Antibiotic efficacy varies based on the infection model and treatment regimen for *Pseudomonas aeruginosa*

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Supplementary Methods, Figures and Tables

SUPPLEMENTARY METHODS

Mouse model. Immunocompetent C57BL/6NCrlBR male mice (8-10 weeks of age) were purchased from Charles River (Calco, Italy), shipped in protective, filtered containers, transported in climate-controlled trucks, and allowed to acclimatize for at least two days in the stabulary prior to use. Mice were maintained in the biosafety level 3 (BSL3) facility at San Raffaele Scientific Institute (Milano, Italia) where 3-5 mice per cage were housed. Mice were maintained in sterile ventilated cages. Mice were fed with standard rodent autoclaved chow (VRFI, Special Diets Services, UK) and autoclaved tap water. Fluorescent lights were cycled 12h on, 12h off, and ambient temperature (23±1°C) and relative humidity (40-60%) were regulated.

For infection experiments, mice were anesthetized by an intraperitoneal injection of a solution of Avertin (2,2,2- tribromethanol, 97%) in 0.9% NaCl and administered at a volume of 0.015 ml/g body weight. Mice were placed in supine position. The trachea was directly visualised by ventral midline, exposed and intubated with a sterile, flexible 22-g cannula attached to a 1 ml syringe. An inoculum of 60 µl of planktonic bacterial cells or 50 µl of agar-bead suspension was implanted via the cannula into the lung. After inoculation, all incisions were closed by suture.

Mice were monitored daily for coat quality, posture, attitude, ambulation, hydration status and body weight. Mice that lost >20% body weight and had evidence of severe clinical disease, such as scruffy coat, inactivity, loss of appetite, poor locomotion, or painful posture, were sacrificed before the termination of the experiments with an overdose of carbon dioxide. Gross lung pathology was noted.

Broncho alveolar lavage fluid (BALF) was extracted with a 22-gauge venous catheter, ligated to the trachea to prevent backflow. The lungs were washed with three one ml of RPMI-1640 (Euroclone) with protease inhibitors (Complete tablets, Roche Diagnostic) and pooled. Quantitative bacteriology on BALF was performed by plating serial dilution on tryptic soy agar (TSA). Total cells present in the BALF were counted using an inverted light optical microscope after diluting an aliquot of the BALF 1:2 with Tuerk solution in a disposable counting chamber. BALF cells were centrifuged at 330 x g for 8 min at 4°C. If the pellet was red, erythrocytes were lysed by resuspending the pellet in 250- 300 µl of RBC lysis buffer diluted 1:10 in ultra-pure distilled water for 3 min. Then, 2-3 ml PBS were added and cells were centrifuged at 330 x g for 8 min at 4°C. The pellet was resuspended in RPMI 1640 10% fetal bovine serum (FBS) at concentration of $1x10^6$ cells/ml, and an aliquot of 170 µl

was pipetted into the appropriate wells of the cytospin and centrifuged at 300 x g for 5 min medium brake. Slides were then stained by Diff-Quik staining using a commercial kit (Medion Diagnostics, code: 726443), according to the manufacturer's instructions. A differential cell count was performed at an inverted light optical microscope.

Lungs were excised aseptically and homogenized in 2 ml PBS added with protease inhibitors using the homogenizer gentleMACS™ Octo Dissociator. One-hundred µl of the homogenates and 10-fold serial dilutions were spotted onto tryptic soy agar (TSA). Colony Forming Units (CFUs) were determined after overnight growth at 37°C.

Infections, treatments and sacrifices in the chronic infection models were all performed in the late morning, while the sacrifices in the acute infection model were performed in the late afternoon. In addition, in all the experiments, mice had been subdivided according to the body weight to have similar mean in all the groups of treatment.

Animal studies were conducted according to protocols approved by San Raffaele Scientific Institute (Milan, Italy) Institutional Animal Care and Use Committee (IACUC #733 and #878) and adhered strictly to the Italian Ministry of Health guidelines for the use and care of experimental animals.

