Anti-*Pseudomonas aeruginosa* IgY antibodies promote bacterial aggregation and internalization in polymorphonuclear neutrophils

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Introduction

Chronic lung infections with *Pseudomonas aeruginosa* (*PA*) in cystic fibrosis (CF) patients are major causes of morbidity and premature death.
Oral treatment with pathogen-specific IgY antibodies poses a promising complement to antibiotics in averting the devastating chronic lung infections with *PA* in CF. Anti-PA IgY antibodies augments the PMN-mediated bacterial killing *in vitro*, indicating a faster bacterial clearance of pathogens in the airways, thus reducing the deteriorating chronic settlement of *PA* in the lungs. The present study aimed at investigating whether IgY-mediated bacterial aggregation improves the internalization of bacteria in polymorphonuclear neutrophils (PMNs).

Conclusion

Anti-*Pseudomonas aeruginosa* IgY antibodies augments bacterial internalization in PMNs and subsequent bacterial killing in vitro. The conferred IgY mediated bacterial aggregation suggest a more promptly and effortless bacterial phagocytosis by PMNs, which may imply a faster bacterial clearance in the CF airways and a mode of action to the observed clinical impact of oral IgY prophylaxis.

Materials and Methods

Anti-*Pseudomonas aeruginosa* IgY solution containing specific anti-*Pseudomonas aeruginosa* (S-IgY) or non-specific (C-IgY) IgY antibodies were obtained from ImmunSystem I.M.S. (Uppsala, Sweden).
***Pseudomonas aeruginosa* PAO1** strain was grown in LB broth. Stationary phase organisms were attained from overnight cultures (ON). To allow aggregation bacteria were mixed with IgY antibodies prior to application.
Neutrophil isolation: PMNs were isolated from whole blood. Erythrocytes were sedimented in 5% dextran and the leucocyte-enriched plasma was layered on Lymphoprep (Axis-Shield, Norway) and centrifuged. After hypertonic lyses, the purified PMNs were re-suspended in Krebs-Ringer buffer supplemented with 10mM glucose (KRB).
Respiratory burst assay: Aliqouts of isolated PMNs were added to wells in a 96-well microtiter plate (Nunc). Phagocytosis was commenced by adding *PA* supplemented with either C-IgY or S-IgY. Fc-receptor blocking was performed by pretreating PMNs with anti-CD16, anti-CD32, anti-CD64 (Abcam) for 15 min at 37°C prior to analysis. Luminol was added to the wells and the luminol-enhanced chemiluminescence analyzing phagocyte-derived reactive oxygen species (ROS) was measured using a luminometer (Wallac 1420 Victor2, Perkin Elmer) at 37°C for 1 hour.
Bactericidal assay: Measuring the loss of bacterial viability over time by mixing (non)-IgY opsonized bacteria and PMNs and plating diluted samples overnight followed by colony counting. Samples were removed at 0 min and 60 min for bacterial killing determination.
FACS: Fluorescent PAO1 (green fluorescent protein [GFP]) were mixed with either S-IgY or C-IgY and allowed to aggregate at room temperature for 1 h before size estimation with FACScanto (BD biosciences).
Indirect immunofluorescense microscopy: PAO1 cultured in LB medium was mixed with S-IgY and incubated at 37°C for 1 h. After washing twice with PBS, TexasRed-conjugated rabbit anti-chicken IgG (Abcam) was added and incubated at 37°C for 1 h. Samples were washed twice with PBS and resuspended in PBS. The solution was smeared on microscope slide and air-dried followed by a drop of DAPI stain and mounting of coverslip. Immunofluorescent micrographs of specimens were obtained using a fluorescent microscope.
Bacterial internalization by PMN: Internalization of bacteria was examined by confocal scanning laser microscopy (CSLM). Aliquots of overnight cultures of fluorescent PAO1 (green fluorescent protein [GFP]) were added to a microtiter plate and mixed with either S-IgY or PBS (control) and allowed to aggregate for 1 h at room temperature. Freshly purified PMNs or PBS for control were added and incubated for 30 min. at 37°C preceding the microscopy.

