**Materials and Methods**

**Preparation of Human neutrophilic granulocytes**

Venous blood of healthy adult volunteers was drawn following the procedures approved by the National Ethical Committee (31937-7/2020/EÜIG). Neutrophilic granulocytes were isolated with dextran sedimentation followed by a 62.5 V/V% Ficoll gradient centrifugation (Beckman Coulter Allegra X-15R, 700 g, 20 min, 22°C). The obtained samples contained more than 95% neutrophils and less than 0.5% eosinophils (data not shown).

**Experimental conditions**

In each experiment, the following conditions were used to measure neutrophil functions: In a 5% CO2 environment, Hank’s balanced salt solution (HBSS, HyClone) was used, supplemented with 25 or 50 mM NaHCO3 (Sigma-Aldrich). As adequate osmotic controls, we used HBSS supplemented with 25 or 50 mM NaCl (Biolab) in an ambient air environment. Plain HBSS in a 5% CO2 and ambient air environments was used for pH control samples. All experiments were performed at 37 °C.

**Viability assessment**

For the measurement of viability, 3×106 PMN cells were incubated with 1 µl of FITC conjugated Annexin V antibody (BD Biosciences) and 1 µl of propidium iodide (PI, ThermoFisher Scientific) in 500 µl final volume using the above-described conditions. Afterward, samples were transferred into Eppendorf tubes, centrifuged, and washed twice with ice-cold HBSS. Finally, 5,000 cells were measured in each sample using a Cytoflex flow cytometer. During evaluation, double negative cells were considered viable, PI single positive cells as necrotic cells, annexin V single positive neutrophils as early apoptotic while PI and annexin V double positive cells as late apoptotic PMN.

**Measurement of Phagocytosis**

GFP expressing *S. aureus* (USA300) was a kind gift from Professor William Nauseef (University of Iowa, USA). Approx. 109 bacteria (one mL bacteria solution at OD600nm=1.0) were opsonized with 10% pooled human serum (derived from 3 donors) for 20 minutes at 37°C and then washed three times with HBSS. After that, 6x105 neutrophils were co-incubated with 3×107 opsonized *USA300* (1:50 ratio) for 20 minutes at 37°C with 600 rpm shaking in the above-mentioned experimental conditions. Before incubation and every 5 minutes (at 0, 5, 10, 15, and 20 min.), 20% of the samples were taken out and fixed using 4% paraformaldehyde (Sigma-Aldrich). The ratio of phagocytosing cells and the phagocytosis capacity (the median fluorescence intensity of phagocytosing cells) was determined using a Cytoflex flow cytometer (Beckman Coulter) by measuring 5,000 cells/sample.

For the visual representation of phagocytosis, polymorphonuclear neutrophils (PMNs) were co-incubated with green fluorescent, serum opsonized, *S. aureus* as previously described, centrifuged onto coverslips (ThermoFisher Scientific) in 6 well plates (Biofil), fixed with 4% PFA for 15 minutes, permeabilized with 0,1% Tween-20 (Sigma-Aldrich) for 5 minutes and then filamentous actin was stained with Alexa568 conjugated phalloidin (1:50, 20 min.). After each step, samples were washed with 1 mL of cold PBS. Finally, microscopic slides were prepared, and images were captured using a Zeiss LSM510 laser scanning confocal microscope equipped with a 63x/1.3 oil immersion objective.

**Determination of the spreading capability of neutrophils**

To prepare an immune complex surface, nunc MaxiSorp microtiter plates (ThermoFisher Scientific) were coated with 20 µg/mL human serum albumin (HSA, Sigma-Aldrich), diluted in bicarbonate buffer (35 mM sodium bicarbonate, 15 mM sodium carbonate) overnight at 4 °C, then washed three times with HBSS, and blocked with 10 % fetal bovine serum (FBS, Capricorn Scientific) for one hour at room temperature. After another washing step, the anti-HSA antibody (Sigma-Aldrich) diluted in HBSS at 10 µg/mL concentration was added to the wells for 1 hour. To measure the background, antibody-free HBSS was added. After washing the plates, 105 PMNs resuspended in the previously described different solutions were added to the wells, and the plates were incubated in 5% CO2 or ambient air for 20 minutes. Afterward, cells were fixed with 4% PFA, and images were captured with a Leica (Wetzlar, Germany) DMI 6000B inverted microscope with a 20× phase contrast objective, connected to a Leica DFC480 CCD camera. For each different condition, two parallel wells were prepared and photographed. During evaluation, all cells were counted and identified as spread or non-spread in one field of view per well by an observer blinded to the experiment.

