Pathophysiology of staphylococcal bone and joint infections

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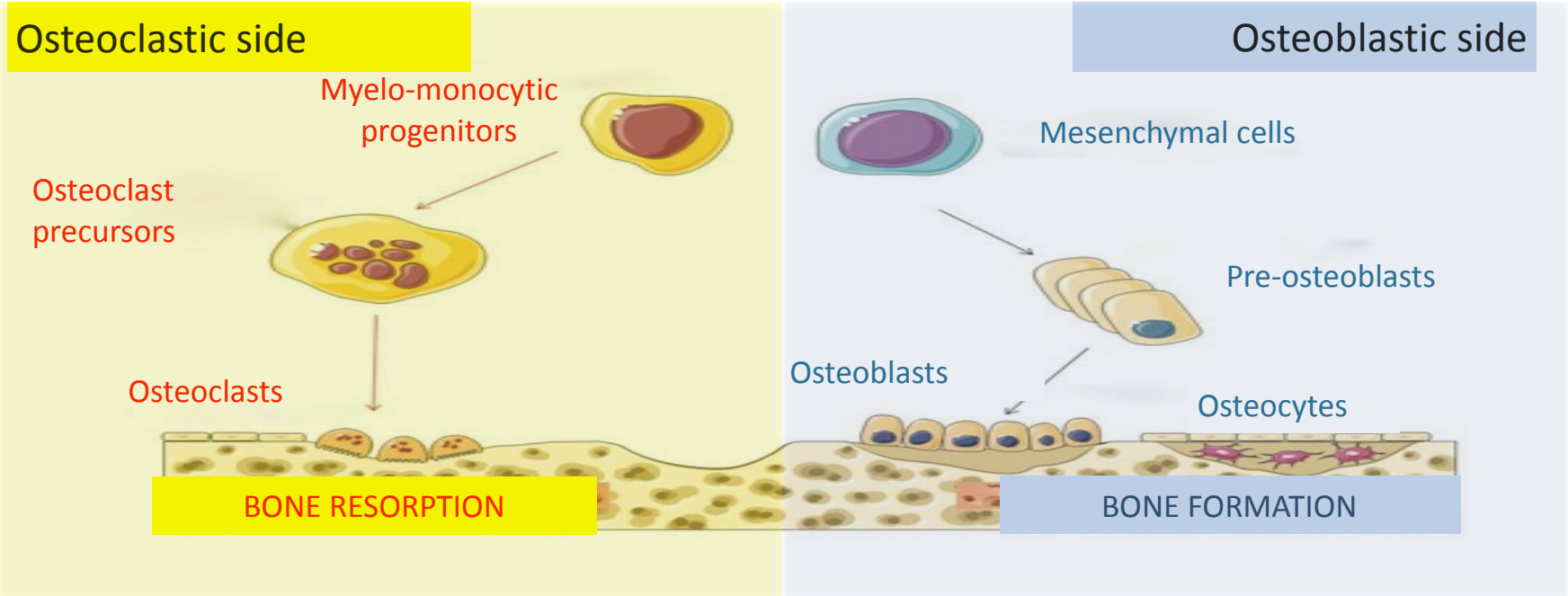
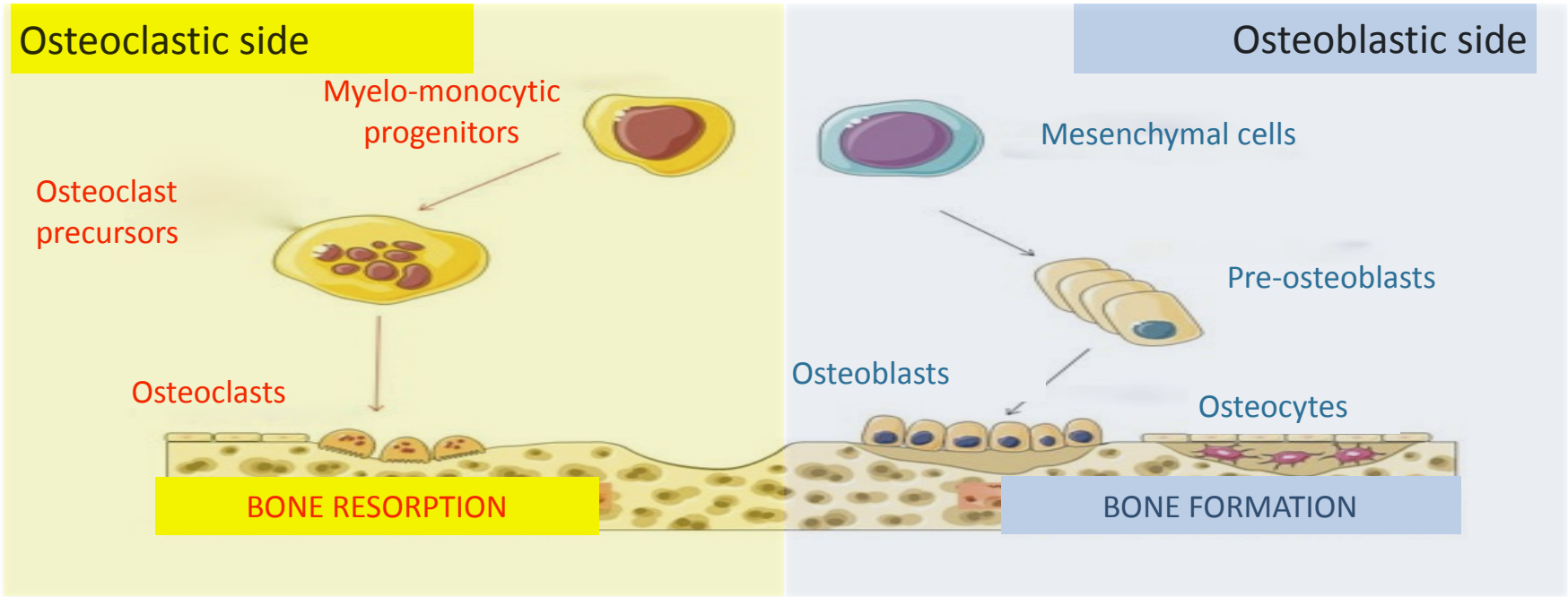
Lyon, France

Bone tissue and infection



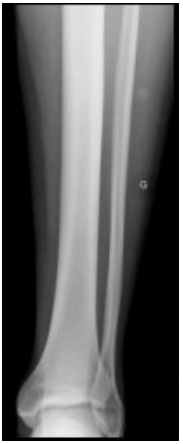
Bone tissue and infection

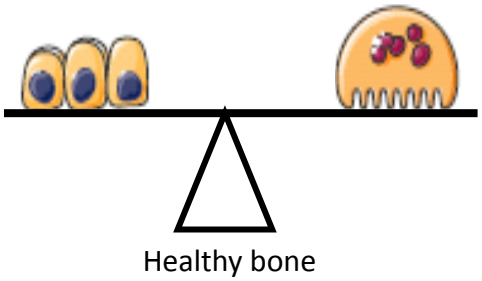
Bone tissue and infection



Physiological context

Healthy bone

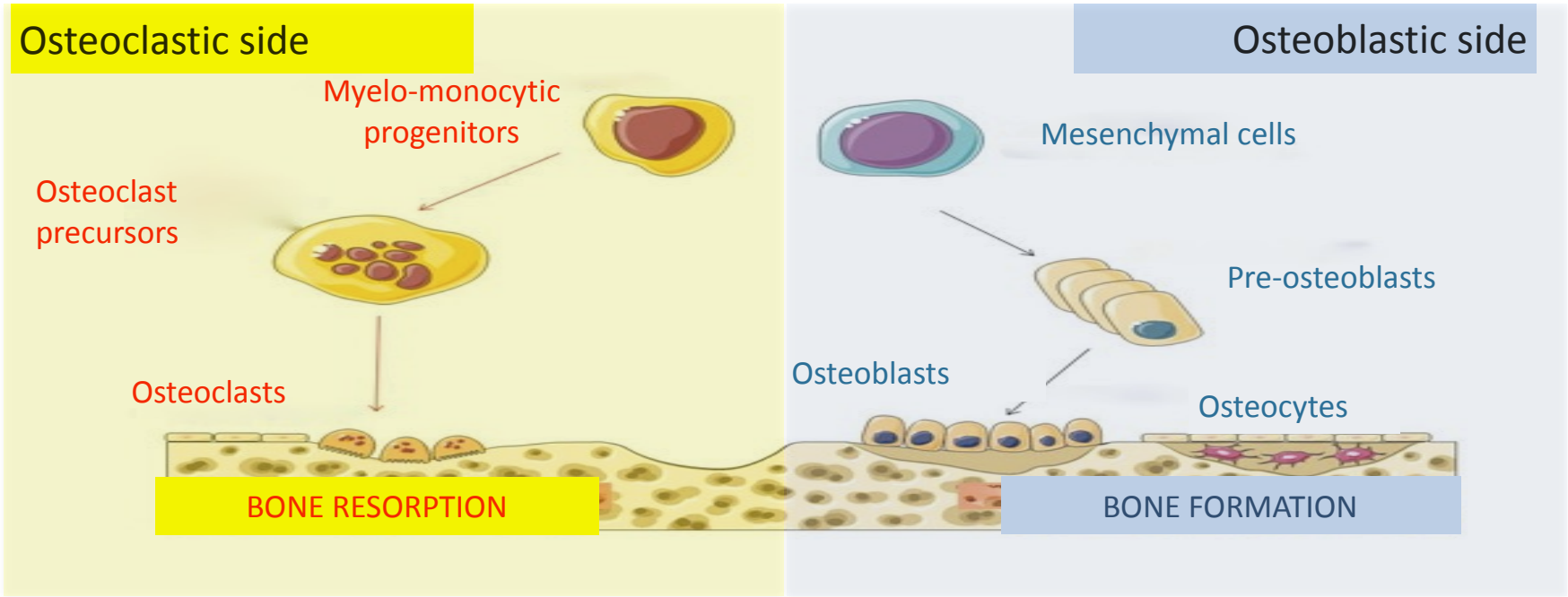




Healthy bone

Balance osteoblast/osteoclast

Bone tissue and infection



Physiological context

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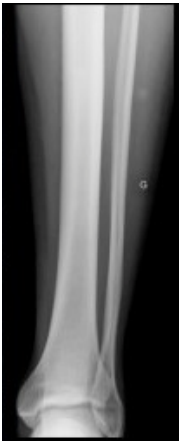
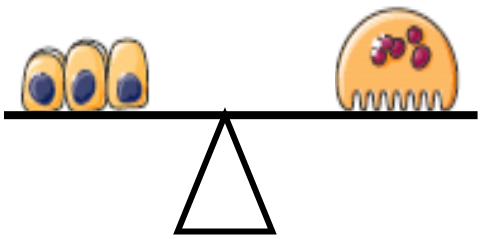
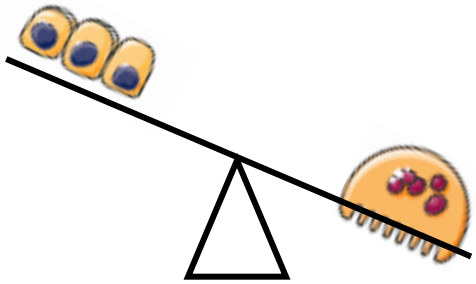