Results

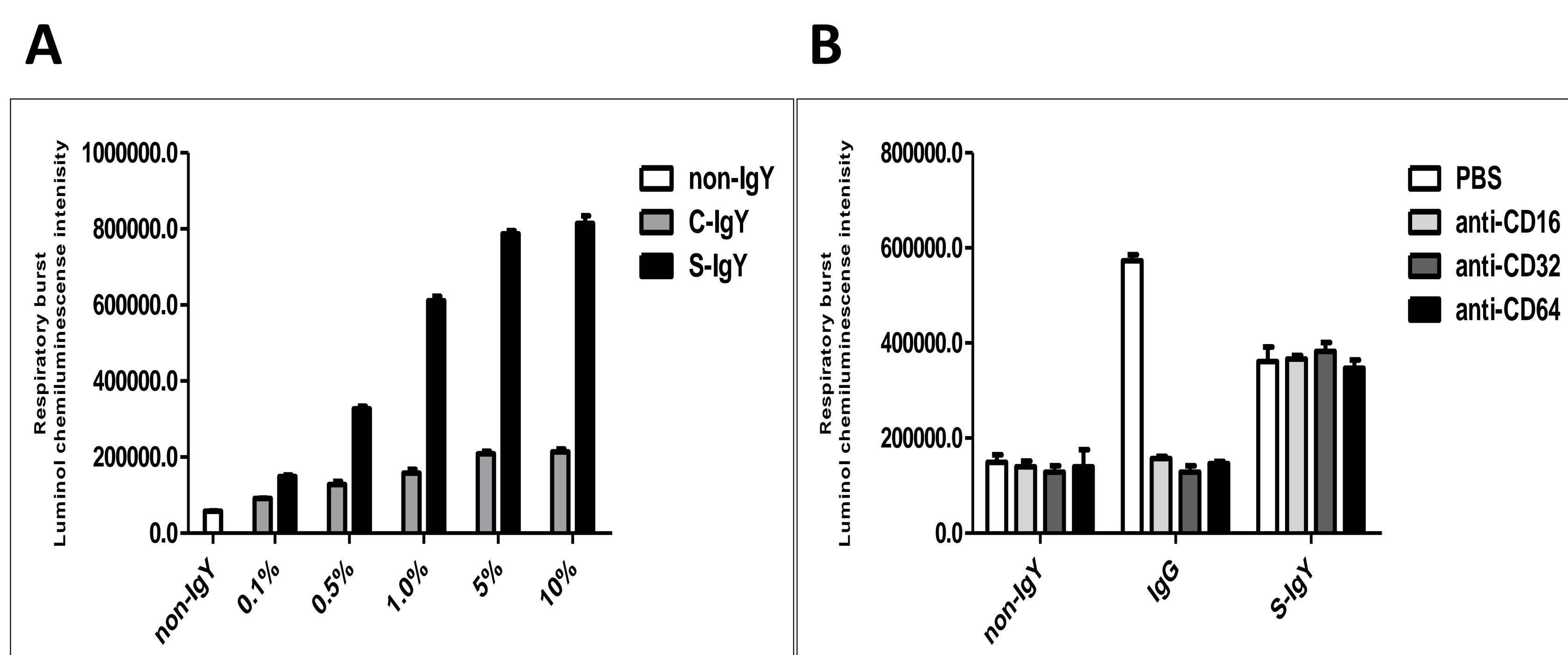

Figure 1. **Respiratory burst. (A) Cumulative burst from PMNs phagocytizing PAO1 opsonized with S-IgY or C-IgY after 1 h. (B) Effect of Fc-receptor blocking on the cumulative burst from PMNs phagocytizing PAO1 opsonized with S-IgY or IgG. Results are depicted as the mean chemiluminescense intensity.**