**Measurement of the F-actin amount in PMN**

To measure the amount of F-actin in PMNs during phagocytosis, neutrophils were co-incubated with GFP-expressing *S. aureus* for 10 min as previously described (see measurement of phagocytosis). Cells were fixed with 4% PFA solution for 15 minutes, permeabilized with PBS containing 0,1% Tween-20 for 5 minutes and F-actin was stained using Alexa568 conjugated phalloidin in 1:50 dilution (after each step, cells were washed twice with 1 mL of cold PBS). Afterward, samples were measured by flow cytometry. Using a complex gating strategy based on the engulfed bacteria's green fluorescence and F-actin's red fluorescence, we determined the relative amount of F-actin in phagocytosing and non-phagocytosing PMNs.

**Assessment of Superoxide Production**

For the measurement of superoxide production, lucigenin was added to neutrophils at a 50 μg/mL concentration and in each well, 2×105 cells were stimulated using opsonized zymosan (OPZ, 500 μg/mL) on a 96-well, white, flat-bottom plate (Greiner, Frickenhausen, Germany) in the previously described conditions. Luminescence intensity was measured every 30 seconds for 1 hour on a 450-550 nm spectra range on a CLARIOstar luminometer (BMG Labtech, Ortenberg, Germany). The basal luminescence of neutrophils was also measured without opsonized zymosan for each condition.

**Bacterium survival assay**

To measure the bacterium-killing capacity of neutrophils, *S. aureus* bacteria were opsonized with pooled human serum as described at the measurement of phagocytosis section, and then 5×106 neutrophils were co-incubated with 5×107 MSSA (1:10 ratio) for 40 minutes with 100 rpm shaking in the previously described conditions (additionally, each sample was supplemented with 4v/v% Luria-Bertani (LB) broth. Following this, 2,5 ml stopping solution (0.5 mg/ml saponin in HBSS) was used to lyse the neutrophils and then, samples were frozen at -80 ̊C for 20 minutes, and thawed at 37 ̊C. Afterward, samples were inoculated to LB broth on a 96-well microtiter plate (Greiner), in 3-6 parallel wells, and bacterium growth was followed by measuring OD every 3 minutes for 8 hours (Labsystems iEMS Reader MF; Thermo Scientific). Initial bacterium counts, and according to this, the bacterium killing capacity of neutrophils was calculated as previously described (Rada et al. Blood 2004).

**Transmigration assay**

Measurement of neutrophil transmigration capability was performed using a transwell assay. Transwell plates, and inserts (Costar) with 3 µm pore size, were blocked for one hour at 37 ̊C with 10% FBS diluted in HBSS, then washed twice with HBSS. Afterward, for stimulation 100 nM fMLP was prepared in HBSS supplemented with NaHCO3 or NaCl as previously described, and 1 mL of these solutions were added to the wells. Inserts were placed in each well, and neutrophils (2×105 cells in 200 µl volume) in the exact respective solutions were pipetted into the inserts in the case of each sample, then the plates were incubated for 1 hour at 37 ̊C with or without 5% CO2. Following the incubation, the plates were centrifuged (4000 rpm, 3 minutes), the inserts were discarded, and transmigrated cells were collected from the wells and treated with acid phosphatase buffer (0,1 M sodium acetate, 0,1 % acetic acid, 0,1% Triton and 10 µM para-nitrophenyl phosphate, pH=5,3). OD measurement on 450 nm wavelength was performed on 96 well microtiter plates (Greiner) and transmigrated cell numbers were determined using a calibration curve based on serial dilutions of known cell numbers.

**Cytokine production measurement**

To measure cytokine production, 48-well plates (ThermoFisher Scientific) were coated with 10% FBS in HBSS for 1 hour and then washed twice. In each well 3×106 neutrophils and 250 µg OPZ were incubated in 500 µl final volumes for 3 hours using the above-described conditions (‘background’ cytokine production without OPZ stimulus was also measured). Afterward, samples were transferred to Eppendorf tubes, centrifuged at 500 g for 10 minutes and supernatants were collected and stored at -80 ̊C for further measurements (for maximum two weeks). Measurement of IL-8 amounts was performed using an ELISA kit (R&D Systems) according to the manufacturer's instructions.