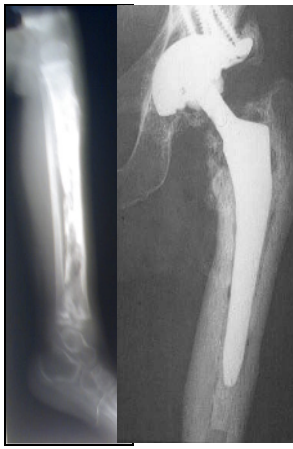
Balance osteoblast/osteoclast

Infectious context

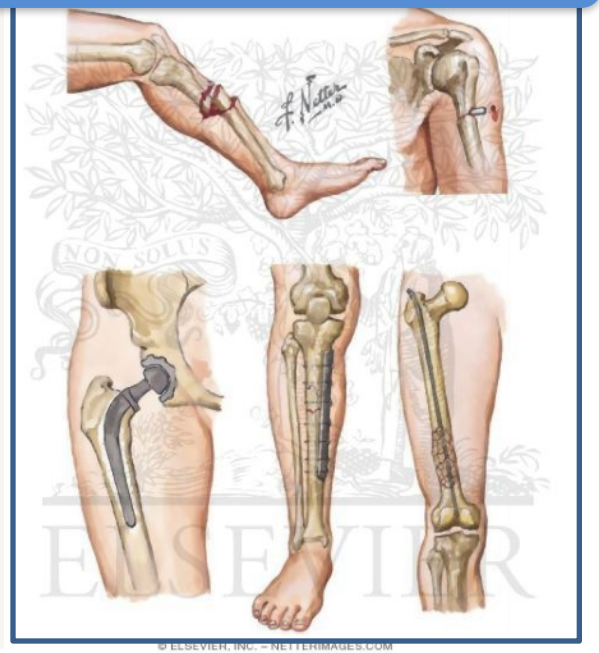


Progressive bone loss/destruction
.prothesis loosening
. loss of function

Infected bone



Bone and joint infections

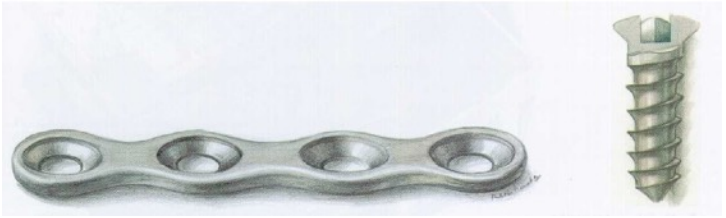


Definitions

- Osteomyelitis
- Spondylodiscitis (vertebra)
- Septic arthritis

- Acute or chronic: delay of 1 month (arbitrary!)
- Implant-associated or not

  prothesis or osteosynthesis



Bone and joint infections

Hematogeneous

during bacteriemia

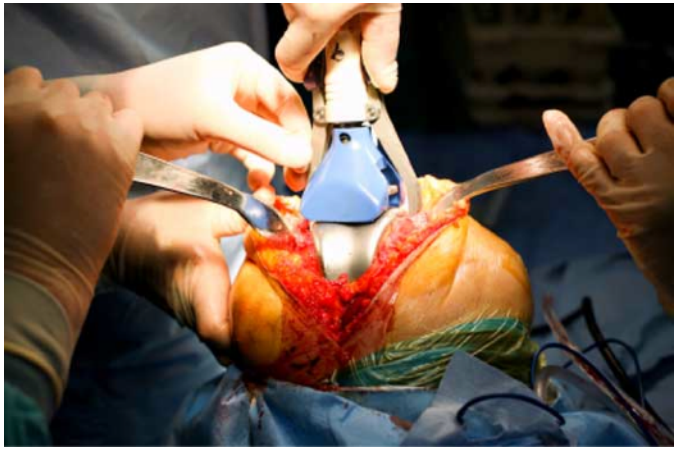
Risk on prosthesis:< 1%

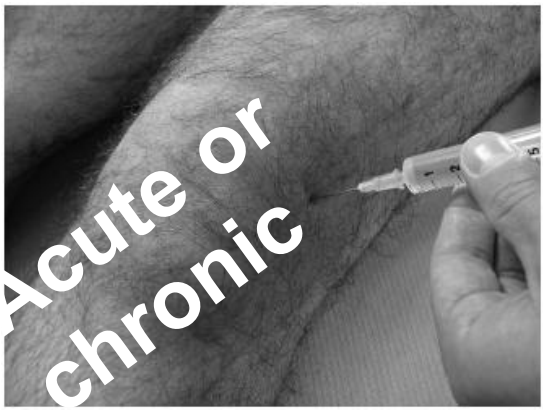
Except for *S. aureus* : 30-40%

Acute

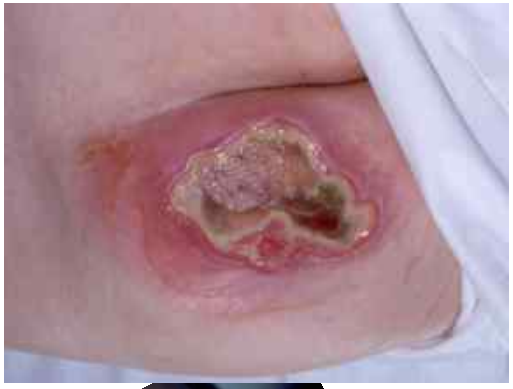
Direct Inoculation

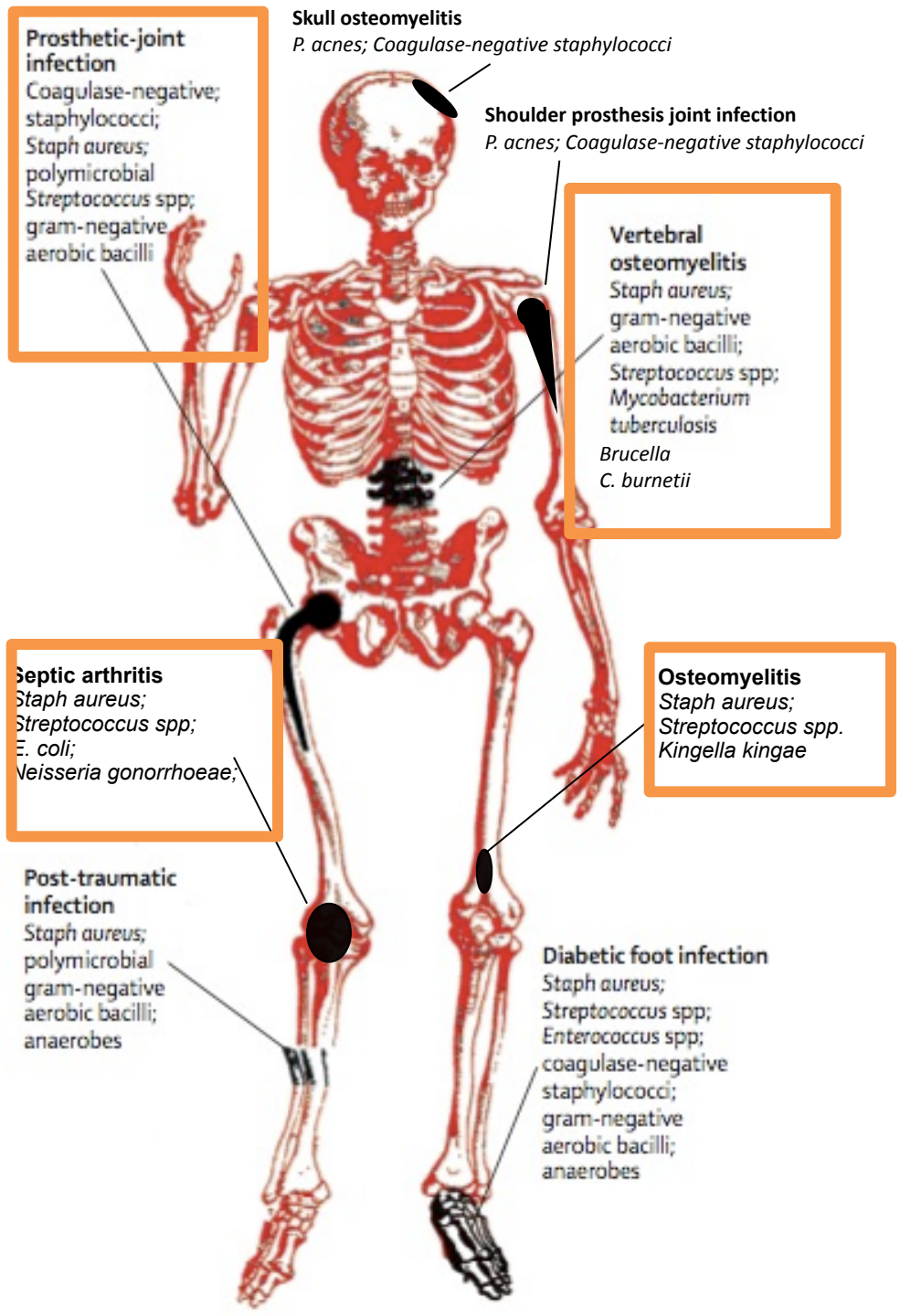
joint puncture/infiltration
open surgery or arthroscopy





Contiguous





Acute BJIs

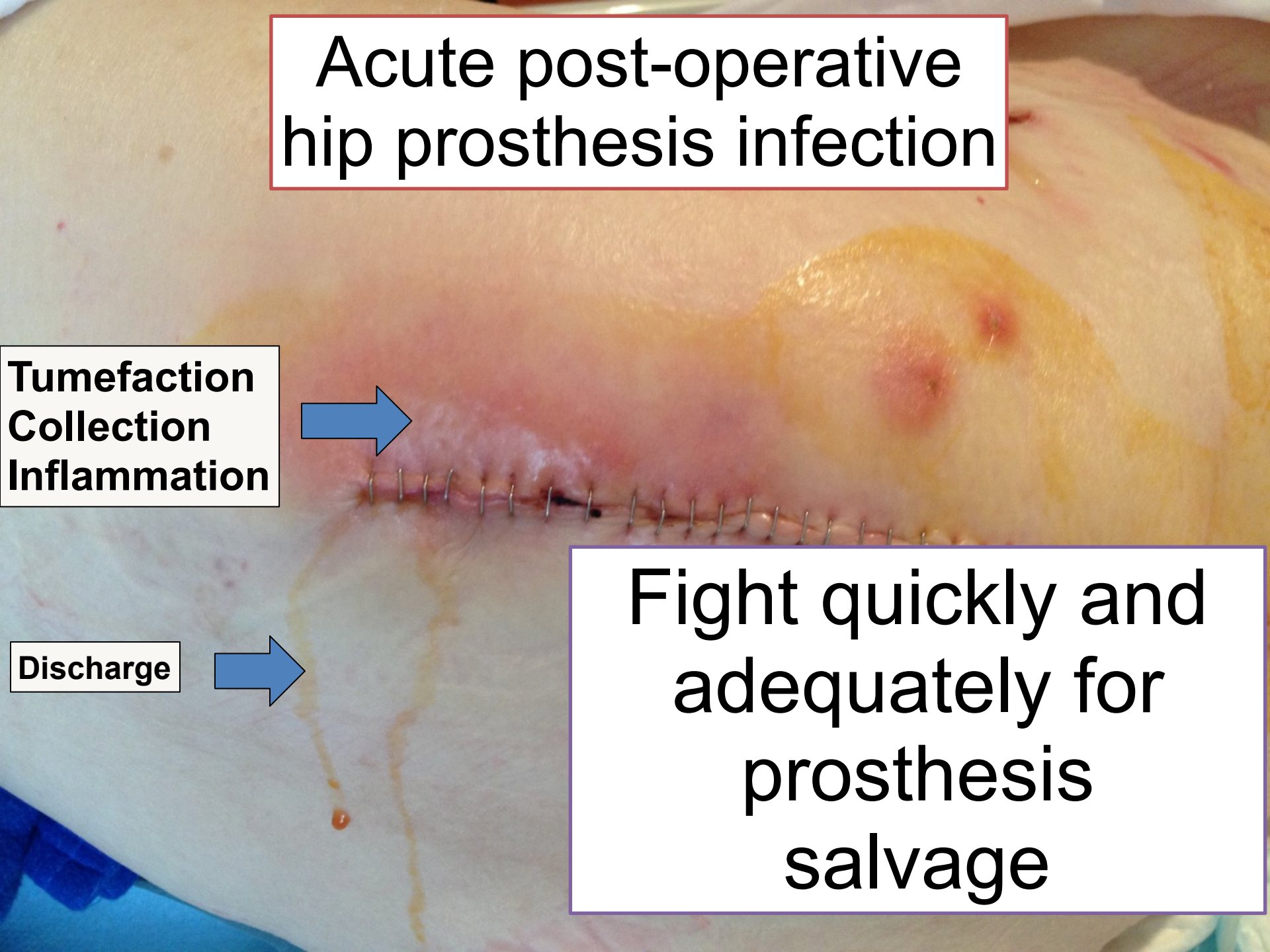
Osteomyelitis in childhood
Hematogenous

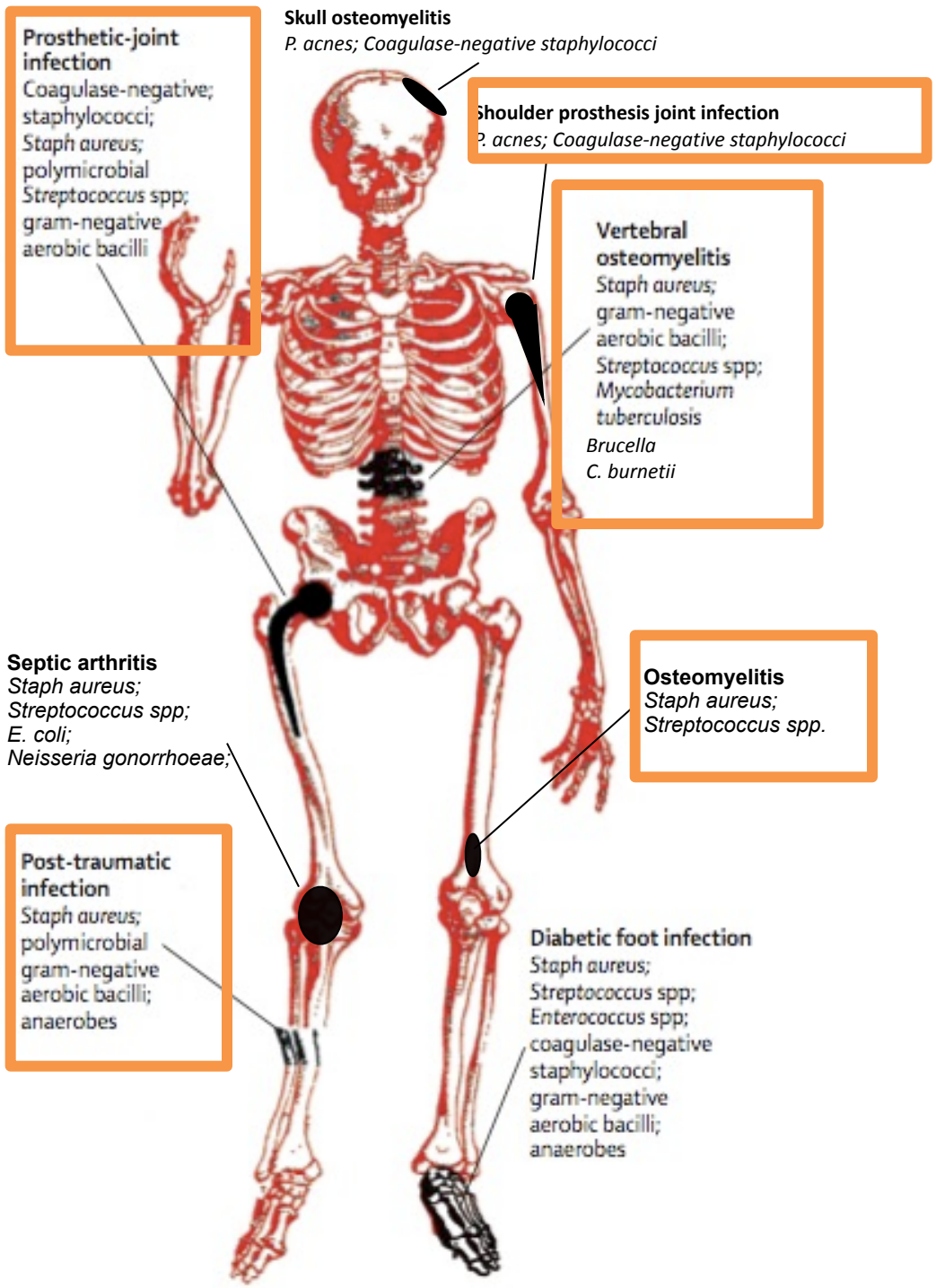
Septic arthritis
Hematogenous
Post-operative
Contigous

Spondylodiscitis
Hematogenous

Spinal implant infection
Hematogenous
Post-operative

Joint prosthesis or osteosynthesis infection
Hematogenous
Post-operative
Contigous


Acute post-operative hip prosthesis infection
Tumefaction
Collection
Inflammation
Discharge
Fight quickly and adequately for prosthesis salvage