Bacteria preparation for acute infection. An aliquot of *P. aeruginosa* PAO1 reference strain from glycerol stocks was streaked for isolation on TSA and incubated at 37°C O/N. Bacterial glycerol stocks were not used more than three times to avoid variability in the animal experiments. One colony was picked from the plate and used to inoculate 5 ml of tryptic soy broth (TSB) (BD, Becton and Dickinson) and placed in a shaking incubator at 37°C 200 rpm O/N. The O/N bacterial suspension was diluted to 0.1 OD/ml in 20 ml of TSB / flask and grown for 3 h at 37°C at 200rpm, to reach the log phase [1, 2]. The bacteria were pelleted by centrifugation (2,700 *g*, 15 min, 4°C), resuspended in sterile phosphate-buffered saline (PBS) and diluted to give the required dose in 60 µl (1×10^6 CFUs). Mice were anaesthetized by intraperitoneal injection as described before, then infected intratracheally (i.t.) with *P. aeruginosa* (planktonic form).

Agar-beads preparation for chronic infection. The agar-beads *P. aeruginosa* mouse model was used [2-4]. An aliquot of *P. aeruginosa* MDR-RP73 clinical strain from glycerol stocks was streaked for isolation on TSA and incubated at 37°C O/N. One colony was picked from the plate and used to inoculate 10 ml of TSB and placed in a shaking incubator at 37°C 200 rpm O/N. The O/N bacterial suspension was diluted to 0.15 OD/ml in 20 ml of TSB /

flask and grown for 4 h at 37°C at 200rpm, to reach the log phase. The bacteria were pelleted by centrifugation (2,700 *g*, 15 min, 4°C) and resuspended in 1 ml PBS (pH 7.4). A starting amount of 2 $x10^9$ CFUs of *P. aeruginosa* was used for inclusion in the agar-beads prepared according to the previously described method [2-4]. Bacteria were added to 9 ml of 1.5% TSA (w/v), prewarmed to 50°C. This mixture was pipetted forcefully into 150 ml heavy mineral oil at 50°C and stirred rapidly with a magnetic stirring bar for 6 min at room temperature, followed by cooling at 4°C with continuous slowly stirring for 20 min. The oil-agar mixture was centrifuged at 4,000 rpm for 15 min to sediment the beads, and washed six times in PBS. The size of the beads was verified microscopically and only those preparations containing beads of 100 µm to 200 µm in diameter were used as inoculum for animal experiments. The number of *P. aeruginosa* CFUs in the beads was determined by plating serial dilutions of the homogenized bacteria-bead suspension on TSA plates. The inoculum was prepared by diluting the bead suspension with PBS to $1x10^7$ CFUs/ml, to inoculate about $5x10^5$ CFU/50µl. *P. aeruginosa* beads were prepared the day before inoculation, stored overnight at 4°C for a maximum of two days. The number of *P. aeruginosa* CFUs in the beads inoculated was determined by plating serial dilutions of the homogenized bacteria-bead suspension at the day of the infection.

Mice treatment with antibiotics. Tobramycin (TOB, code: T1783, Sigma-Aldrich) and colistin (COL, sulphate salt, code: C4461, Sigma-Aldrich) powders were dissolved in water for injection for stock solutions. For efficacy experiments, animals were separated into six groups for acute infection and four groups for chronic infection, with three routes of administration studied in an acute model and two routes of administration studied in chronic model.

TOB and COL therapy was initiated five minutes ("early treatment) or seven days after the infection in the case of "late" treatment during chronic infection, and was repeated once a day for seven days in the chronic infection models. Mice were treated with TOB, COL or vehicle (water) either by local administration using Penn-Century MicroSprayer® Aerosoliser (p.c., volume: 50 µl) or intranasal (i.n., volume: 10µl/nostril) or systemic subcutaneous (s.c., volume: 100µl) administration according to the schedule in **Fig. 1** (stock solutions: TOB p.c. 0.88mg/ml, i.n. 2.2mg/ml and s.c. 4.4mg/ml for acute infection; TOB p.c. 7.04mg/ml, i.n. 17.6mg/ml and s.c. 35.2mg/ml for chronic infection; COL p.c. 0.44mg/ml, i.n. 1.1mg/ml and s.c. 2.2mg/ml for both acute and chronic infections). Endotracheal

administration with p.c. was carried out under 5% isoflurane–oxygen. At different time points, mice were humanely euthanized to determine efficacy of treatment unless the welfare of the animals necessitated earlier termination, in accordance with animal welfare regulations.