**Statistical analysis**

GraphPad Prism 8.0.1 software was used for the data analysis and plotting. A pairwise comparison of experimental conditions was performed with a paired t-test, or two-way ANOVA followed by a Tukey post hoc test, depending on the experiment. All p values <0.05 were considered statistically significant.

**Results**

**HCO3- does not affect the viability of neutrophilic granulocytes.**

First, we tested whether the different conditions used in our experiments affect the viability of primary human polymorphonuclear neutrophils (PMN). The cells were incubated under the previously detailed conditions (refer to the methods section) for one hour and stained with annexin V and Propidium Iodide (PI). Samples were then measured using flow cytometry. The HBSS buffer supplemented with 5% CO2 did not change the ratio of double-negative events representing the viable cells (Fig. 1 A, B). Similarly, there was no observable difference in the number of viable cells when comparing the 25 or 50 mM HCO3- supplemented buffer in a CO2 environment to their osmotic controls (25 and 50 mM NaCl in ambient air, respectively) (Fig. 1 A, B). Furthermore, the addition of HCO3- resulted in no significant difference in necrotic (PI positive) or apoptotic (Annexin V single positive and double positive) cell numbers compared to the appropriate controls (Fig. 1 A, C, D). These results suggest that the observed differences in neutrophil effector functions detailed below are indeed the result of bicarbonate’s modulatory/regulatory effect and not due to altered cell viability.

**HCO3- significantly increases the capacity of phagocytosis.**

First, we investigated the effect of bicarbonate on phagocytosis, one of the most essential effector functions of neutrophils. We co-incubated fluorescently labeled (red) PMN and pooled serum-opsonized, green-fluorescent S. aureus for 20 minutes, visualizing the phagocytosis with confocal microscopy or quantifying it with flow cytometry. In the latter case, we determined the ratio of phagocytosing cells by the presence of green fluorescence in PMN cells. The number of engulfed bacteria, or the phagocytosis capacity, was represented by the median fluorescence of cells in the phagocytosing cell's gate. We observed that the slightly reduced pH of the plain HBSS buffer in a 5% CO2 environment did not affect phagocytosis (Fig. 2 A, B, E). In the presence of 25 or 50 mM HCO3-, the ratio of phagocytosing neutrophils slightly, but not significantly, increased compared to osmotic controls (Fig. 2 C, D). However, both 25 and 50 mM HCO3- remarkably increased the capacity for phagocytosis (significant after 10 or 5 minutes, respectively), indicating that the cells that phagocytosed were able to engulf significantly more bacteria in the presence of HCO3- (Fig. 2 A, F, G).

**Bicarbonate enhances the spreading of PMN on an immune complex surface.**

Neutrophils can bind immune complexes (IC) through the interaction between their Fc receptors and the Fc domain of antibodies. If the IC is surface-bound, neutrophils can spread on this surface, which, like phagocytosis, is an actin polymerization-dependent process. Subsequently, they may engage in what is known as frustrated phagocytosis, which can ultimately lead to the release of reactive oxygen and lytic enzymes (hiv). We incubated PMN on a human serum albumin (HSA)-anti-HSA immune complex surface to explore cell spreading under various experimental conditions. We assessed the ratio of spread neutrophils (Fig 3A, dark cells) against non-spread cells (Fig 3A, yellow, round cells). Similar to the capacity for phagocytosis, the spreading ability of neutrophils was significantly increased by 25 and 50 mM HCO3- compared to their respective osmotic controls. Five percent carbon dioxide did not change the ratio of spread PMN, indicating that bicarbonate has a pH-independent enhancing effect on this aspect of neutrophil function (Fig 3 A, B).