Chronic BJI

Osteomyelitis in childhood relapsing in adulthood
Hematogenous

Spinal implant infection
Post-operative

Post-trauma infections
Post-operative
Contigous

Prosthesis joint or osteosynthesis infection
Hematogenous
Post-operative

Skull bone flap
Post-operative

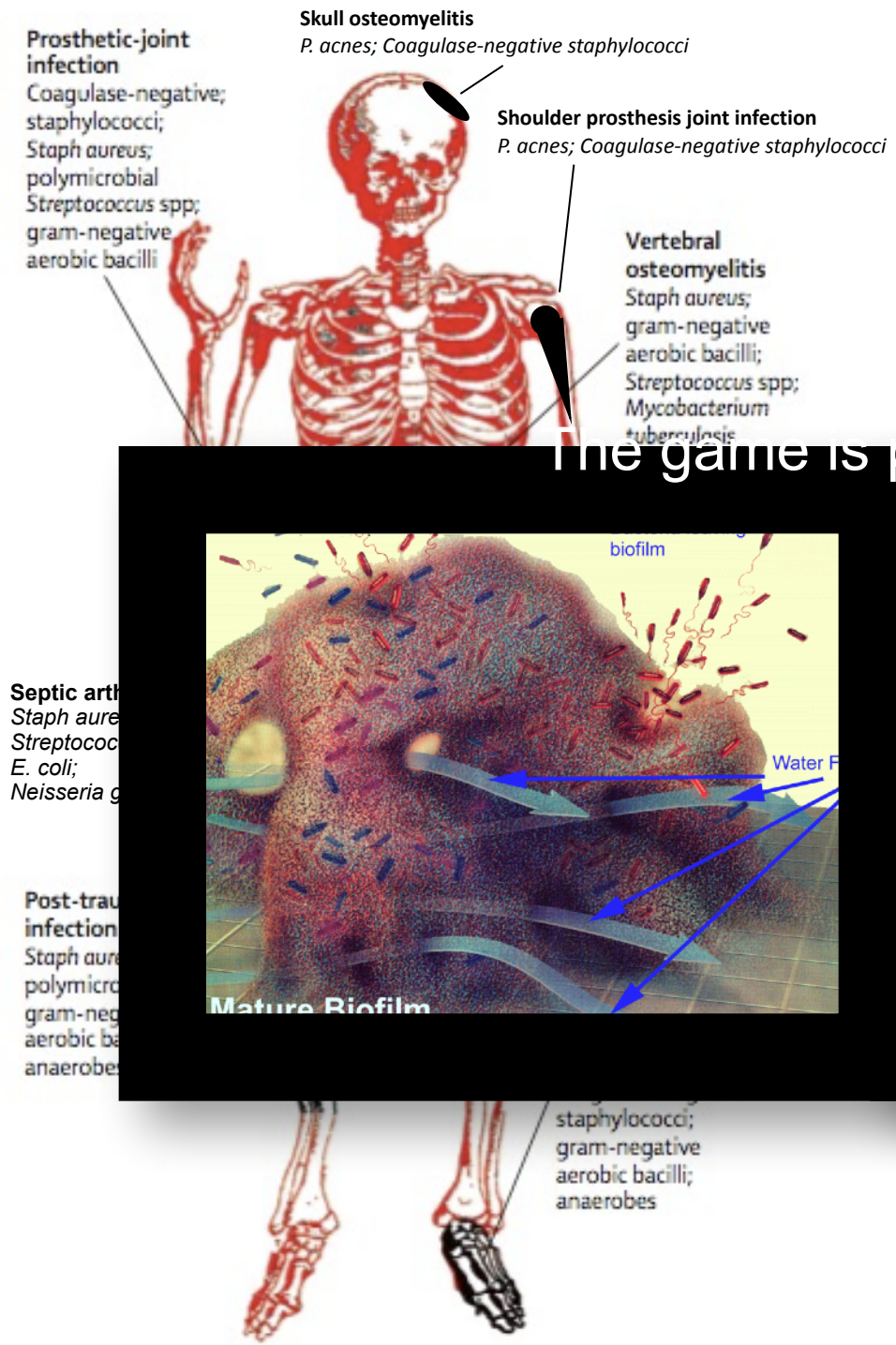
Diabetic foot
Contigous

Chronic BJI

Osteomyelitis in childhood relapsing in adulthood
Hematogenous

Spinal implant infection

Diabetic foot
Contigous



The game is partially lost…

Small colony variants

Pathophysiopathology of BJI

S. aureus = 40% to 70% of BJI

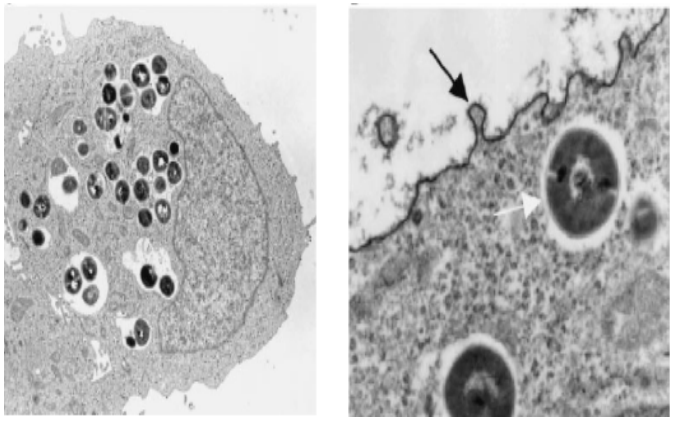
Classical acute virulence

+

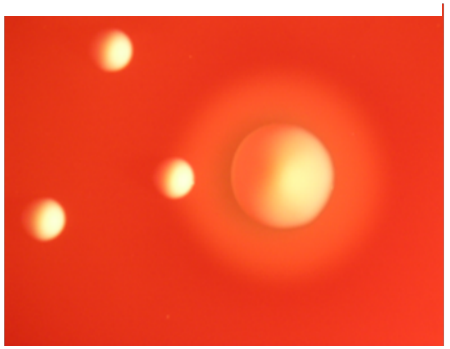
Recurrence et chronicity ++

Biofilm

Internalization



Small Colony Variant (SCV)



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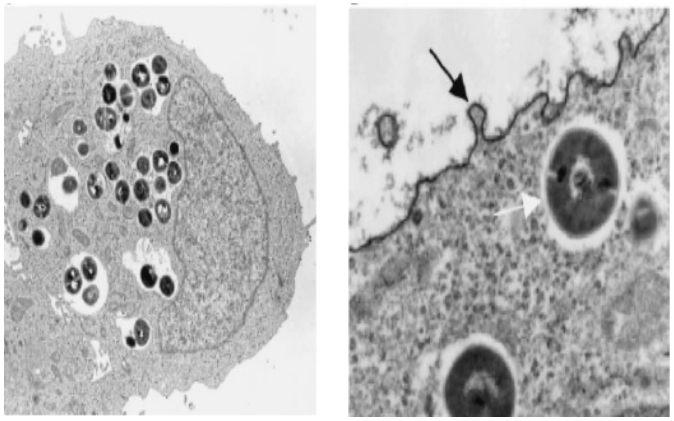

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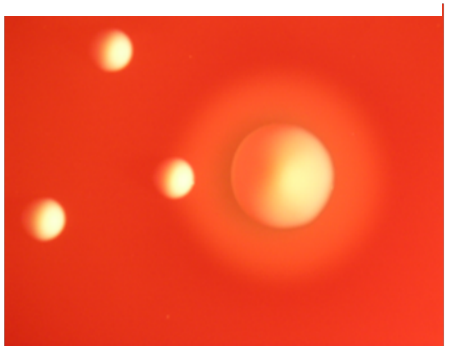
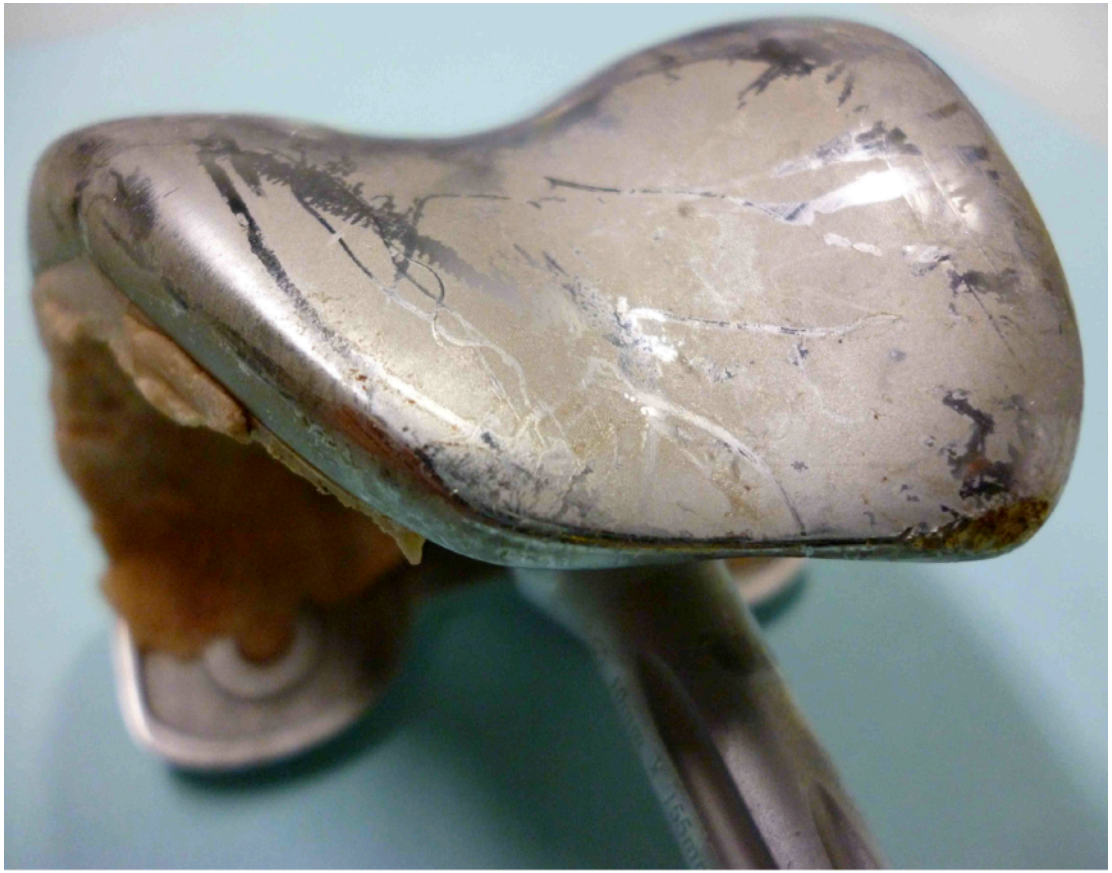


Biofilm

Biofilm on prothesis (true life !)



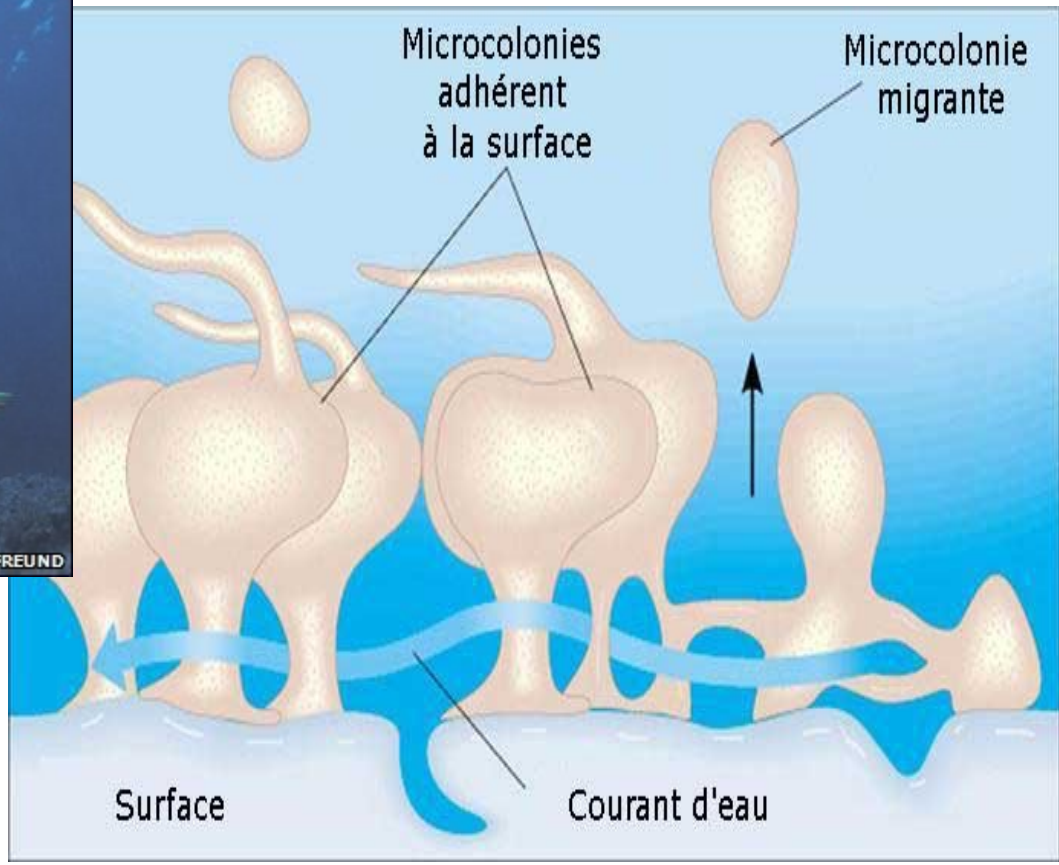
Biofilm

scotch tape

Biofilm

scotch tape

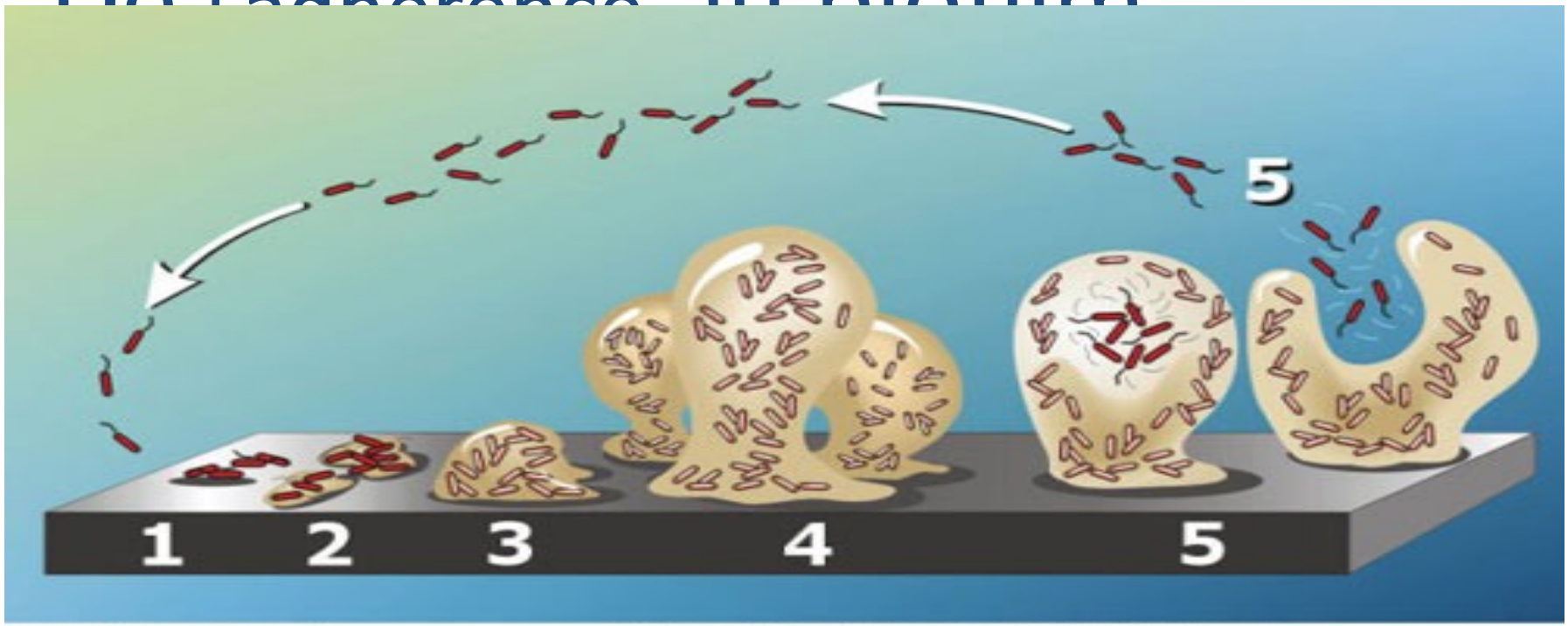
Biofilm

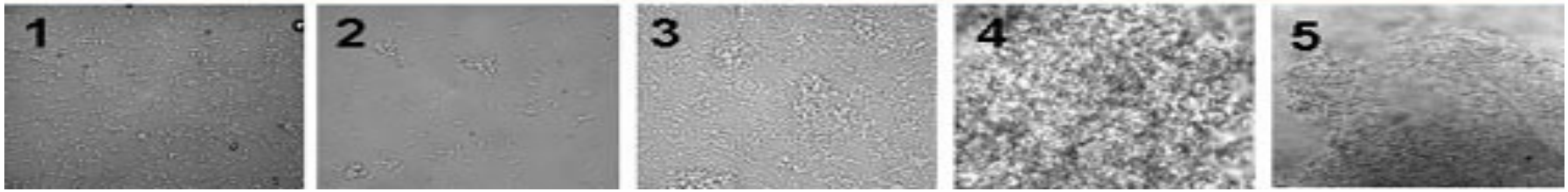


Each bacteria is in a different microenvironment

From adherence to Biofilm

De l'adhérence au biofilm





Polymer surface: hydrophobicity, AtlE, Aae and teichoic acids

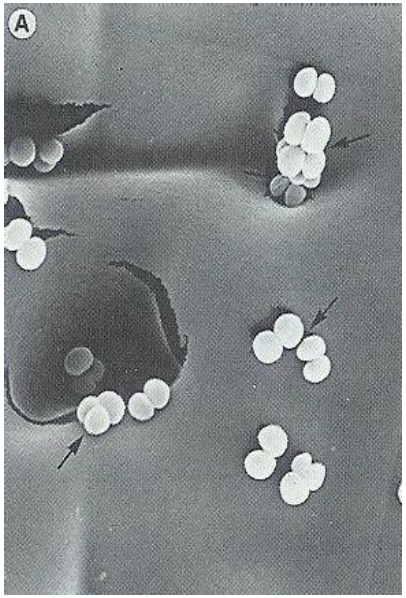
Host matrix proteins: SdrF, SdrG, SdrH, Ebp, AtlE and Aae

PNAG, teichoic acids, Bap and Aap

PSMs? Proteases?