Pharmacokinetics measurements. Mice were infected with planktonic bacteria and, five minutes after, TOB and COL were administered by systemic or local delivery. At specific time-points, after euthanasia, the diaphragm was cut with exposure of the lungs. Blood was recovered by cardiac puncture in K3-EDTA tubes. Plasma was separated by centrifugation and stored at -80°C. Lungs were recovered in 2ml PBS, homogenized as described above and stored at -80°C. For TOB PK, a solution of 10% TCA in 1/1 water ACN with 50ng/mL Antypirine was used as precipitating agent. 200µl of cold precipitation agent were added to the plasma or homogenate samples (50µl). Samples were vortexed and centrifuged for 15min at 5°C. Supernatant was transferred into a 96 plate and analyzed on a HPLC Shimadzu LC20 coupled with an API 4500 Triple Quadrupole ABSciex with an ESI source. The column was a Kinetex C18 2,6µm, 100A 75*3mm at 35°C (Phenomenex); mobile phases 0.025%HFBA in water (A) and Acetonitrile (B) with a gradient from 0.5min at 98% A to 5% A at 2.5min and isocratic mode at 5% A until 4min at 400µl/min. TOB MRM transitions: 468/163.3 Da Dwell time 150msec DP 60 volts, CE 30 volts; Antipyrine 189.2/77.1 Da Dwell time 150ms DP 36 volts CE 42 volts. Analytical range plasma 1 - 1000 ng/mL and 5 - 2000ng/mL in lung homogenate.

For COL PK, samples were prepared as previously described for TOB with 150 µl of precipitating agent 10% TCA in 1/1 water/ACN. Column was a Kinetex XB C18 5µm, 100A 50*2.1 mm (Phenomenex) at 35°C; mobile phases 0.1% Formic acid in water (A) and 0.1% Formic acid in Acetonitrile (B) with a gradient from 98% A at 0.5min to 40% A in 5 min then to 5% A at 6min and isocratic mode at 5 % until 6.5min at 300µl/min. COL MRM transitions: 386.1/ 101.1 Da, Dwell time 150msec DP 65 volts, CE 22 volts. Analytical range 1-1000 ng/mL in plasma and from 10 to 2000 ng/mL in lung homogenate.

TABLES

Table S1. *In vitro* activity of tobramycin (TOB) and colistin (COL) against PAO1 and MDR-RP73 *P. aeruginosa* strains. MIC values (µg/mL) were determined by the microdilution method in cation-adjusted Müller-Hinton (MH-II) broth, according to the CLSI guidelines [5]. Doses for TOB and COL for efficacy in animal models were adjusted according to MICs. The ratio between local and systemic doses was 1:10 [6].

Strain	**TOB MIC**	**Route of administration and dose**		**COL MIC**	**Route of administration and dose**	
		Local	**Systemic**		**Local**	**Systemic**
PAO1	0.25	2 mg/kg	20 mg/kg	0.125	1 mg/kg	10 mg/kg
MDR-RP73	2	16 mg/kg	160 mg/kg	0.125	1 mg/kg	10 mg/kg

Table S2. Bacterial load and incidence of bacterial clearance upon tobramycin (TOB) and colistin (COL) treatment in murine models of acute and chronic *P. aeruginosa* lung infection.