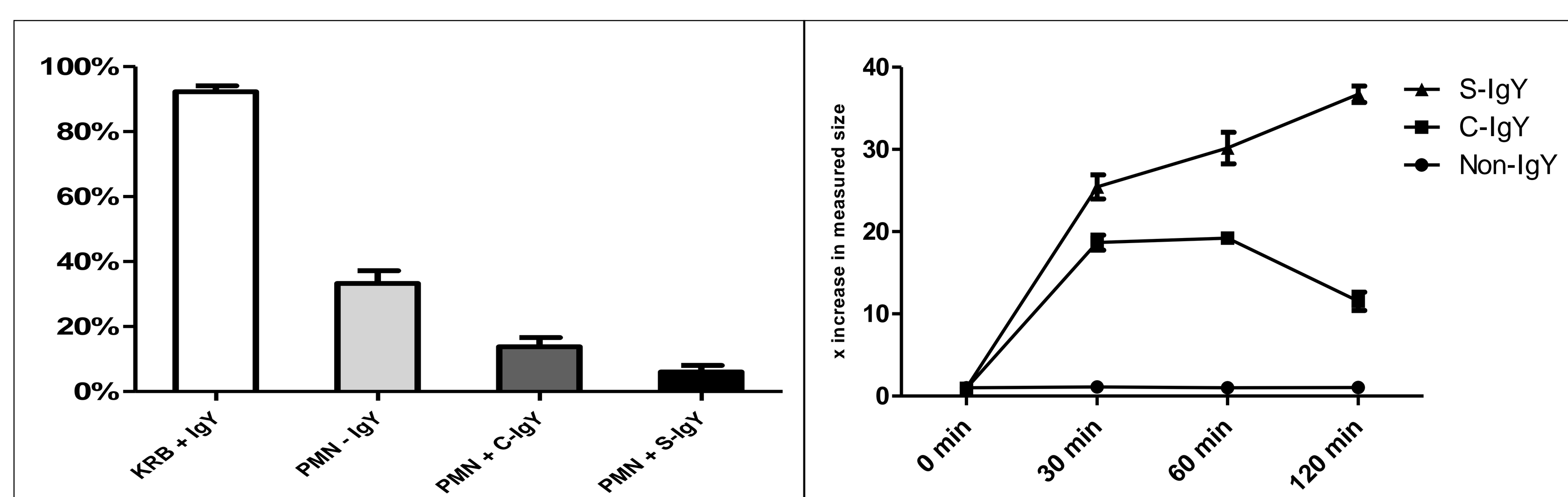

Figure 2: **Bacterial killing capacity by PMNs. The percentage of viable PAO1 opsonized with S-IgY or C-IgY after 1 h of PMN-mediated phagocytosis.**

Figure 3: **FACS results show the increase in measured size in a time period of 2 h. PAO1 ([GFP]) is mixed with either S-IgY or C-IgY and mean size is estimated by FACS.**