From adherence to Biofilm

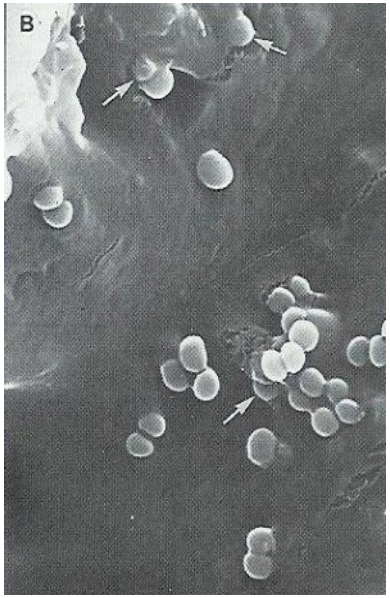
Bactéries IOA = +/- biofilm / intracellulaire



2 h

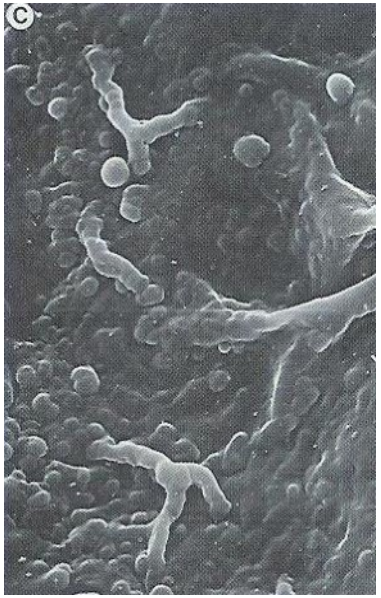
Fixation des S. aureus

sur des irrégularités à la surface du matériel



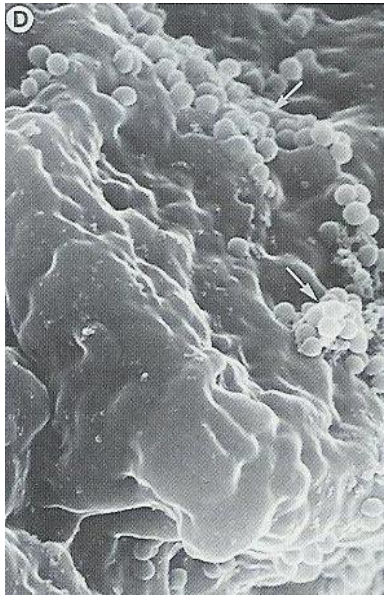
4 h

Début de fabrication du "slime "



8 h

La surface du matériel est recouverte par une couche épaisse de "slime"

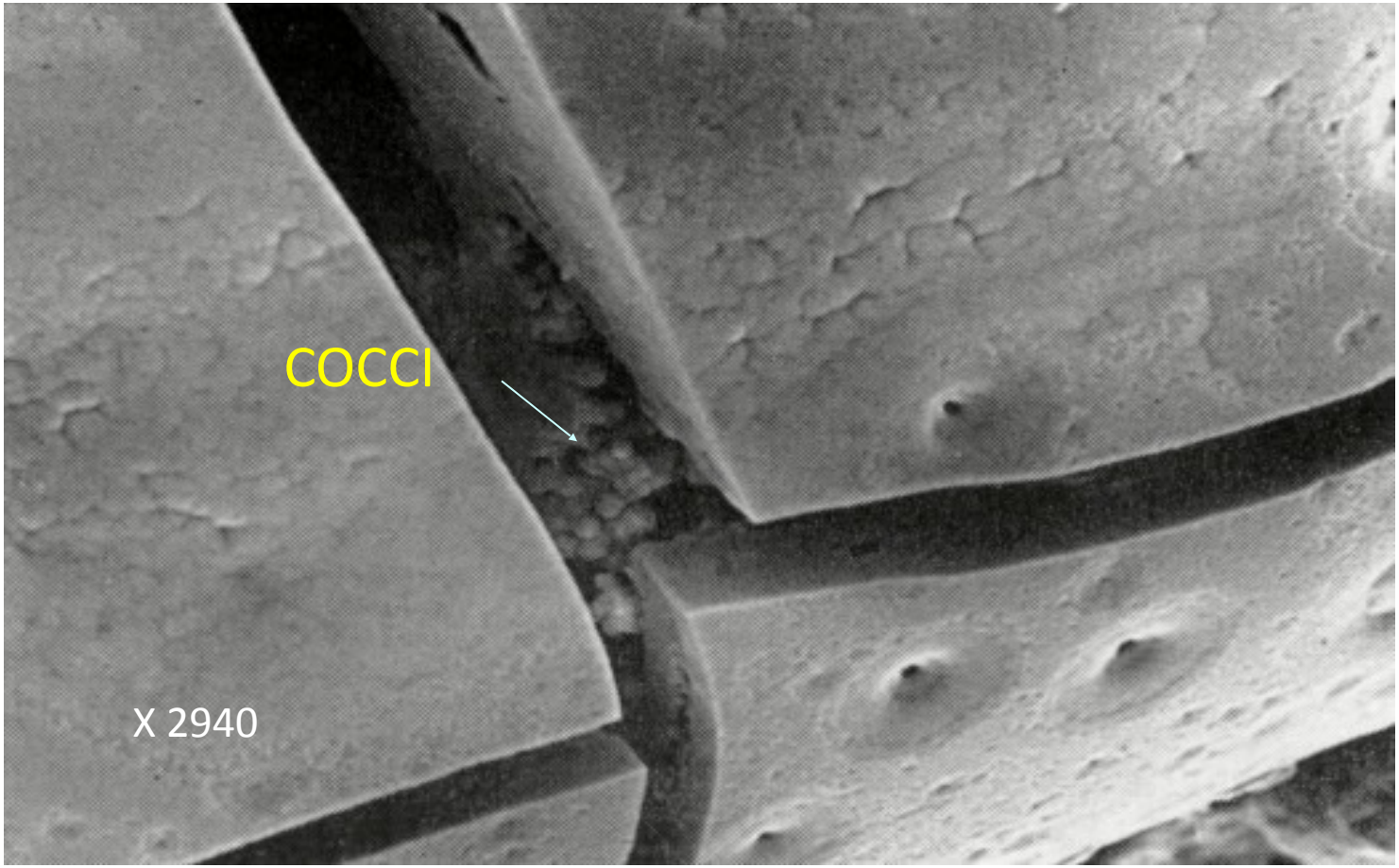


24 h

Des bactéries émergent du biofilm, libres et prêtes à se fixer ailleurs

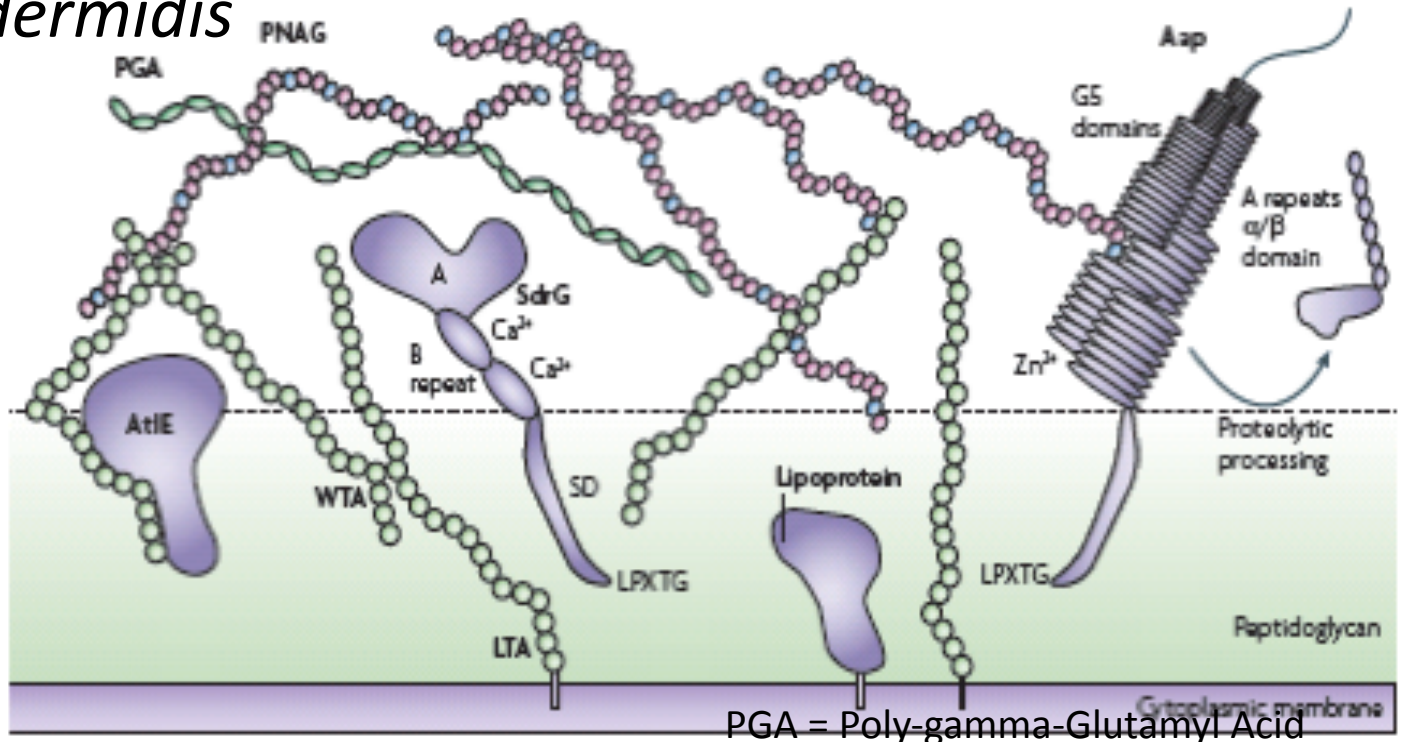
Olson, et al. J. Biomed Mater Res **1988**

From adherence to Biofilm



From adherence to Biofilm

S. epidermidis



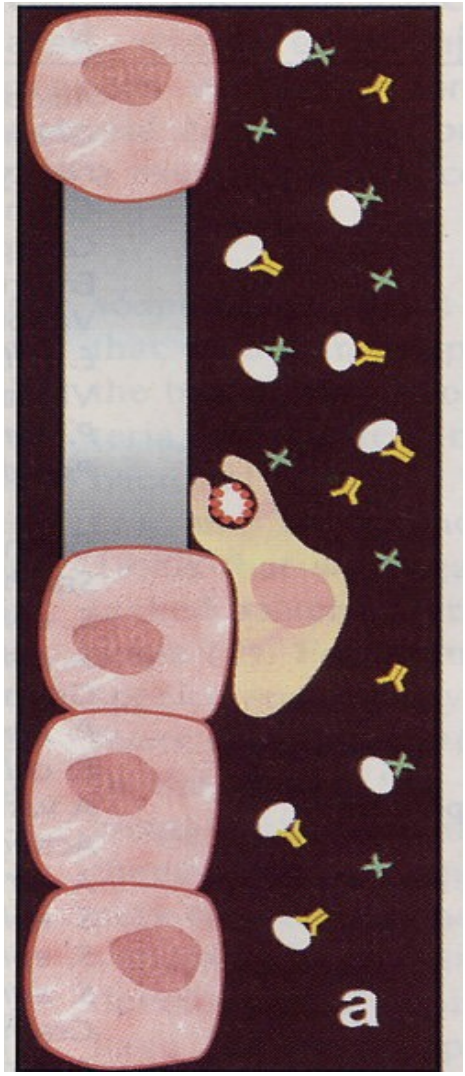
Aap = Adhesion-associated-protein
AtlE = Autolysine E
Bap = Bone-associated-protein
LTA = Lipo-Teichoic Acid

PGA = Poly-gamma-Glutamyl Acid
PNAG = Poly-N-acetyl-glucosamine ;
Sdr = Serin domain repeat
WTA = Wall Techoic Acid

+ free DNA + human/bacterial proteins + polysaccharides = GLUE

Otto, M. Nat Rev Microbiol, 2009.

Biofilm and prosthetic materials



Planctonic bacteria

. Elimination by the mechanisms of natural defense : antibodies, phagocytes

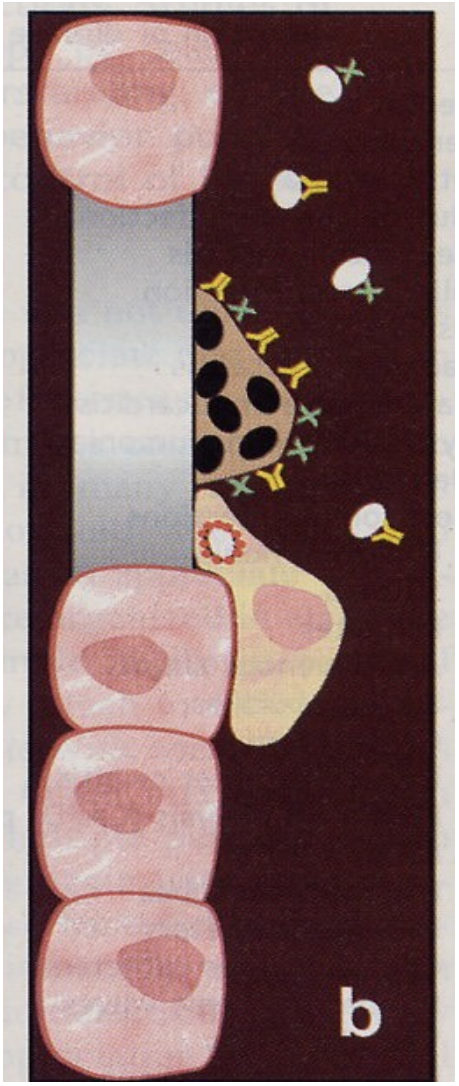
. Susceptibility to antibiotics

× Antibiotics Anticorps Enzymes in phagocytes

planctonic bacteria

JW Costerton (1999) Science, 284:1318-1322

Biofilm and prosthetic materials



Bacteria and bifiofilm= adhesion on biotic or abiotic surfaces then adhesion bacteria/bacteria

. Resistance to antibodies

. Resistance to phagocytes

. Resistance to most of teh antibiotics

× Antibiotics

Anticorps

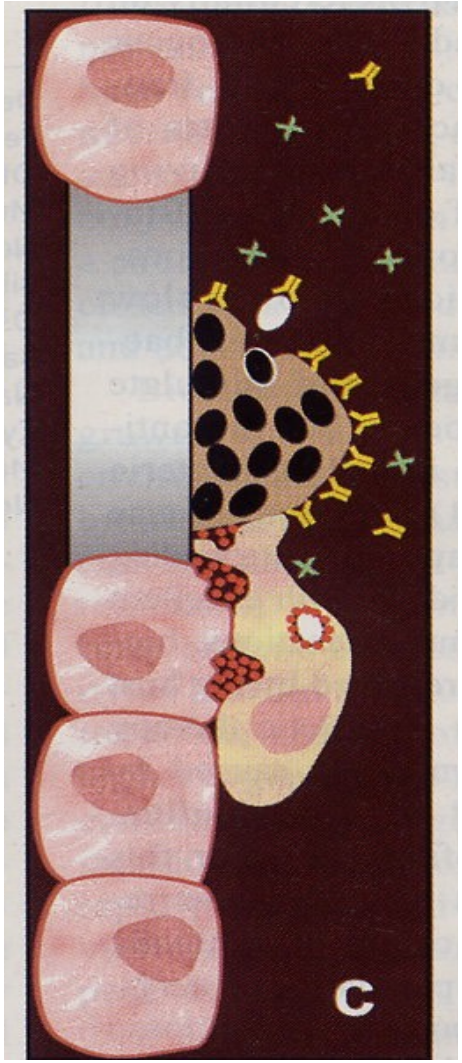
Enzymes in phagocytes

planctonic bacteria

bacteria in biofilm

JW Costerton (1999) Science, 284:1318-1322

Biofilm and prosthetic materials



- phagocytes attracted by bacterial biofilm
- unefficient phagocytosis

but local release of lysosomial enzymes from phagocytes unable to destroy biofilm matrix