Infection model / Treatment schedule	Administration Route	CFUs[a]				% of clearance[b]			
		Vehicle	TOB	Vehicle	COL	Vehicle	TOB	Vehicle	COL
Acute	p.c.	7.1×10^3	1	4.6×10^4	1.8×10^2	0	67	0	0
	i.n.	2.1×10^4	4.1×10^3	4.9×10^3	8.0×10^3	0	12	0	0
	s.c.	2.1×10^4	3.3×10^1	7.8×10^3	2.0×10^2	0	50	0	0
Chronic / early	p.c.	4.0×10^3	1	1.0×10^3	1.3×10^2	0	80	10	36
	s.c.	4.3×10^3	0	2.8×10^3	1.3×10^3	0	100	8	0
Chronic / late	p.c.	2.0×10^3	1.5×10^3	1.8×10^3	1.3×10^3	9	25	0	17
	s.c.	8.8×10^3	4.5×10^3	8.9×10^3	4.0×10^3	0	8	0	0

[a] Data represent median values of lung CFUs in mice

[b] Incidence of bacterial clearance

11 **FIGURES**
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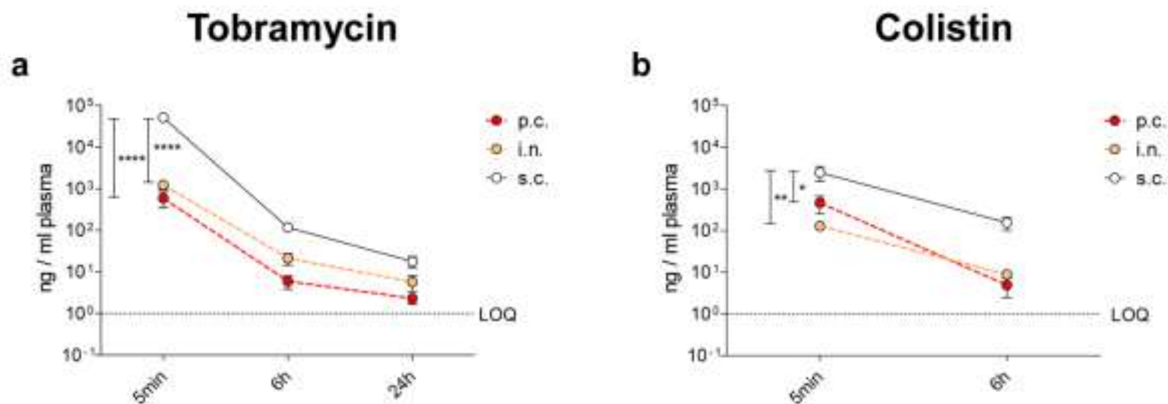

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16 **Supplementary Fig. S1. Pharmacokinetics of tobramycin (TOB) and colistin (COL) in**
17 **murine plasma during acute *P. aeruginosa* airway infection**. C57BL/6NCrlBR male mice
18 (8 to 10 weeks of age) were infected intratracheally with $1x10^6$ CFUs of planktonic PAO1.
19 Five minutes after infection, 2 mg/kg TOB (**a**), 1 mg/kg COL (**b**) or vehicle was administered
20 via the aerosol (p.c.) or intranasal (i.n.) route. Alternatively, 20 mg/kg TOB (**a**), 10 mg/kg
21 COL (**b**) or vehicle was administered via the subcutaneous (s.c.) route. The mice were
22 sacrificed five min, six and 24 hours after treatment and the concentration of antibiotics was
23 measured in the plasma. Data represent mean values ± standard errors of the mean
24 (SEMs). The limits of quantification are indicated (LOQs). The data were pooled from two
25 independent experiments (*n*=4-8). Statistical significance is indicated as follows: **, *P*<0.05;
26 ** *P*<0.01; ****, *P*<0.0001.
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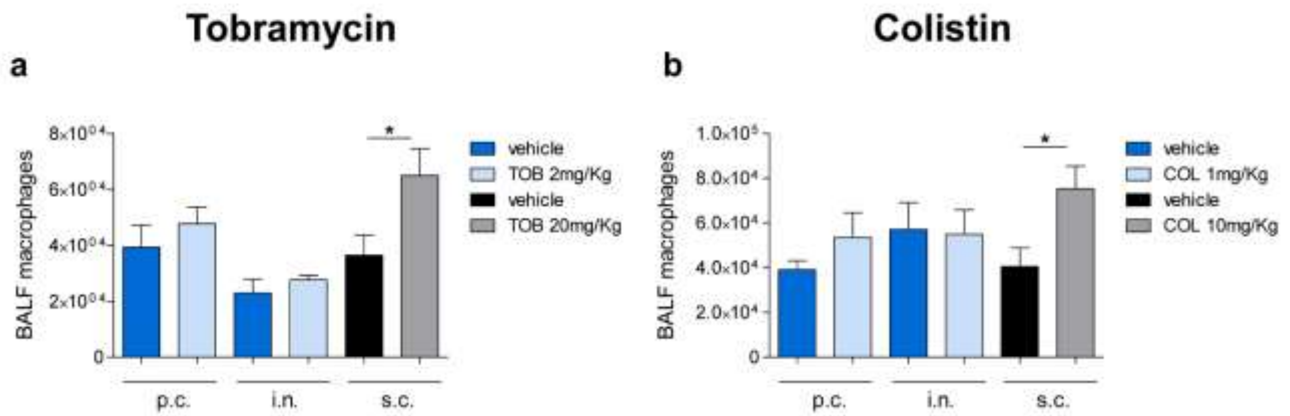