**During phagocytosis, the filamentous actin amount of neutrophils is elevated by HCO3-.**

We conducted similar phagocytosis experiments as described above to investigate the reasons for the enhanced phagocytosis capacity and spreading of PMN in HCO3--supplemented buffer. After 10 minutes of co-incubation with S. aureus, we fixed and stained the cells for F-actin and analyzed the samples using flow cytometry. To evaluate our data, we employed a three-step gating strategy. First, we selected the intact cells on the FSC-SSC dot plot and constructed two gates based on FITC fluorescence within this selection. FITC-negative cells did not phagocytose any bacteria, while FITC-positive cells phagocytosed at least one bacterium (Fig. 4 A, B). Finally, we measured the mean Alexa568 fluorescence in these two gates (non-phagocytosing and phagocytosing PMN), which reflects the F-actin amount (Fig. 4 C-F). As expected, the F-actin levels indicated approximately a 2-fold increase in phagocytosing cells compared to non-phagocytosing ones in each case (Fig. 4 D, F). In non-phagocytosing PMN, there was no noticeable difference in F-actin amount between HCO3--supplemented and osmotic control samples (Fig. 4 C, D). Interestingly, in the case of the phagocytosing cells, while CO2 alone did not influence the F-actin level of PMN, the presence of both 25 and 50 mM HCO3- significantly increased the F-actin amount in neutrophils compared to the osmotic control NaCl (Fig. 4 E, F). These results indicate that the previously observed elevated phagocytosis capacity arises from increased actin polymerization during phagocytosis in the presence of HCO3-.

**Bicarbonate enhances the superoxide production of PMN.**

The phagocytosis-related superoxide production of neutrophils was measured using a lucigenin-based luminescence assay. Cells were stimulated with pooled serum opsonized zymosan, and superoxide production was monitored for 1 hour (Fig. 5 A). The maximal superoxide production (the peak of the luminescence curve) was significantly reduced by CO2 alone (Fig. 5 B). However, supplementing the buffer with 25 or 50 mM HCO3- in a CO2 environment not only eliminated this difference but also increased the maximal superoxide production of PMN compared to the osmotic controls (Fig. 5 B). We also calculated the superoxide production rate, represented by the RLU change per second on the steepest ascending sections of the curves. Similar to the maximal superoxide production, the rate of superoxide production was diminished by CO2 alone, but it was enhanced when bicarbonate was added to the samples (Fig. 5 C).

**The *S. aureus* killing of neutrophils is more effective in the presence of HCO3-.**

To measure the culmination of these functions, which ultimately manifest in the pathogen-killing capacity of PMN, we used a bacterial survival assay (hiv.). Neutrophils were co-incubated with opsonized S. aureus for 40 minutes, after which PMN cells were lysed, and surviving bacteria were grown in LB broth while OD was measured. CO2 alone caused no significant difference in the percentage of surviving bacteria; however, the presence of both 25 and 50 mM HCO3- in a 5% CO2 environment resulted in a decreased number of living bacteria compared to the respective osmotic control NaCl (Fig. 6). It should be noted that in these measurements, rather than NaCl decreasing, HCO3- increased the bacteria-killing capacity of PMN (Fig. 6).

**Bicarbonate does not affect the in vitro transmigration and IL-8 production of PMN.**

In vitro transmigration was measured using transwell plates with a 3 µm pore size in response to the bacterial chemotactic oligopeptide Formyl-Methionine-Leucine-Phenylalanine (fMLP). This system models the extravasation of neutrophils during bacterial infection. In the absence of fMLP stimulus, the ratio of transmigrated cells was minimal (around 5%). After one hour of stimulation, approximately 60-70% of the cells transmigrated across all samples, and neither CO2 nor HCO3- alone caused a significant difference in the number of transmigrated neutrophils (Fig. 7 A).

PMNs were activated with pooled serum opsonized zymosan for 3 hours under the previously described conditions, and the IL-8 levels were measured from the supernatants of the samples. Although no effect of 25 mM HCO3- was observed, 50 mM HCO3- led to a decreased tendency in IL-8 production in PMNs (Fig. 7 B), likely due to the prolonged alkaline conditions (pH ≈ 7.7 for 3 hours).

**Discussion**

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**Bicarbonate enhances the superoxide production of PMN.** This is probably due to the slightly acidic pH in these samples, which aligns with the literature (hiv.).

**The *S. aureus* killing of neutrophils is more effective in the presence of HCO3-.** Neutrophils' capability to eliminate bacteria depends on several effector functions, including phagocytosis, superoxide production, degranulation, and NET formation (hiv.).

**The *S. aureus* killing of neutrophils is more effective in the presence of HCO3-.** Both the addition of NaCl and HCO3- increases the osmotic burden of neutrophils, which is detrimental to the effector functions (hiv.), however, in the bicarbonate supplemented samples, there was no such decrease in bacteria killing, thus these differences are significant and, in our opinion, biologically relevant as well.