× Antibiotics

Anticorps

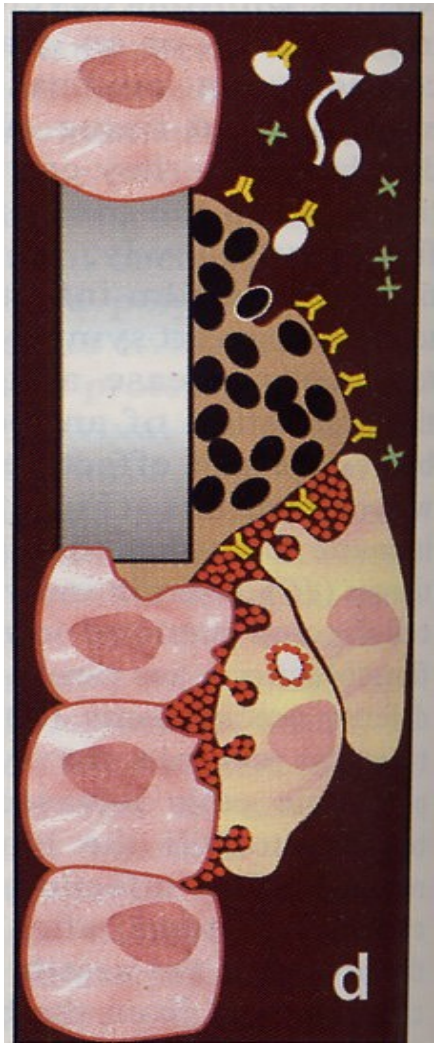
Enzymes in phagocytes

planctonic bacteria

bacteria in biofilm

JW Costerton (1999) Science, 284:1318-1322

Biofilm and prosthetic materials



. local release of lysosomial enzymes from phagocytes unable to destroy biofilm matrix

Tissue damages around prosthesis = loosening of prothesis

. Swarming of bacteria from biofilm

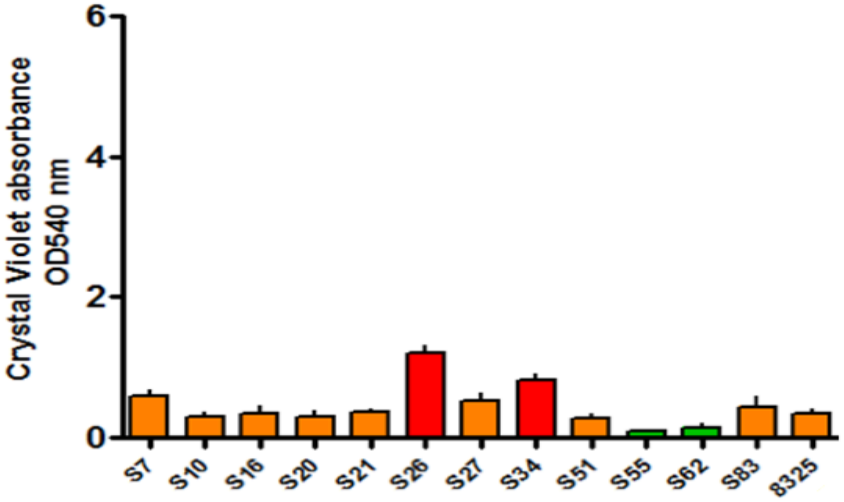
× Antibiotics Anticorps Enzymes in phagocytes

planctonic bacteria bacteria in biofilm

JW Costerton (1999) Science, 284:1318-1322

Determination of biofilm formation : which relevance ?

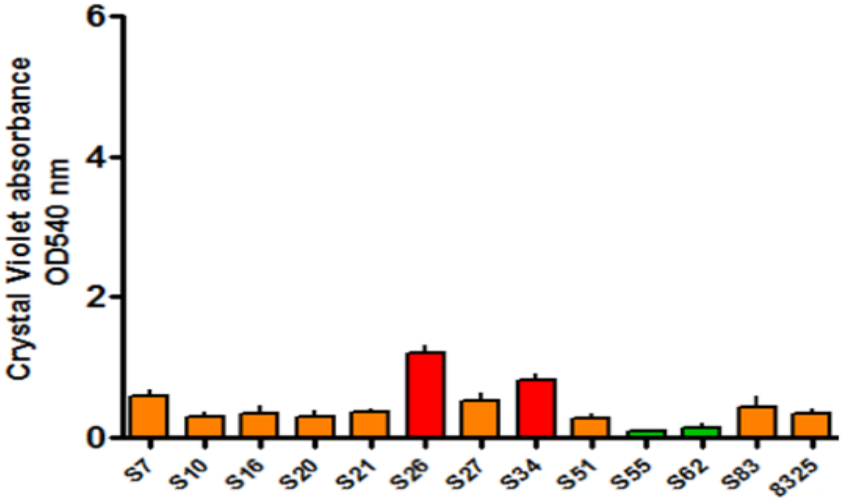
In BHI+ 1% glucose after 48H



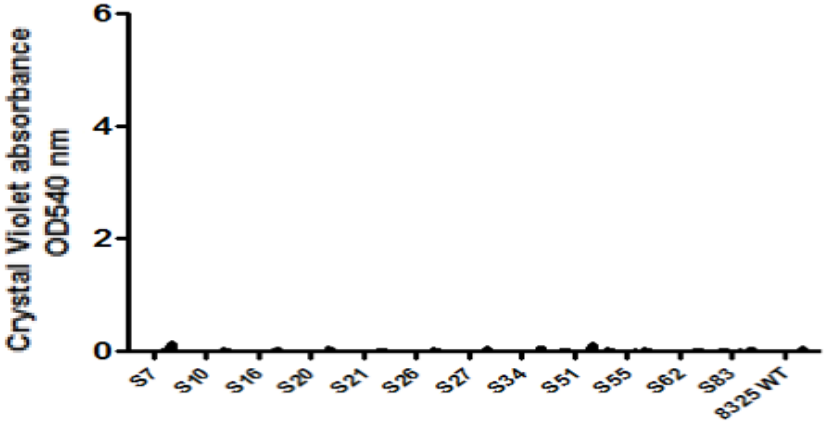
Clinical MSSA isolatescollected in patient suffering from BJI

Determination of biofilm formation : which relevance ?

In BHI+ 1% glucose after 48H



In human sera after 48H/D30



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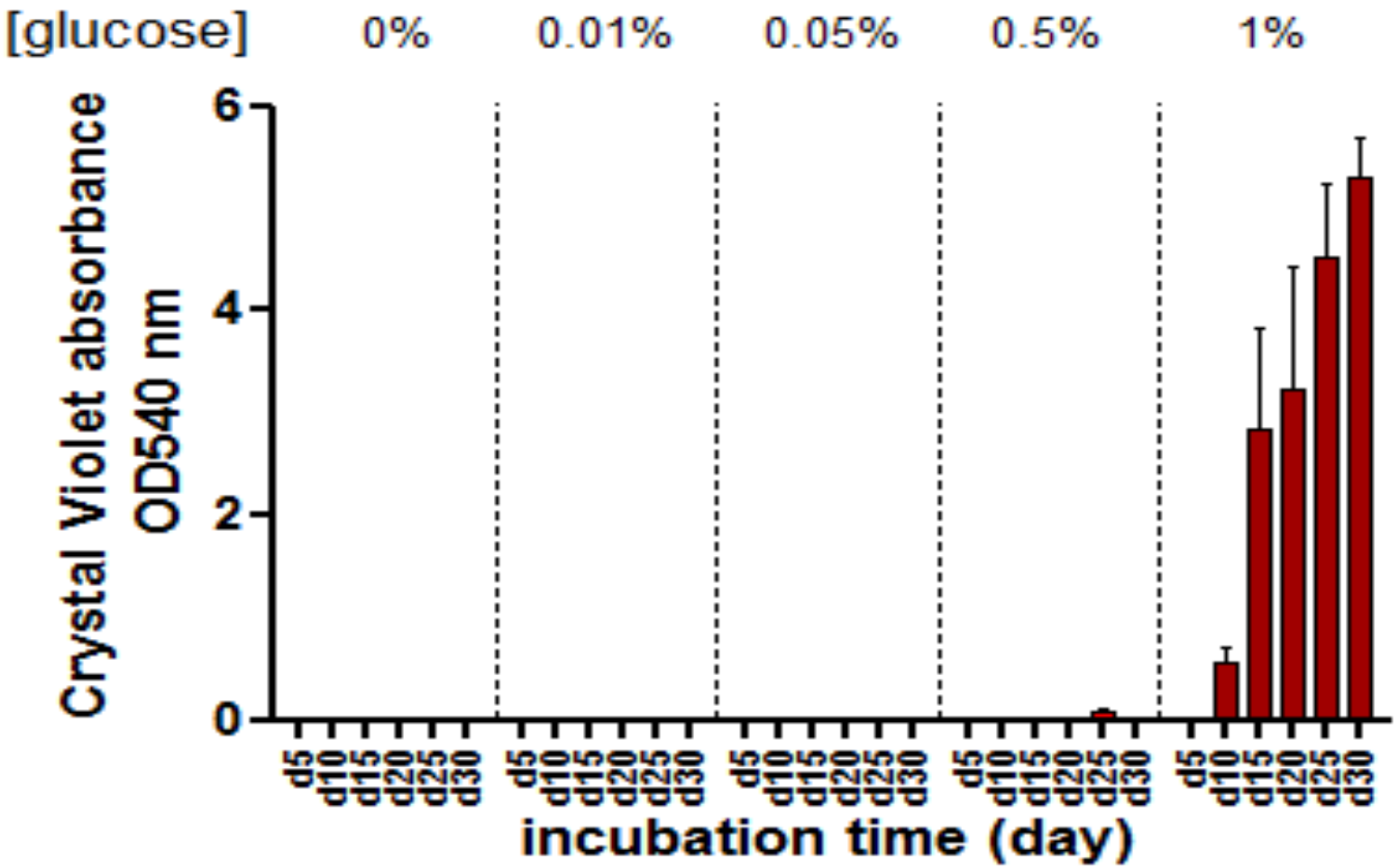

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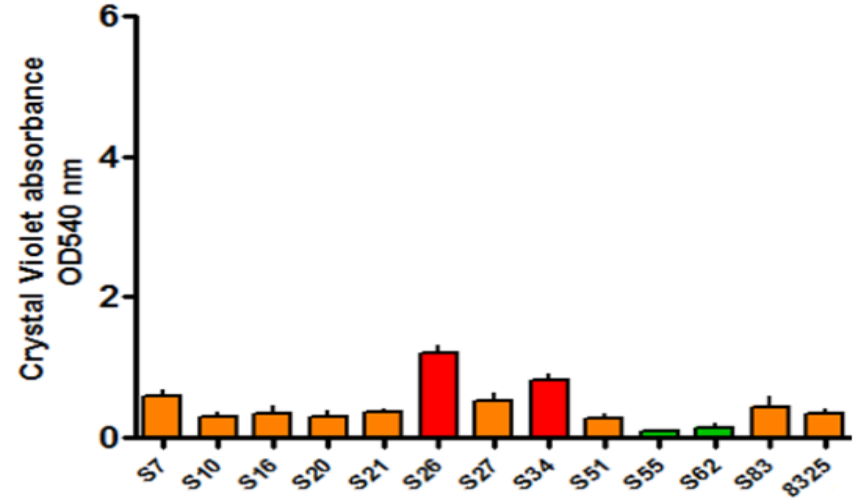

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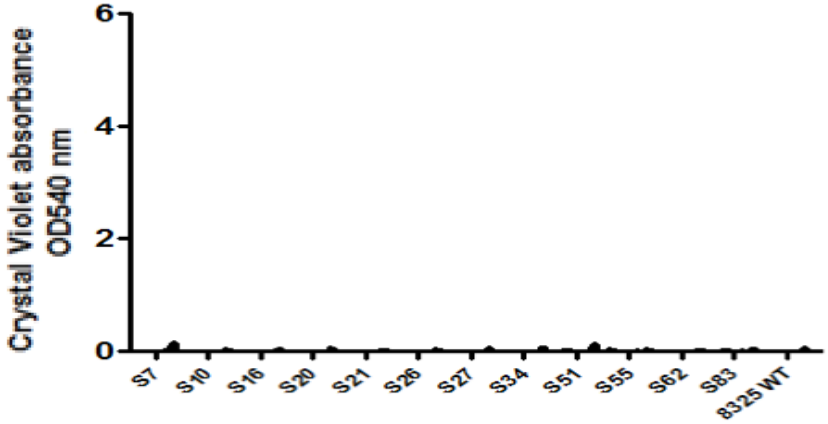
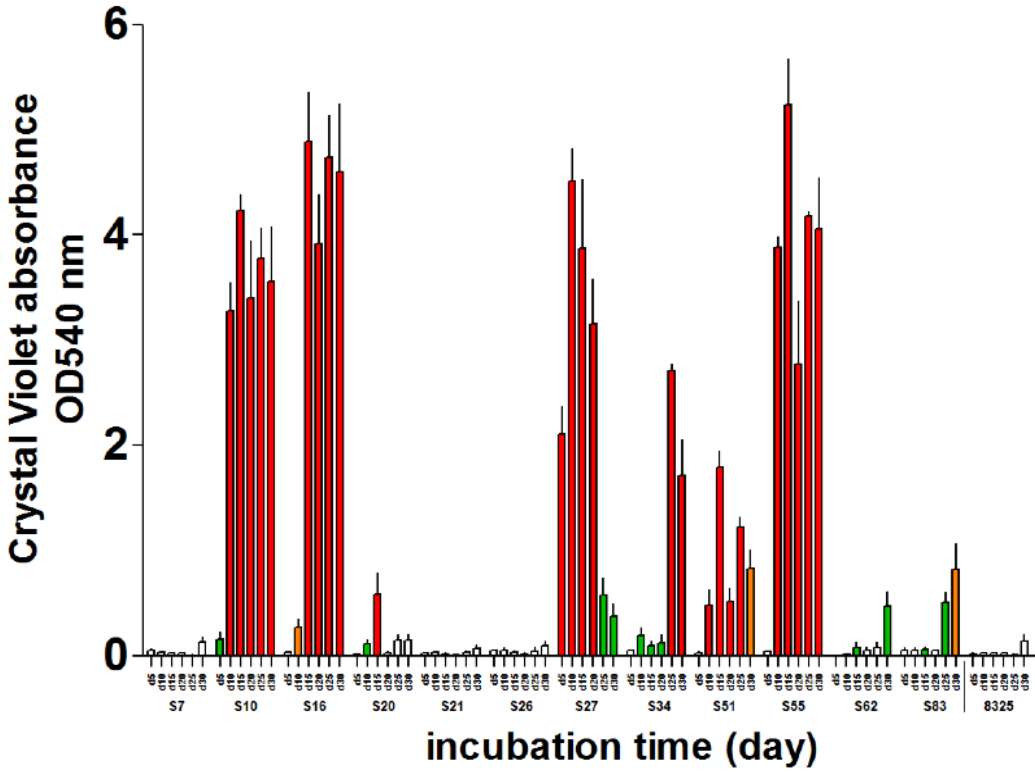


In human sera + 1% Glusoce after D2/D10/D15/D20/D25/D30



Determination of biofilm formation : which relevance ?