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31 **Supplementary Fig. S2. Murine macrophages after tobramycin (TOB) and colistin**
32 **(COL) treatment in acute *P. aeruginosa* airway infection.** C57BL/6NCrlBR male mice (8
33 to 10 weeks of age) were infected intratracheally with $1x10^6$ CFUs of planktonic PAO1. Five
34 minutes after infection, 2 mg/kg TOB (**a**), 1 mg/kg COL (**b**) or vehicle was administered via
35 the aerosol (p.c.) or intranasal (i.n.) route. Alternatively, 20 mg/kg TOB (**a**), 10 mg/kg COL
36 (**b**) or vehicle was administered via the subcutaneous (s.c.) route. The mice were sacrificed
37 after six hours and the bronchoalveolar lavage fluid (BALF) was collected. Macrophage
38 counts were performed in BALF. Data represent the mean values $\pm$ SEMs. The data were
39 pooled from two independent experiments (*n*=7-8). Statistical significance is indicated as
40 follows: *, $P<0.05$.

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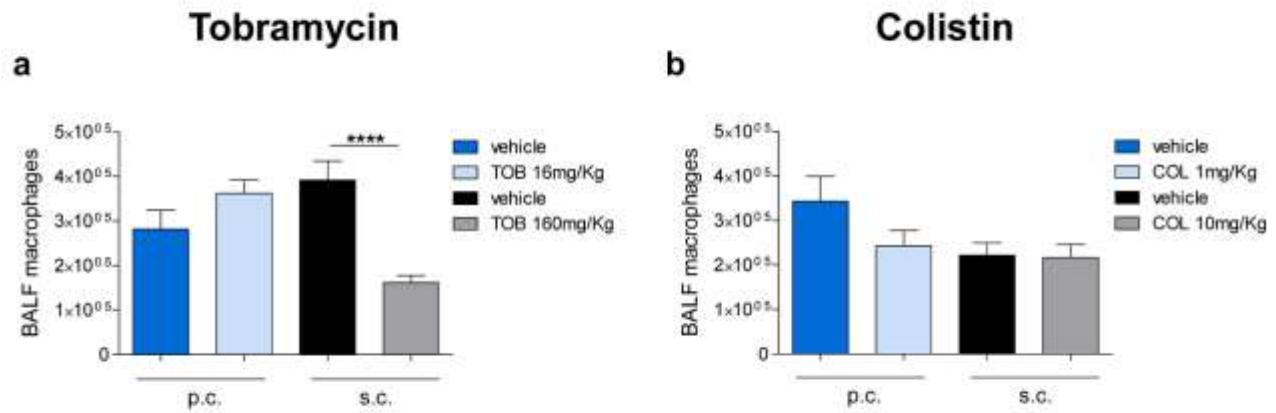

