NADH and Amide I	Bound and free NADH, proteins	(2, 6)
2856		2854		CH <sub>2</sub> symmetric stretch	Lipids	(4)
2886				CH stretching	Lipids	(4)
2897				CH stretching	Lipids	(4)
			2914	CH stretching	Protein, lipids	(7)
	2947/2951			CH stretching	Proteins	(4)
		2992		=CH asymmetric stretch	Lipids	
<b>Monocytes</b>						
<i>In vitro</i>		<i>In-vivo</i>				
<i>Non-infected</i>	<i>Infected</i>	<i>Non-infected</i>	<i>Infected</i>	<i>Raman assignments</i>	<i>Biomolecule</i>	<i>References</i>
791				Phosphate backbone vibrations	Nucleic acid	(1, 5)



1005			Phenylalanine	Proteins	(1, 5)
1100			PO <sub>2</sub> backbone C-N str	Nucleic acid	(1, 4)
1235			Amide III (coupled N-H/C-H deformations)	Proteins(7)	(1)
1344/1340			G(DNA/RNA)	Nucleic acid	(1)
	1443		CH <sub>2</sub> CH <sub>3</sub> deformation	carbohydrates and lipids	(2)
	1556		C-C stretching Tryptophan	Proteins	
1589			C and G of DNA	Nucleic acid	(1)
	1597		Guanine/Adenine	Nucleic acid	(1, 5)
	1629		Amide I (β-sheet structure)	Proteins	
	2850/2852	2844	CH <sub>2</sub> symmetric stretching of lipids	Lipids	(4)
	2893/2886		CH stretching	Lipids	(4)
2947/2951		2948	CH stretching	Proteins	(4)
2988			=CH asymmetric stretch	Lipids	(4)
		2974	CH <sub>2</sub> symmetric stretch	All proteins, carbohydrates, and lipids	(4)

**Table S2.** Interpretation of biochemical information from Raman spectra in neutrophils, monocytes, and lymphocytes infected by bacterial pathogens (*S. aureus*, *K. pneumoniae*) and fungi (*C. albicans*).

<b>Neutrophils</b>						
<i>In-vitro</i>		<i>In-vivo</i>		<i>Raman assignments</i>	<i>Biomolecule</i>	<i>References</i>
<i>Bacteria</i>	<i>Fungi</i>	<i>Bacteria</i>	<i>Fungi</i>			
	698					
	764			Trp	proteins	(1, 5)
1010				Phenylalanine	Proteins	(1, 5)
1095				PO <sub>2</sub> backbone C-N str	Nucleic acid	(1, 5)
1265				Amide III random coil	Proteins	(5)
1302				CH <sub>3</sub> CH <sub>2</sub> deformation	All protein, lipids, and carbohydrate s	(1)
1335				DNA, Tyr, Trp	Nucleic acid, Proteins	(8)
1451		1438		CH <sub>2</sub> CH <sub>3</sub> deformation	All proteins, carbohydrates, and lipids	(2)
	1560			CC stretching Tryptophan	Proteins	(2)
		1578		Guanine/Adenine	Nucleic acid	(1)
	1593			Guanine/Adenine	Nucleic acid	(1)
1625				Amide I (β-sheet structure)	Proteins	(3)
1669			1662	Amide I (α-helix structure)	Proteins	(1, 5)
2849/2856		2858		CH <sub>2</sub> symmetric stretching	Lipids	(4)
	2934		2930	CH stretching	Proteins	(4)
	2951			CH <sub>3</sub> asymmetric stretching	Proteins	(4)