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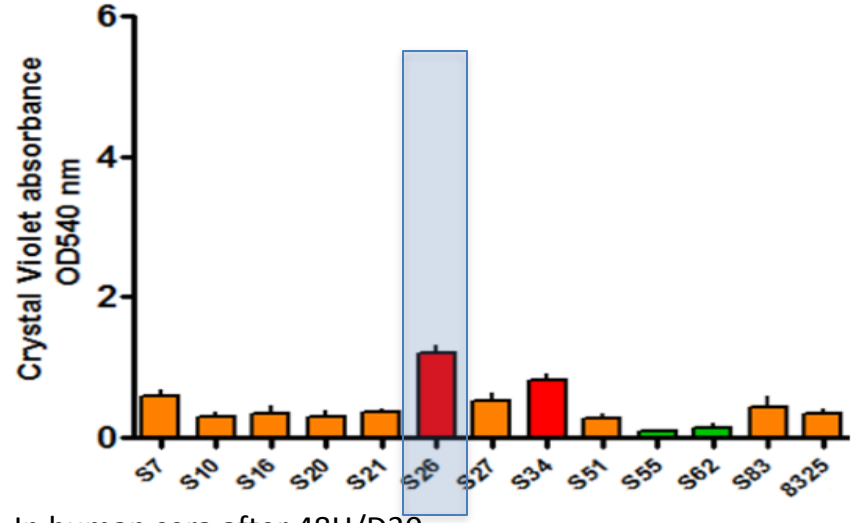
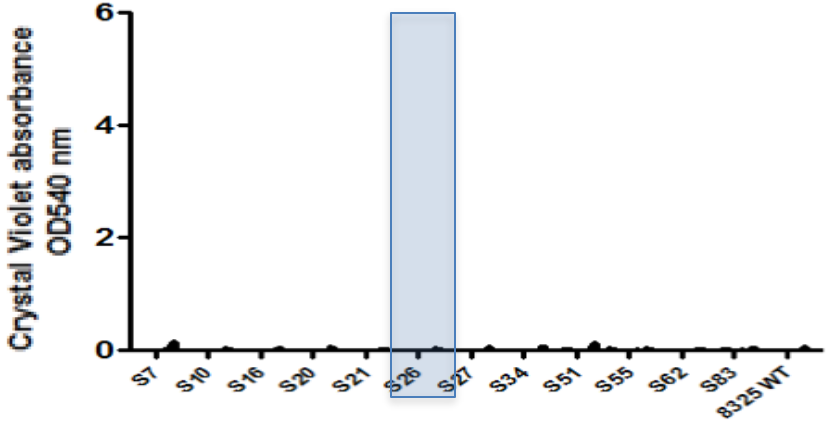
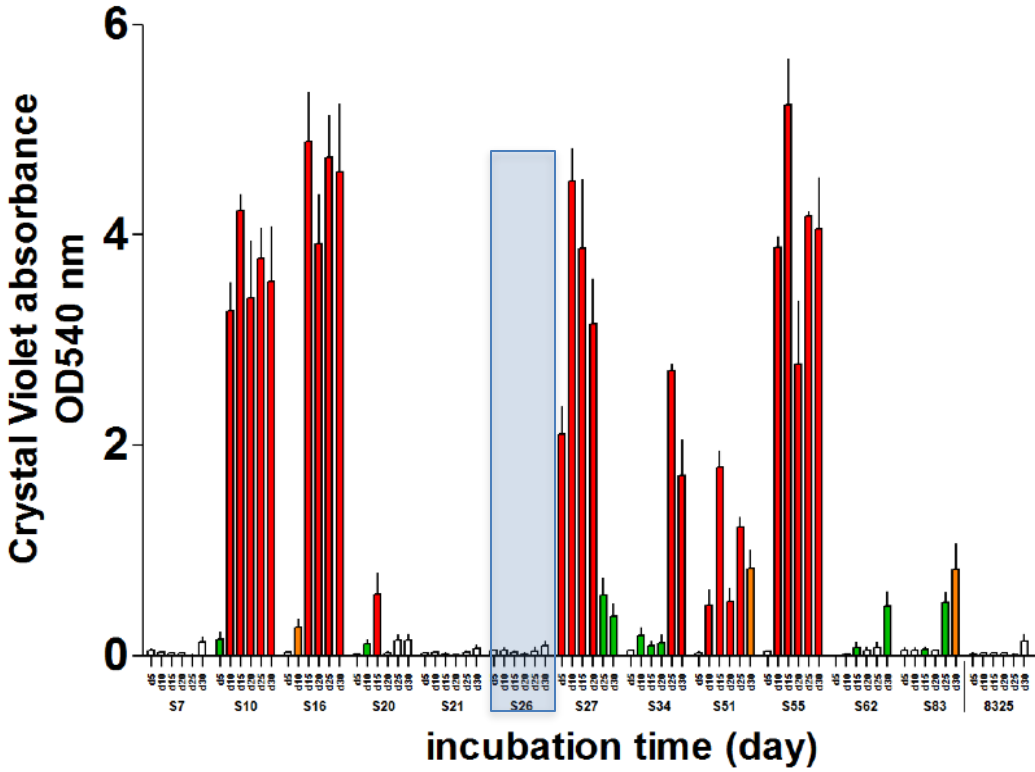


In human sera after 48H/D30



In human sera + 1% Glusoce after D2/D10/D15/D20/D25/D30



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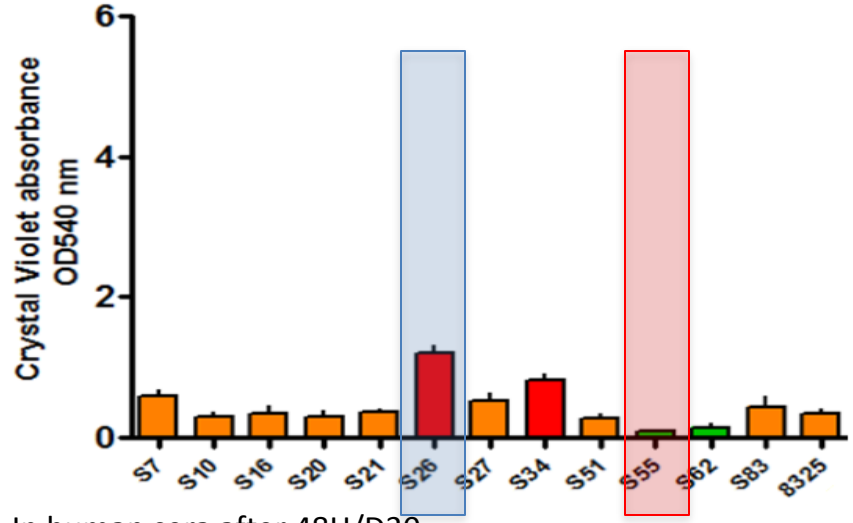
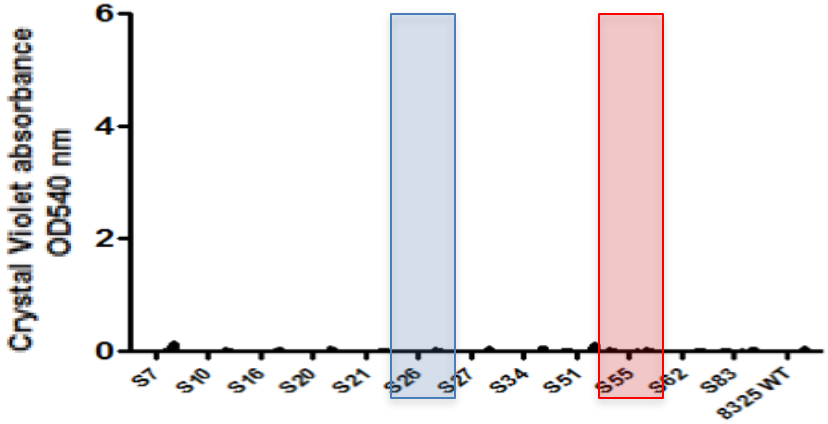
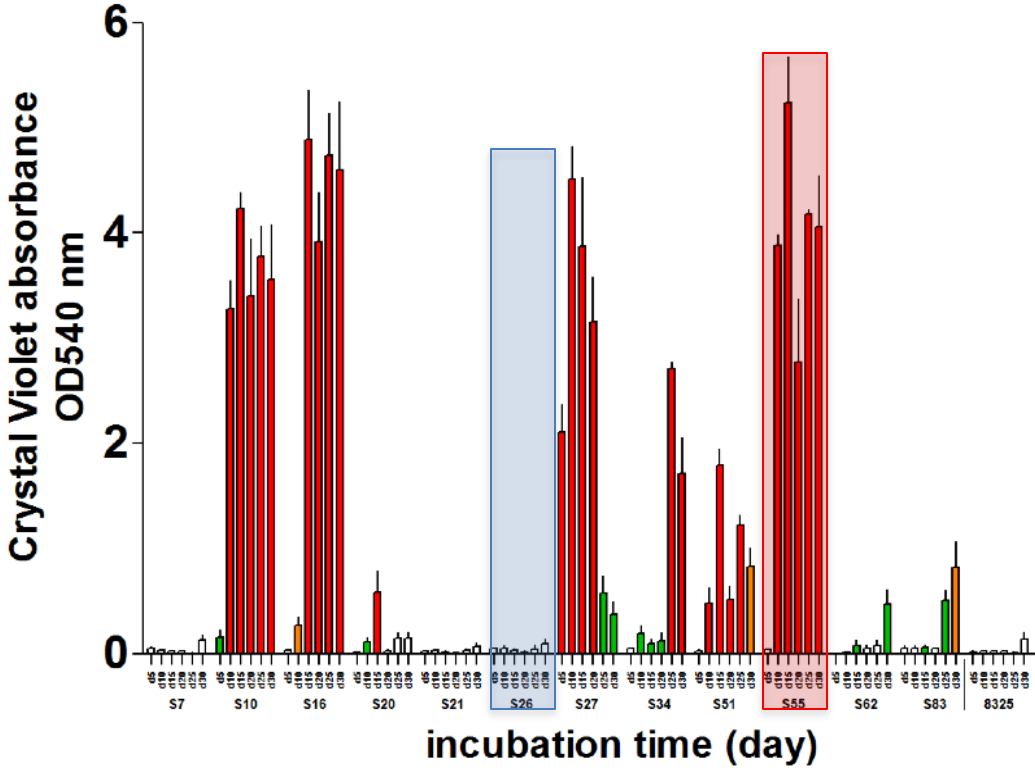


In human sera after 48H/D30



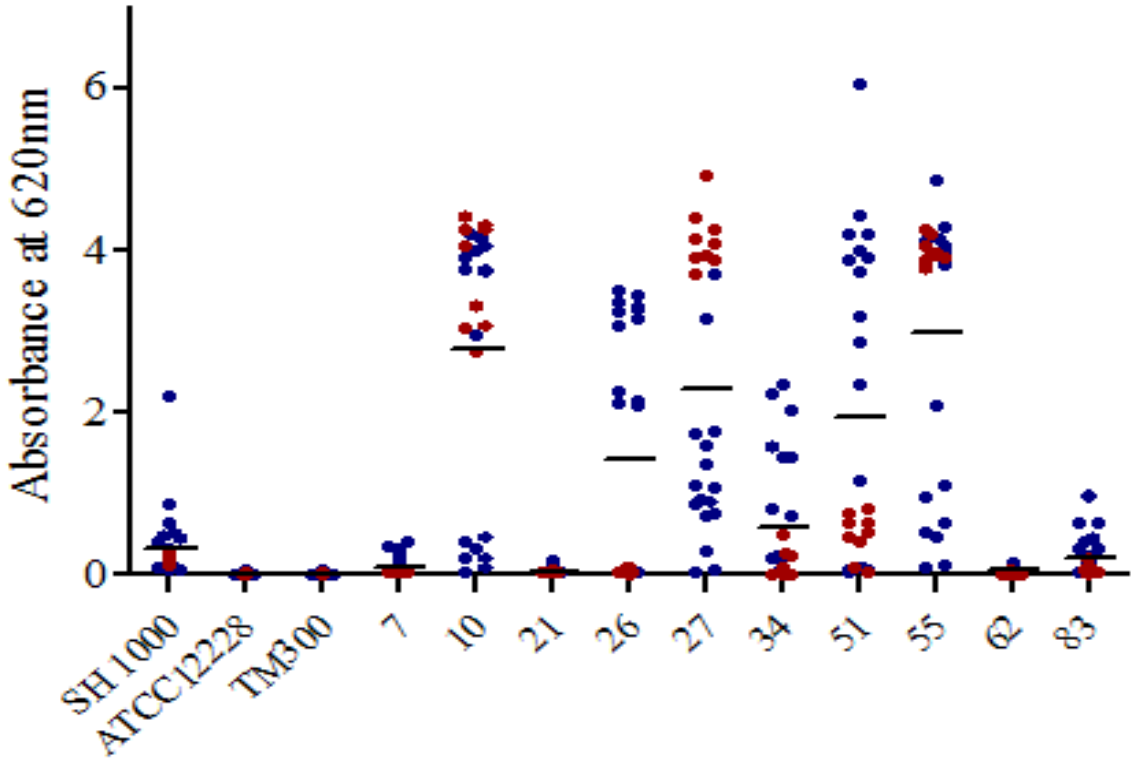
In human sera + 1% Glusoce after D2/D10/D15/D20/D25/D30



Determination of biofilm formation : which relevance ?

In BHI after 48H

In human sera + 1% Glusoce after D30



Relevance of data ? in vivo predictive value of data?

Standardisation needed ? But which conditions ?