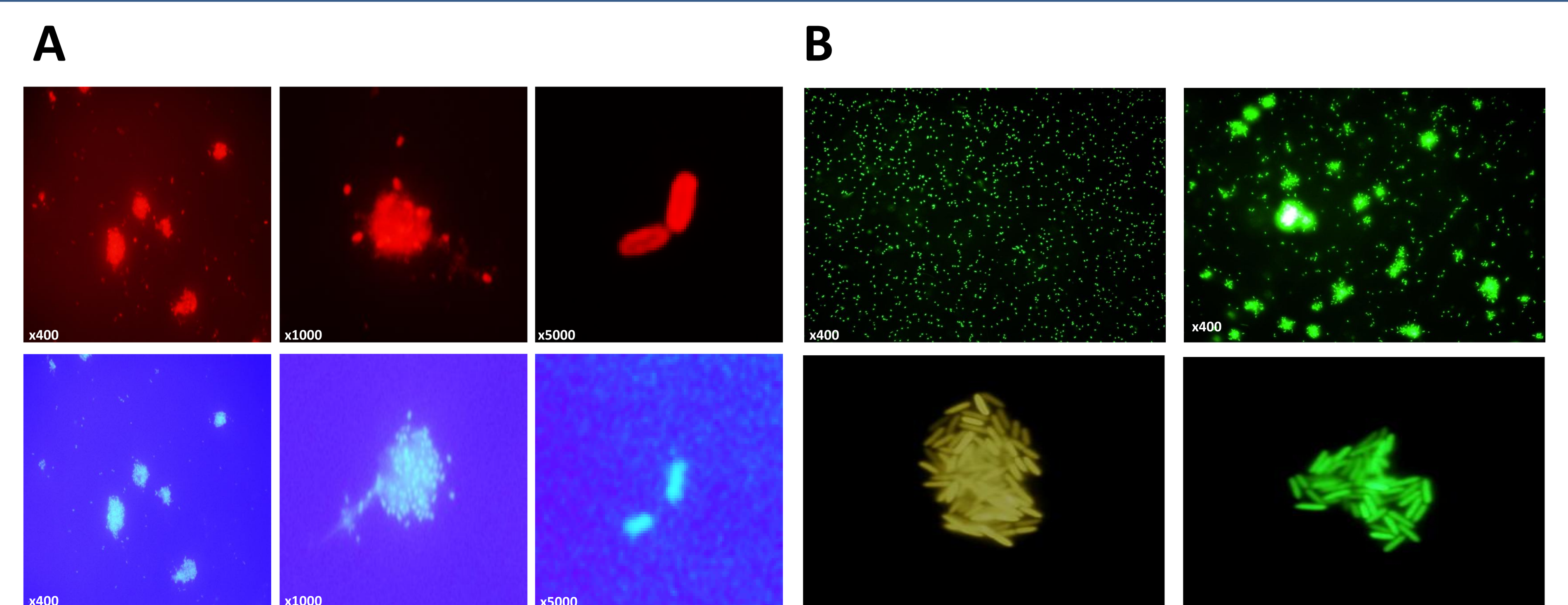

Figure 4. **(A) Indirect immunofluorescense microscopy of PAO1 opsonized with S-IgY. Top images visualize TexasRed-conjugated rabbit anti-chicken IgG detecting S-IgY antibodies. Bottom images display the corresponding DAPI stain. (B) The time-dependent development of S-IgY mediated aggregates of PAO1 ([GFP]). Upper left image shows dispersed bacteria prior to addition of S-IgY. Upper right image shows the formation of bacterial aggregates 30 min after S-IgY is added. Bottom images demonstrate close-ups of bacterial aggregates 2h after S-IgY addition.**

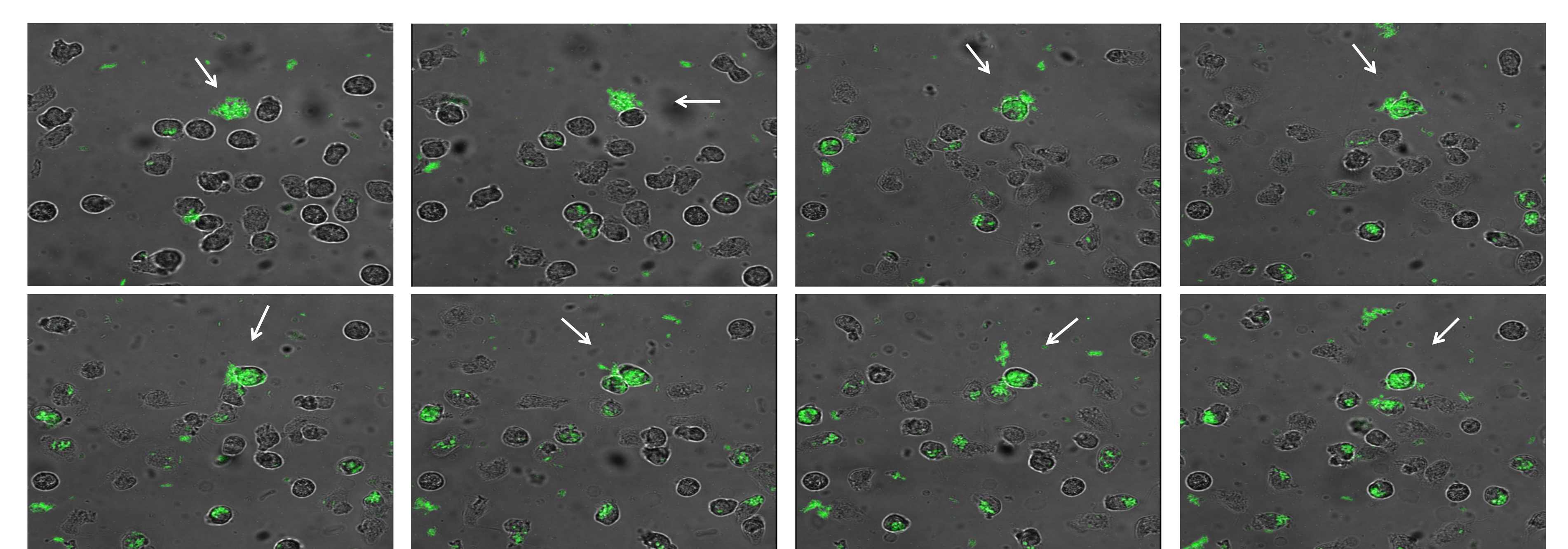

Figure 5: **Time-lapse microscopy of PMNs phagocytizing PAO1 during a period of 20 min. S-IgY opsonized PAO1 ([GFP] were allowed to aggregate 1 h prior to addition of PMNs.**

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