<b>Lymphocytes</b>						
<i>In-vitro</i>		<i>In-vivo</i>				
<i>Bacteria</i>	<i>Fungi</i>	<i>Bacteria</i>	<i>Fungi</i>			<i>References</i>
791		788		Phosphate backbone vibrations	Nucleic acid	(1, 5)
			860	Ribose ring vibration	Nucleic acid	(4)
	1010			Phenylalanine	Proteins	(1, 5)
	1104			PO <sub>2</sub> - in nucleic acids	Nucleic acid	(1)
		1236		Amide III coupled N-H/C-H deformations	Proteins	(1, 4)
	1256			Amide III coupled N-H/C-H deformations	Proteins	(1)
		1304		CH <sub>3</sub> CH <sub>2</sub> deformation	All protein, lipids, and carbohydrates	(1)
	1348			G (DNA/RNA)	Nucleic acid	(1)
		1438		CH <sub>2</sub> CH <sub>3</sub> deformation	All proteins, carbohydrates and lipids	(2)
	1459/1451			CH <sub>2</sub> CH <sub>3</sub> deformation	All proteins, carbohydrates, and lipids	(5)
1496				C and G of DNA	Nucleic acid	(4)
		1580		Cytosine and Guanine of DNA	Nucleic acid	(1)
	1613			Amino acids	Proteins	(5)
	1621		1622	Amide I (β-sheet structure)	Proteins	(3)
	1661			Amide I (α-helix structure)	Proteins	(1, 5)
	1677			NADH and Amide I (beta-sheet)	Bound and free NADH	(2, 6)
			1744	C=O ester stretching, non-saturated	Lipids	(9)
		2830		CH <sub>2</sub> symmetric stretching	Lipids	(4)
2852				CH <sub>2</sub> symmetric stretching	Lipids	(4)
2876				CH <sub>3</sub> symmetric stretching	Lipids	(4)
	2883			CH stretching	Lipids	(4)
			2908	CH stretching	lipids	(4)
	2930			CH stretching	Proteins	(4)

<b>Monocytes</b>						
<i>In-vitro</i>		<i>In-vivo</i>				
<i>Bacteria</i>	<i>Fungi</i>	<i>Bacteria</i>	<i>Fungi</i>			<i>References</i>
	675			Guanine (DNA/RNA) C-C twist	Nucleic acid	(4, 5)
	791			Phosphate backbone vibrations	Nucleic acid	(1, 5)

953			Backbone DNA	Nucleic acid	(2)
1014/1010			Phenylalanine	Proteins	(1, 5)
	1100		PO <sub>2</sub> backbone C-N str	Nucleic acid	(1)
1248			Amide III coupled N-H/C-H deformations	Proteins	(1, 4)
1310		1310	CH <sub>3</sub> CH <sub>2</sub> deformation	Proteins, lipids, and carbohydrates	(1)
	1381		Guanine, Adenine ring vibrations	Nucleic acid	(10)
1455/1451			CH <sub>2</sub> CH <sub>3</sub> deformation	All proteins, carbohydrates, and lipids	(2)
	1492		C and G of DNA	Nucleic acid	(4)
		1548	CC stretching Tryptophan	Proteins	(2)
1593			Guanine/Adenine	Nucleic acid	(1)
1609			Amino acids	Protein	(5)
1661			Amide I ( $\alpha$ -helix structure)	Proteins	(1, 5)
1673			NADH and Amide I	Protein	(2, 6)
2859		2842	CH <sub>2</sub> symmetric stretching	Lipids	(4)
2886			CH stretching	Lipids	(4)
	2940	2936	CH stretching	Proteins	(4)
2998			=CH asymmetric stretch	Lipids, fatty acids	

**Table S3.** Interpretation of biochemical information from Raman spectra in monocytes infected by *K. pneumoniae* and *S. aureus*.

Neutrophils						
<i>In-vitro</i>		<i>In-vivo</i>		<i>Raman assignments</i>	<i>Biomolecule</i>	<i>References</i>
<i>K. pneumoniae</i>	<i>S. aureus</i>	<i>K. pneumoniae</i>	<i>S. aureus</i>			
		1000		Phenylalanine	Proteins	(1)
		1312		CH <sub>3</sub> CH <sub>2</sub> deformation	Proteins, lipids, and carbohydrates	(1)
	1348			G (DNA/RNA)	Nucleic acid	(1)
		1372		G, A ring vibrations	Nucleic acid	(2)
1443	1438			CH <sub>2</sub> CH <sub>3</sub> deformation	All proteins, carbohydrates, and lipids	(2)
		1554		CC stretching Tryptophan	Proteins	(2)
1593				Guanine/Adenine	Nucleic acid	(1)
1637/1641		1648		Amide I	Proteins	(1)
2852		2842		CH <sub>2</sub> symmetric stretching	Lipids	(4)
	2954			CH <sub>3</sub> asymmetric stretching	Proteins	(4)