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43 **Supplementary Fig. S3. Murine macrophages after "early" treatment with tobramycin**
44 **(TOB) and colistin (COL) during chronic *P. aeruginosa* airway infection.**
45 C57BL/6NCrlBR male mice (8 to 10 weeks of age) were infected with $5x10^5$ CFUs of MDR-
46 RP73, embedded in agar-beads, by intratracheal inoculation. Treatment, started five
47 minutes after infection, was performed daily for seven days by aerosol (p.c.) with vehicle, 16
48 mg/kg TOB (**a**) or 1 mg/kg COL (**b**) or by subcutaneous (s.c.) administration with vehicle,
49 160 mg/kg TOB (**a**) or 10 mg/kg COL (**b**). The mice were sacrificed at day seven post-
50 infection and the bronchoalveolar lavage fluid (BALF) was collected. Macrophage counts
51 were performed in BALF. Data represent the mean values ± SEMs. The data were pooled
52 from two independent experiments (*n*=11-16). Statistical significance is indicated as follows:
53 ****, *P*<0.0001.

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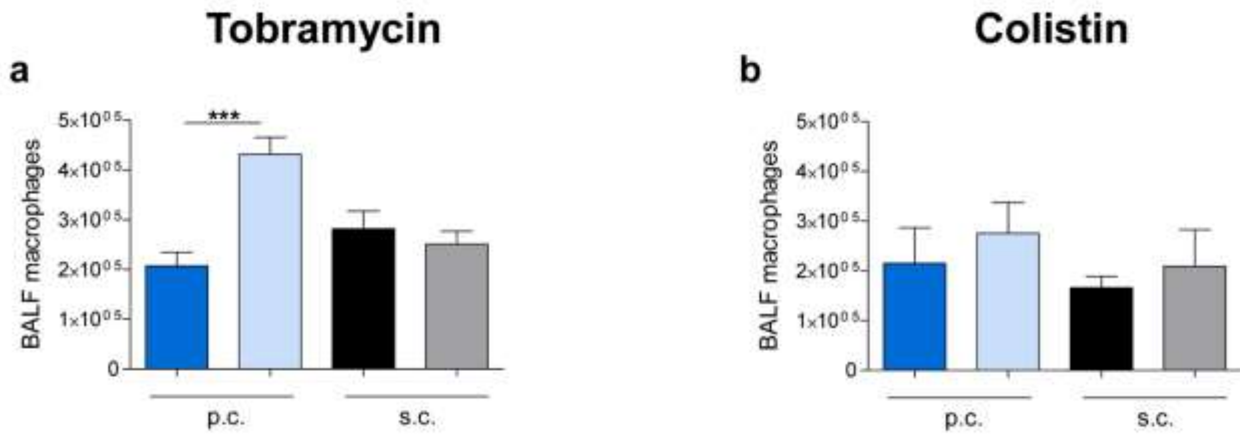

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57 **Supplementary Fig. S4. Murine macrophages after "late" treatment with tobramycin**
58 **(TOB) and colistin (COL) during chronic *P. aeruginosa* airway infection.**
59 C57BL/6NCrlBR male mice (8 to 10 weeks of age) were infected with $5x10^5$ CFUs of MDR-
60 RP73, embedded in agar-beads, by intratracheal inoculation. Treatment, started seven days
61 post-infection, was performed daily for another seven days by aerosol (p.c.) with vehicle, 16
62 mg/kg TOB (**a**) or 1 mg/kg COL (**b**) or by subcutaneous (s.c.) administration with vehicle,
63 160 mg/kg TOB (**a**) or 10 mg/kg COL (**b**). Seven days after the beginning of treatment, which
64 is a total of fourteen days after infection, the mice were sacrificed and the bronchoalveolar
65 lavage fluid (BALF) was collected. Macrophage counts were performed in BALF. Data
66 represent the mean values ± SEMs. The data were pooled from one to two experiments
67 (*n*=5-14). Statistical significance is indicated as follows: ***, *P*<0.001.

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