**Figure Legends**

**Figure 1. PMN viability remains unaffected by the various conditions used in our experiments.** Neutrophils were incubated with fluorescently labeled annexin V and Propidium Iodide (PI) for 1 hour under the previously described conditions, and samples were analyzed using flow cytometry. Representative dot plots illustrate our evaluation strategy: double-negative cells were deemed viable, PI single-positive events were classified as necrotic, annexin V single-positive events were identified as early apoptotic, and PI and annexin V double-positive events were recognized as late apoptotic cells (A). No significant difference in the number of viable (B), apoptotic (C), or necrotic (D) PMNs was observed across the different conditions. n=4; mean + SEM.

**Figure 2. HCO3- significantly enhances the phagocytosis capacity of PMN cells.** For the visual representation of phagocytosis, PMNs were co-incubated with serum-opsonized S. aureus (green) in a 1:50 ratio, either in the presence or absence of HCO3-. The mixture was centrifuged onto coverslips, fixed, and filamentous actin was stained using Alexa568-conjugated phalloidin (red) (A). The kinetics of phagocytosis were measured by flow cytometry. Neutrophils were co-incubated with bacteria for 20 minutes, and 20% of the samples were fixed every 5 minutes. The ratio of phagocytosing cells only slightly increased in the presence of HCO3- (B-D); however, there was a remarkable increase in the phagocytosis capacity of PMNs (A, E-G). \*\*:p<0.01, \*\*\*:p<0.001, \*\*\*\*:p<0.0001; n=5; mean+SEM.

**Figure 3. Bicarbonate enhances PMN spreading on an immune complex surface.** The HSA-anti-HSA immune complex surface was established on microtiter plates, after which neutrophils were added for 20 minutes under the previously described experimental conditions. Subsequently, cells were fixed, images were captured (A), and the percentage of spread cells was calculated (B). Both 25 and 50 mM bicarbonate significantly increased the ratio of spread PMN compared to their respective osmotic controls, while CO2 alone resulted in only a slight, non-significant increase (A, B). Scale bars represent 25 µm. \*\*:p<0.01; n=4; mean+SEM.

**Figure 4. The amount of F-actin is increased by HCO3- during phagocytosis.** Serum opsonized, GFP-expressing S. aureus was co-incubated with PMN, and F-actin was stained with Alexa568 conjugated phalloidin. Samples were analyzed using flow cytometry. After identifying intact PMN cells on the FSC-SSC dot plot (A), gates were established for non-phagocytosing and phagocytosing PMN based on the cells' green fluorescence (B). The F-actin amount was determined within these gates by measuring the intensity of red fluorescence (C, E). The amount of F-actin in non-phagocytosing cells remained unchanged (C, D). However, in phagocytosing PMN, the fluorescence of F-actin was significantly increased in the presence of bicarbonate (E, F).\*:p<0.05; n=7; mean+SEM.

**Figure 5. Bicarbonate enhances superoxide production in neutrophils.** The impact of bicarbonate on the superoxide production of PMNs was assessed using a lucigenin-based luminescence assay. PMNs were stimulated with serum-opsonized zymosan, and superoxide production was monitored for 1 hour either with or without bicarbonate. Representative measurement (A). At a concentration of 50 mM, bicarbonate significantly boosted the maximum superoxide production of the cells (B). The rate of superoxide production was notably higher in the presence of 25 mM bicarbonate compared to the osmotic control (C) \*:P<0.05; n=8; mean+SEM.

**Figure 6. HCO3- enhances the bacterial killing capacity of PMN.** Serum-opsonized S. aureus was co-incubated with PMN for 40 minutes under different conditions, after which the neutrophils were lysed, and samples were inoculated into LB broth. OD was monitored for 8 hours, and the surviving bacterial count was determined. Both 25 and 50 mM of HCO3- significantly improved the killing capacity of PMN compared to osmotic controls. \*:p<0.05; n=9; mean+SEM.

**Figure 7. In vitro neutrophil transmigration and IL-8 production are not influenced by HCO3-.** Transwell plates and inserts were blocked with FBS, and fMLP was added to the wells in the various solutions before PMN cells were transferred into the inserts. After 1 hour of incubation with or without 5% CO2, the transmigrated cells were collected from the wells, lysed, and the cell counts were determined. Bicarbonate did not impact the transmigrating capability of neutrophils. n=6; mean+SEM (A). Plates were blocked with FBS, and neutrophils were stimulated with pooled serum opsonized zymosan for 3 hours under different conditions. The supernatants were collected, and IL-8 concentration was measured by ELISA. Bicarbonate did not influence the IL-8 production of neutrophils. n=8; mean+SEM (B).