### Lymphocytes

<i>In-vitro</i>		<i>In-vivo</i>		<i>Raman assignments</i>	<i>Biomolecule</i>	<i>References</i>
<i>K. pneumoniae</i>	<i>S. aureus</i>	<i>K. pneumoniae</i>	<i>S. aureus</i>			
	791			Phosphate backbone vibrations	Nucleic acid	(1)
			860	Ribose ring vibration	Nucleic acid	(4)
	1100			PO <sub>2</sub> <sup>-</sup> in nucleic acids	Nucleic acid	(1)
		1236		Amide III coupled N-H/C-H deformations	Proteins	(1, 4)
		1304		CH <sub>3</sub> CH <sub>2</sub> deformation	All protein, lipids, and carbohydrates	(1)
	1340/1348			G (DNA/RNA)	Nucleic acid	(1)
	1381			Guanine, Adenine ring vibrations	Nucleic acid	
1438/1447		1440		CH <sub>2</sub> CH <sub>3</sub> deformation	All proteins, carbohydrates, and lipids	(2)
	1487			C and G of DNA	Nucleic acid	(4)
	1581	1580		Cytosine and Guanine of DNA	Nucleic acid	(1)
	1677/1685			NADH and Amide I (beta-sheet)	Bound and free NADH	(2, 6)
2840/2852				CH <sub>2</sub> symmetric stretching	Lipids	(4)
2883/2886			2886	CH stretching	Lipids	(4)
	2951			CH <sub>3</sub> asymmetric stretching	Proteins	(4)

### Monocytes

<i>In-vitro</i>		<i>In-vivo</i>		<i>Raman assignments</i>	<i>Biomolecule</i>	<i>References</i>
<i>K. pneumoniae</i>	<i>S. aureus</i>	<i>K. pneumoniae</i>	<i>S. aureus</i>			
791				Phosphate backbone vibrations	Nucleic acid	(1, 5)
	1010	998		Phenylalanine	Proteins	(1, 5)
1104/1100				PO <sub>2</sub> in nucleic acids	Nucleic acid	(1)
1306				CH <sub>3</sub> CH <sub>2</sub> deformation	All protein, lipids, and carbohydrates	(1)
1340				Guanine (DNA/RNA)	Nucleic acid	(1)
		1370		Guanine, Adenine ring vibrations	Nucleic acid	
1381						
	1406					
1447				CH <sub>2</sub> CH <sub>3</sub> deformation	All proteins, carbohydrates, and lipids	(2)
1492				C and G of DNA	Nucleic acid	(4)

	1556	1550	CC stretching Tryptophan	Proteins	(2)
	1593		Guanine, Adenine	Nucleic acid	(1)
	1625		Amide I (C-O stretching)	Proteins	(5)
	1645		Amide I (alpha-helix)	Protein,	
	1677		NADH and Amide I (beta-sheet)	Bound and free NADH	(2, 6)
2856		2842	CH <sub>2</sub> symmetric stretching	Lipids	(4)
		2872	CH <sub>3</sub> symmetric stretching	Lipids	(4)
2897			CH stretching	Lipids	(4)
	2924		CH stretching	Proteins	(4)
	2940	2946	CH stretching	Proteins	(4)

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**Table S4.** Number of patients, measured (and analyzed) cells per patient and subtypes used to compare *in-vitro* data and *in vivo* data. Leukocyte subtype was assigned by visual inspection of nuclei morphology using Kimura staining after Raman measurement. Thus, not equal numbers of cells were measured in each subpopulation.