Pathophysiopathology of BJI

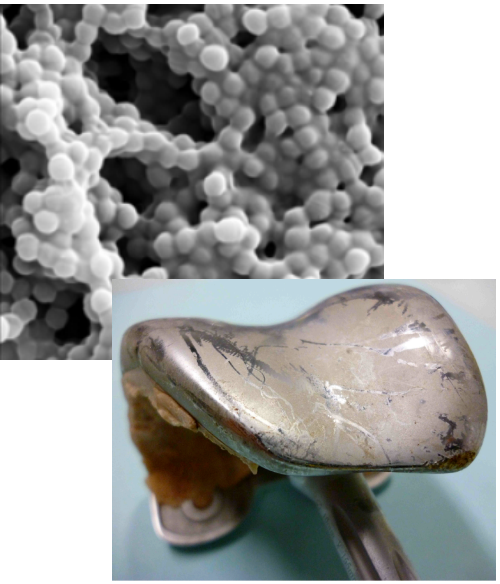
S. aureus = 40% to 70% of BJI

Classical acute virulence

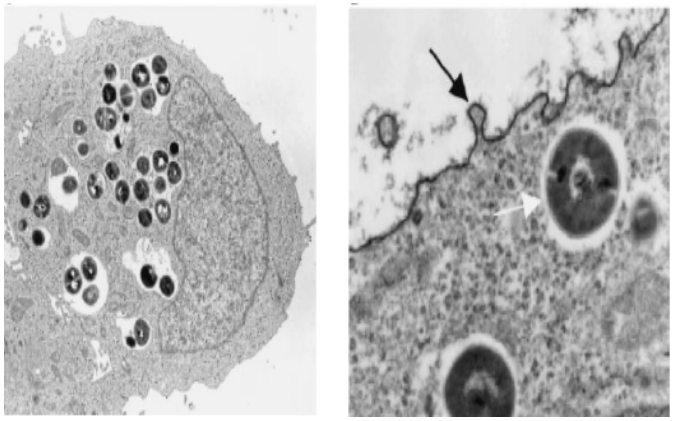
+

Recurrence et chronicity ++

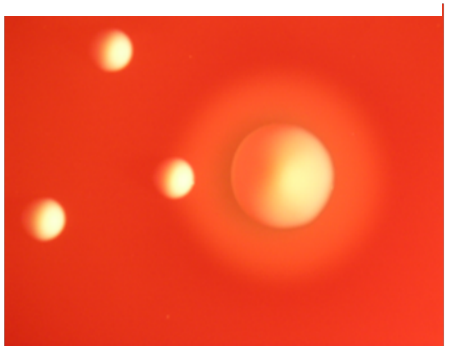
Biofilm



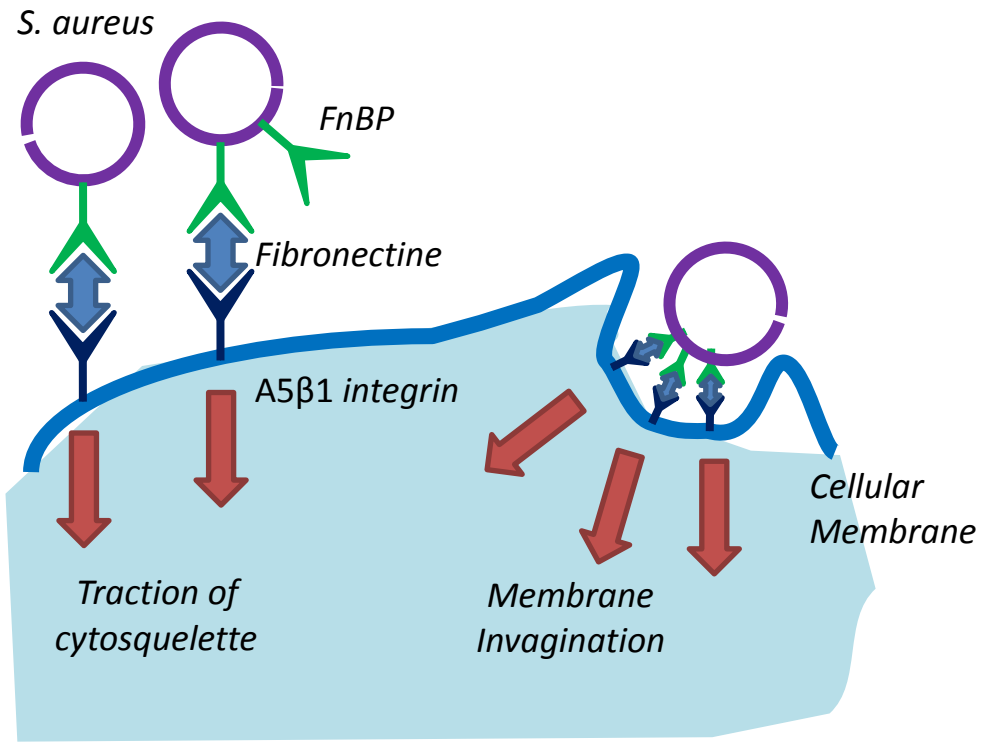
Internalization

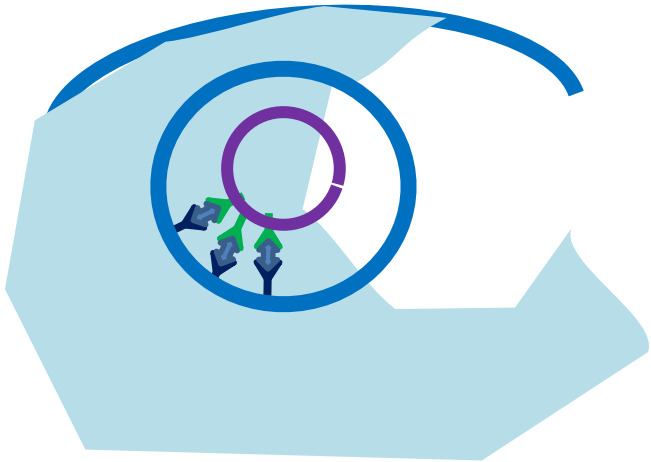


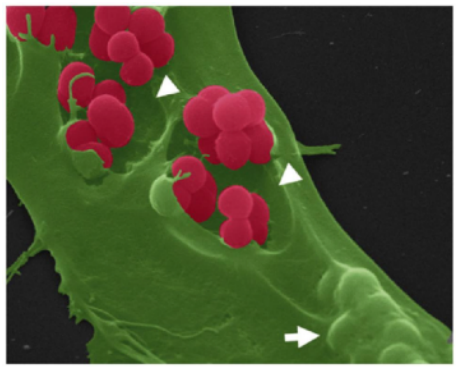
Small Colony Variant (SCV)



Osteoblast invasion by *S. aureus*



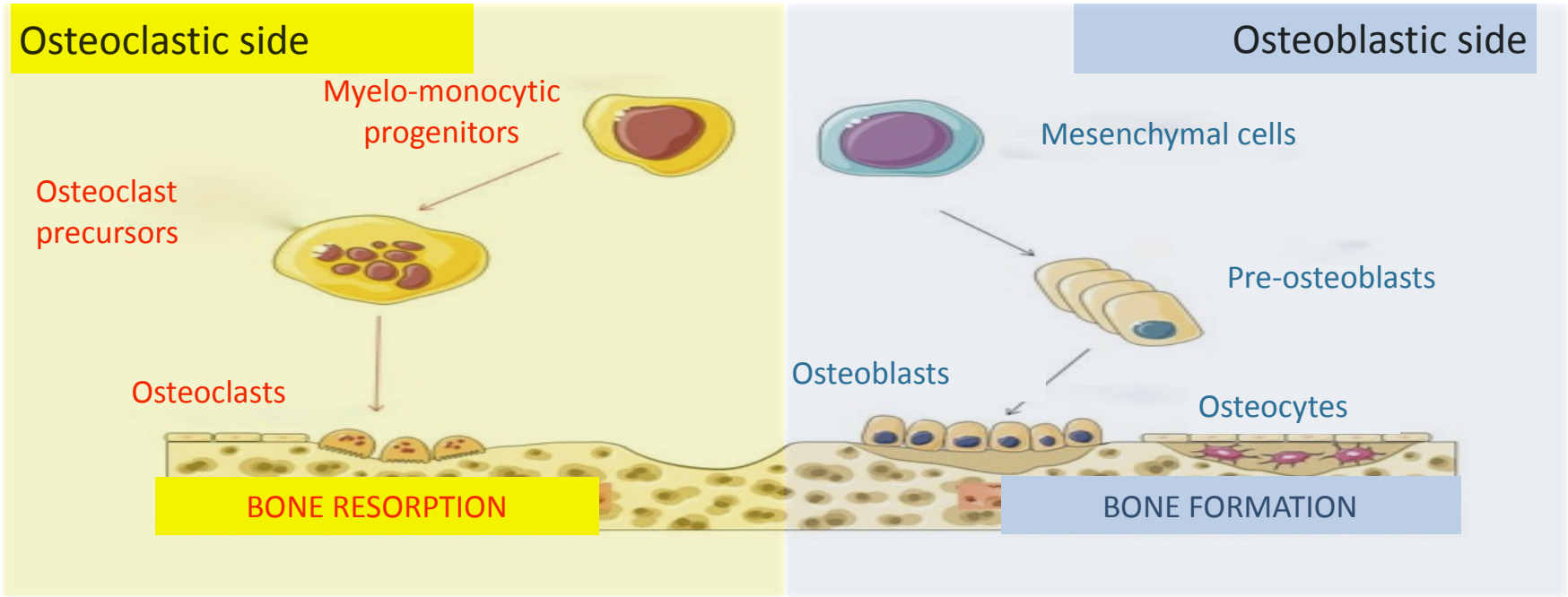




Electronic microscopy
Hoffmann *et al.*, Eur J Cell Biol 2011

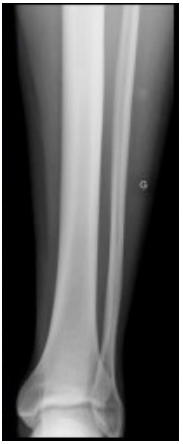
Invasion of host cells is thought to result in a **bacterial sanctuary**

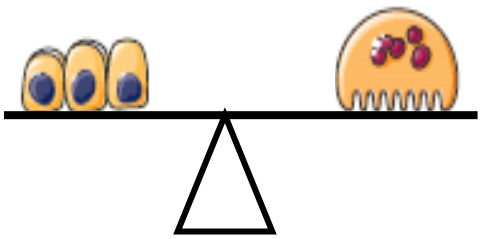
Bone tissue and infection



Physiological context

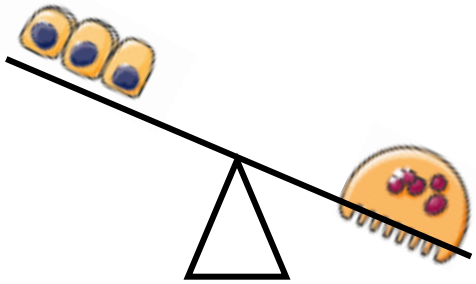
Healthy bone





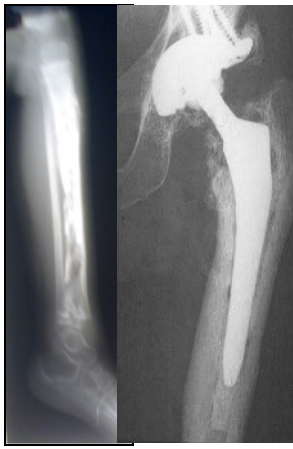
Balance osteoblast/osteoclast

Infectious context

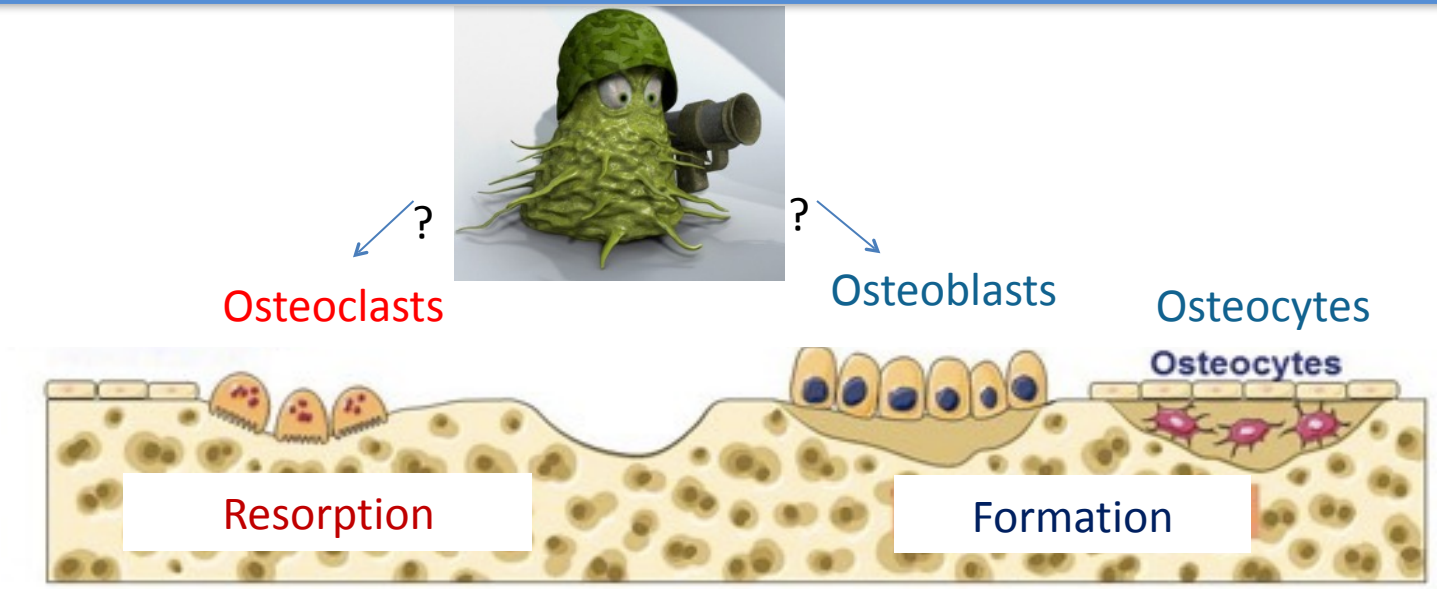


Progressive bone loss/destruction
.prothesis loosening
. loss of function

Infected bone



Unresolved questions



Loss of homeostasy due to

Loss of osteoblast function (destruction, reduction of mineralisation) ?

and/or gain of osteoclast function (increase resorption) ?

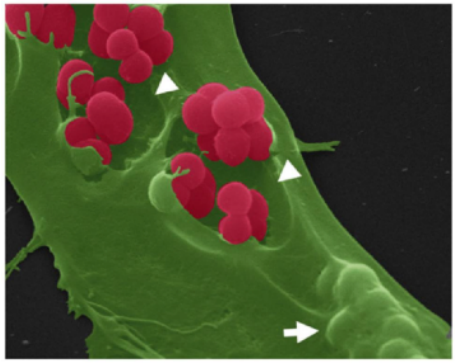
Consequences of intracellular interaction

S. aureus / osteo**b**lasts ?

S. aureus / osteo**c**lasts ?

Intracellular *S. aureus* and osteoblasts

Invade to escape or invade to kill ?



Intracellular *S. aureus* and osteoblasts

Model CA-MRSA / HA-MRSA

PVL, alpha-toxine and PSMs

Intracellular *S. aureus* and osteoclasts

Invasion of precursors

Impact on osteoclastogenesis

Impact on bone resorption

MRSA as study model of BJIs : two clinical patterns

HA-MRSA

- Post-surgical infections
- Periprosthetic infections
- Indolent / relapsing / chronic infections

CA-MRSA

Panton-Valentine leukocidin (PVL)+

- Acute osteomyelitis (children++)
- Rapid progression with sepsis
- Major inflammation, bone destruction, involvement of surrounding soft tissue

Underlying mechanisms ?
Genotypic differences ?

↓

Optimization of therapeutic strategies

Working hypothesis

- Interactions of CA- and HA-MRSA with osteoblasts might differ
  - CA-MRSA => host cell damage
  - HA-MRSA => intracellular survival

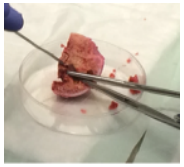
Objectives

- Compare CA- and HA-MRSA virulence phenotypes in an ex vivo model of osteoblast intracellular infection
- Main readouts:
  - Host cell damage
  - Viable intracellular bacterial load

Osteoblast model

Osteoblasts

MC3T3 cells
MOI:50



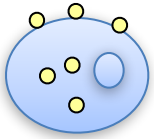
Human primary osteoblasts
MOI:250

Bacteria culture

24h 37°C

Infection

Invasion (2h)

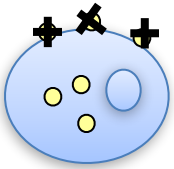


Cellular lysis

H2

Adhesion
Agar plate counting of extracellular bacteria

Lysostaphin



H24- H48

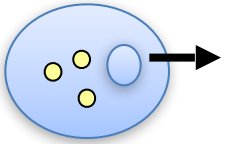
Cytotoxicity
Quantification of LDH release

Internalization
Agar plate counting of intracellular bacteria

H3

H48

Immune response
Quantification of cytokine concentrations in cellular supernatant (Luminex)



Cellular lysis

H24-H48-H72

Persistance
Agar plate counting of intracellular bacteria

Materials

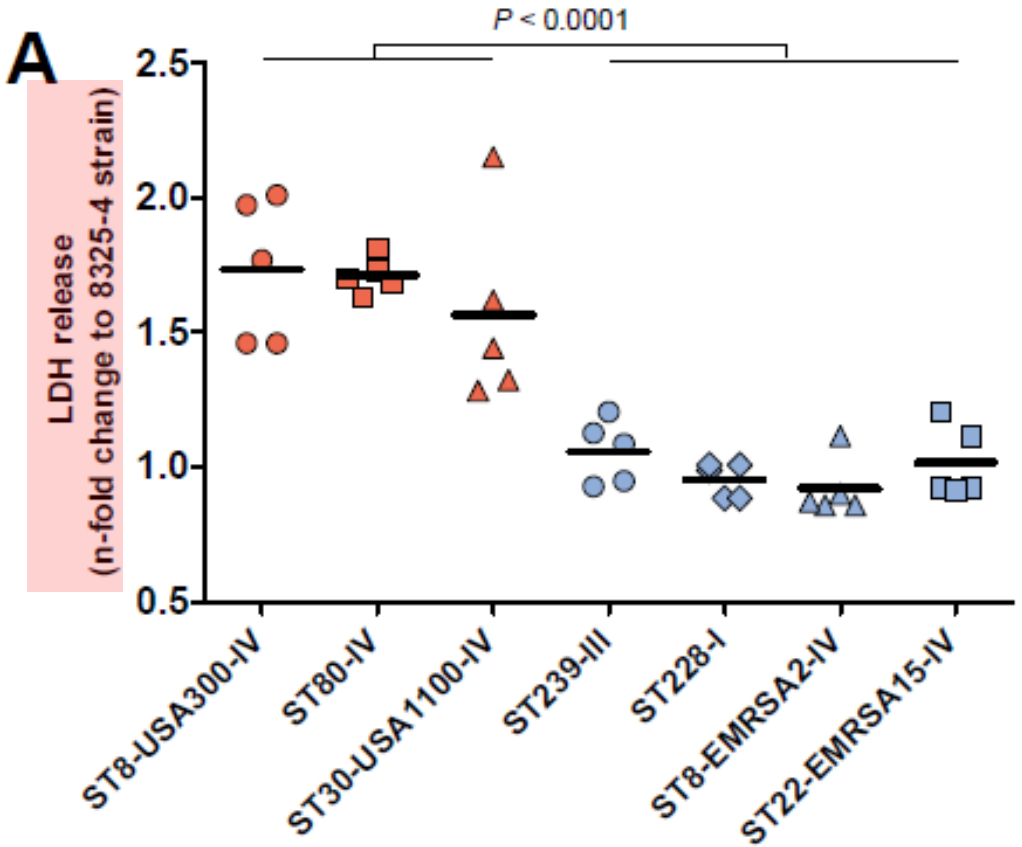
Bacterial strains

- 7 lineages, 5 strains in each lineage
  - CA-MRSA, n=15 / HA-MRSA, n=20

	Genotype	Origin	HA/CA	Seq. type	*agr*	SCC*mec* type
CONTROL	**8325-4**	-	-	8	1	- (MSSA)
CA-MRSA	**ST80**	France	CA	80	3	IV
CA-MRSA	**ST30**	Australia, Japan, New Zealand, Samoa, Singapour	CA	30	3	IV
CA-MRSA	**USA300**	US	CA	8	1	IV
HA-MRSA	**EMRSA-2**	France	HA	8	1	IV
HA-MRSA	**ST228**	Switzerland	HA	228	2	I
HA-MRSA	**ST239**	Poland, Greece, Bulgaria	HA	239	1	III
HA-MRSA	**EMRSA-15**	UK, Germany, Austria	HA	22	1	IV