	Sterile inflammation *			Infected *						
	number of patients	Pat. ID	Measured (analysed)# cells	Number of patients	Pat. ID	Measured (analysed)# cells	sepsis-3 classification	Pathogen and focus	Used to differentiate bacteria vs. fungi	Used to differentiate Gram-positive vs. Gram-negative
Neutro- phils	5	1	14 (14)	7	6	21 (17)	sepsis	<i>S. aureus</i> / blood and abscess	Bacteria	Gram-positive
					7	11 (10)	sepsis	<i>E. faecium</i> + <i>E.coli</i> / blood+abdomen	Bacteria	Gram-positive
					8	39 (36)	sepsis	CNS+ <i>Streprococcus spp</i> / abscess	Bacteria	Gram-positive
					9	29 (28)	sepsis	<i>K. pneumonia</i> /lung	Bacteria	Gram-negative
		2	22 (19)		10	22 (22)	sepsis	<i>Lactobacillius spp</i> +Candida / blood+abdomen	Fungi	-
		3	9 (9)			37 (36)	sepsis	<i>E. faecium</i> +Candida / abdomen	Fungi	-
		4	12 (11)			23 (22)	infection	<i>K. oxytoca</i> / urine	Bacteria	Gram-negative
		5	44 (23)							
Σ#cells	5		101 (76)	7		182 (171)				
Lympho- cytes	4	1	1 (1)	6	10	4 (4)	sepsis	<i>Lactobacillius spp</i> +Candida / blood+abdomen	Fungi	Gram-positive
					11	3 (2)	sepsis	<i>E. faecium</i> + Candida / abdomen	Fungi	Gram-positive
					13	2 (2)	sepsis	<i>Proteus spp.</i> + <i>E. coli</i> / blood+abdomen	Bacteria	Gram-negative
		2	4 (3)		14	3 (3)	sepsis	<i>E. coli</i> / abdomen	Bacteria	Gram-negative
		3	6 (6)		15	2 (2)	sepsis	<i>E. coli</i> / urine	Bacteria	Gram-negative
		4	8 (8)		16	1 (1)	infection	<i>E. coli</i> / urine	Bacteria	Gram-negative
		Σ#cells	4			19 (18)	6		15 (14)	
Mono- cytes	3	1	3 (3)	6	6	5 (5)	sepsis	<i>S. aureus</i> / blood+abscess	Bacteria	Gram-positive
		2	2 (2)		7	4 (4)	sepsis	<i>E. faecium</i> + <i>E.coli</i> / blood+abdomen	Bacteria	Gram-positive
		4	6 (5)		8	4 (4)	sepsis	CNS+ <i>Streprococcus spp</i> / abscess	Bacteria	Gram-positive
					12	2 (2)	infection	<i>K. oxytoca</i> / urine	Bacteria	Gram-negative
					10	4 (3)	sepsis	<i>Lactobacillius spp</i> + Candida / blood+abdomen	Fungi	-
		11	1 (1)		sepsis	<i>E. faecium</i> + Candida / abdomen	Fungi	-		
Σ#cells	3		11 (10)	6		20 (19)				

\* For detailed group assignment see the original publication of the clinical trial (11), sterile inflammation refers to inflammation due to non-infectious origin (e.g. surgery),

# For cases when the whole cell scan was considered background according to the automated quality check described in subsection 4.7 (main manuscript), the scans were excluded from analysis and the actual number of analyzed cells is specified in parentensis.

**Table S5.** Number of cells measured for each treatment and from each donor. 100 spectra/cell were used for analysis. For donor 6 only neutrophils were analyzed because isolated PBMC's were not enough in number to continue the experiment.

	Leukocytes sub-population	Non-infected	<i>C. albicans</i>	<i>K. pneumoniae</i>	<i>S. aureus</i>
Donor 1	Neutrophils	10	10	12	10
	Lymphocytes	14	11	10	12
	Monocytes	10	4	5	10
Donor 2	Neutrophils	13	10	10	10
	Lymphocytes	10	15	11	10
	Monocytes	11	10	10	10
Donor 3	Neutrophils	10	11	10	10
	Lymphocytes	15	10	10	11
	Monocytes	10	10	10	10
Donor 4	Neutrophils	10	11	9	10
	Lymphocytes	13	12	12	10
	Monocytes	10	10	10	10
Donor 5	Neutrophils	11	13	10	13
	Lymphocytes	18	10	11	11
	Monocytes	10	10	10	10
Donor 6	Neutrophils	10	10	19	10

## References

1. Neugebauer U, Clement JH, Bocklitz T, Krafft C, Popp J. Identification and differentiation of single cells from peripheral blood by Raman spectroscopic imaging. *J Biophotonics*. 2010;3(8-9):579-87.
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