Results

Cytotoxicity of CA- and HA-MRSA after 24h

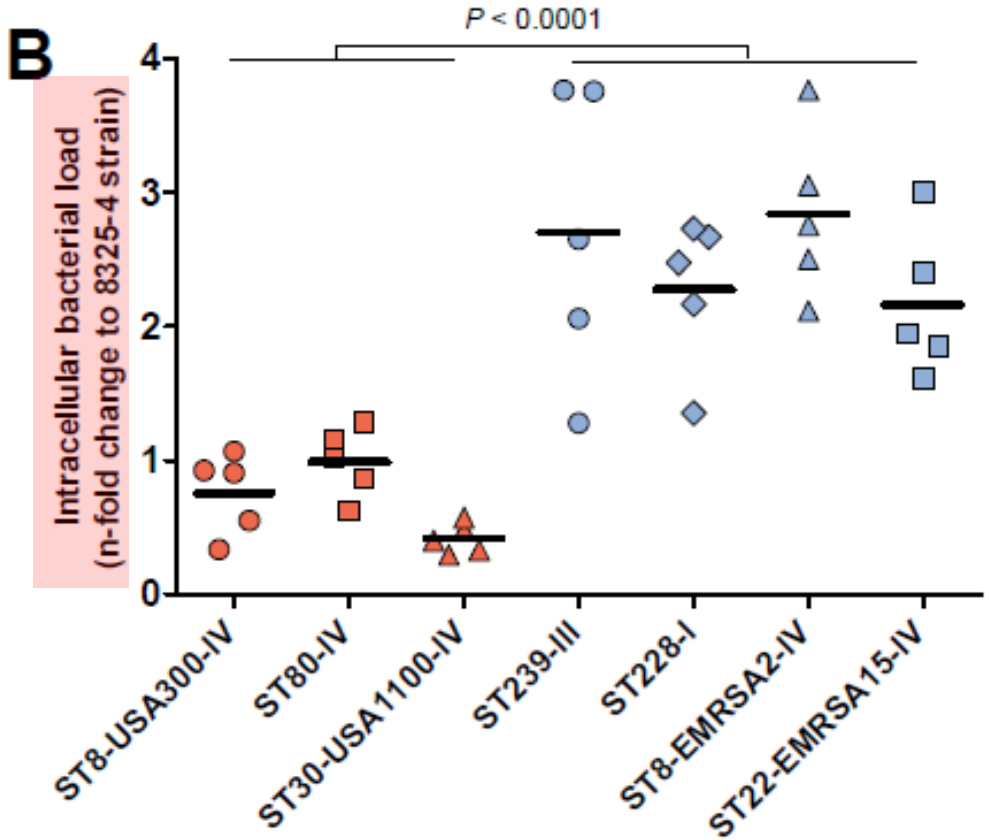


Damage to infected cells: **CA-MRSA** >> **HA-MRSA**

Rasigade et al. PLoS One 2013

Results

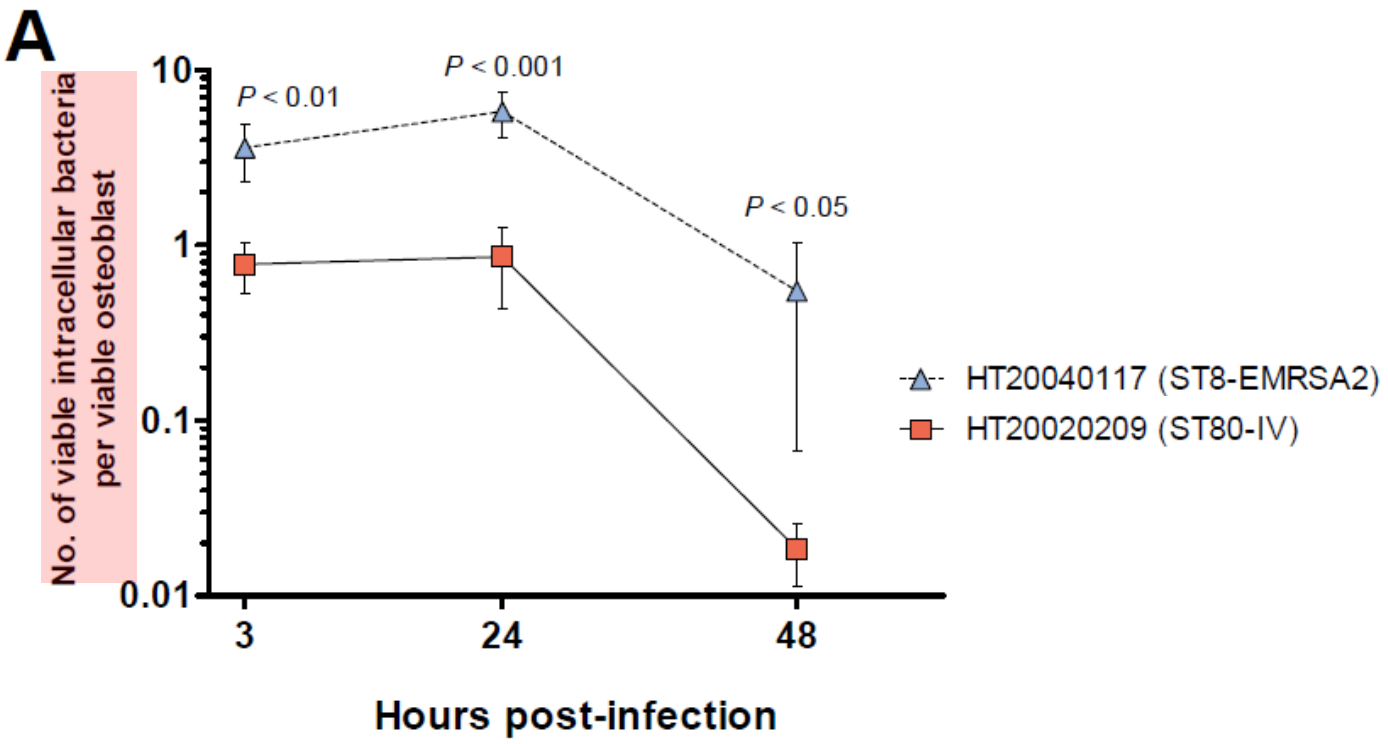
Intracellular persistence of CA- and HA-MRSA after 24h



Intracellular persistence: **HA-MRSA** >> **CA-MRSA**

Results

Kinetics experiments

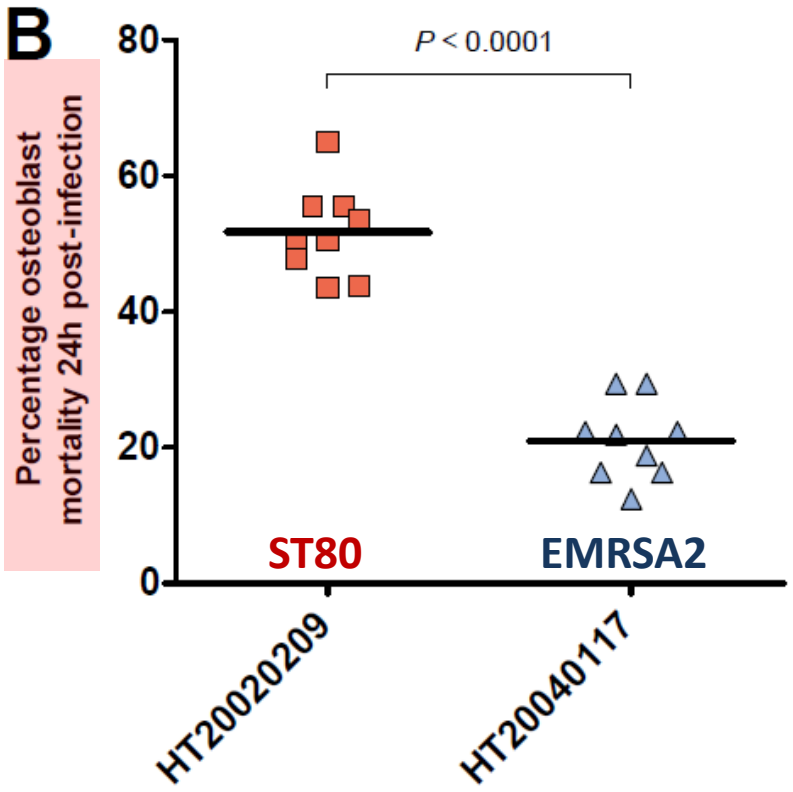


Bone cells bear ≈ **1 CA-MRSA / cell** vs ≈ **5 HA-MRSA / cell**

Differences increase over time

Results

Osteoblast mortality percentage (same experiments)



≈ 1 intracellular CA-MRSA / cell → 50% host cell mortality after 24h

≈ 5 intracellular HA-MRSA / cell → 20% host cell mortality after 24h

Conclusions

CA-MRSA are **less efficient at invading osteoblasts and surviving** inside them than are HA-MRSA

But …

CA-MRSA **cause much stronger damage** to infected osteoblasts than HA-MRSA

Intracellular *S. aureus* and osteoblasts

Model CA-MRSA / HA-MRSA

PVL, alpha-toxine and PSMs

Intracellular *S. aureus* and osteoclasts

Invasion of precursors

Impact on osteoclastogenesis

Impact on bone resorption

Part II.

Role of CA-MRSA virulence determinants in osteoblast damages observed after invasion

Major virulence determinants of CA-MRSA = toxins

We know that

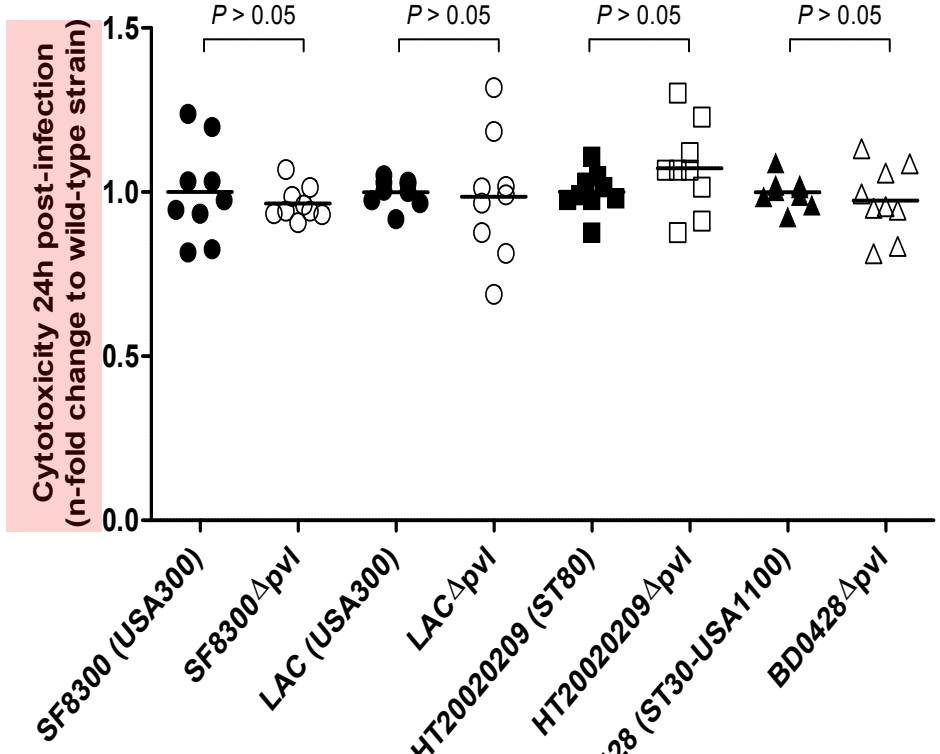
- **Panton-Valentine leukocidin** (PVL)= present +++
- **Alpha-toxin** (HLA) = overexpression
- **Phenol-soluble modulins** (PSMs) = overexpression

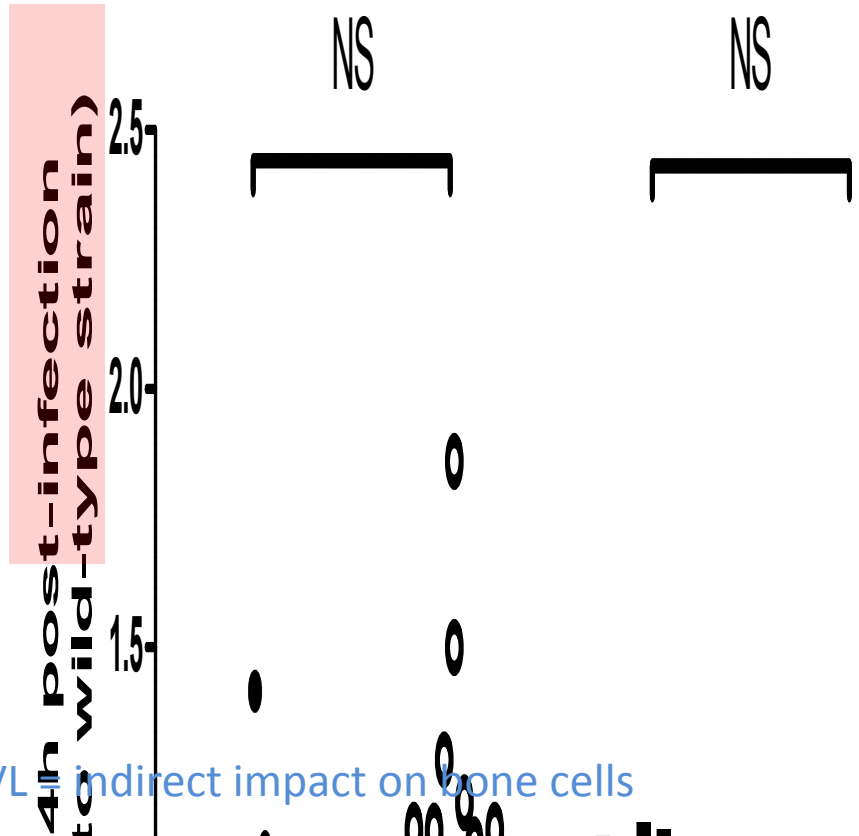
Results

Panton-Valentine leukocidin and alpha-toxin

(loss-of-function approach)

- deletion of ***pvl*** or ***hla*** genes in lineages USA300, ST80 and ST30 **had no impact** on cytotoxicity

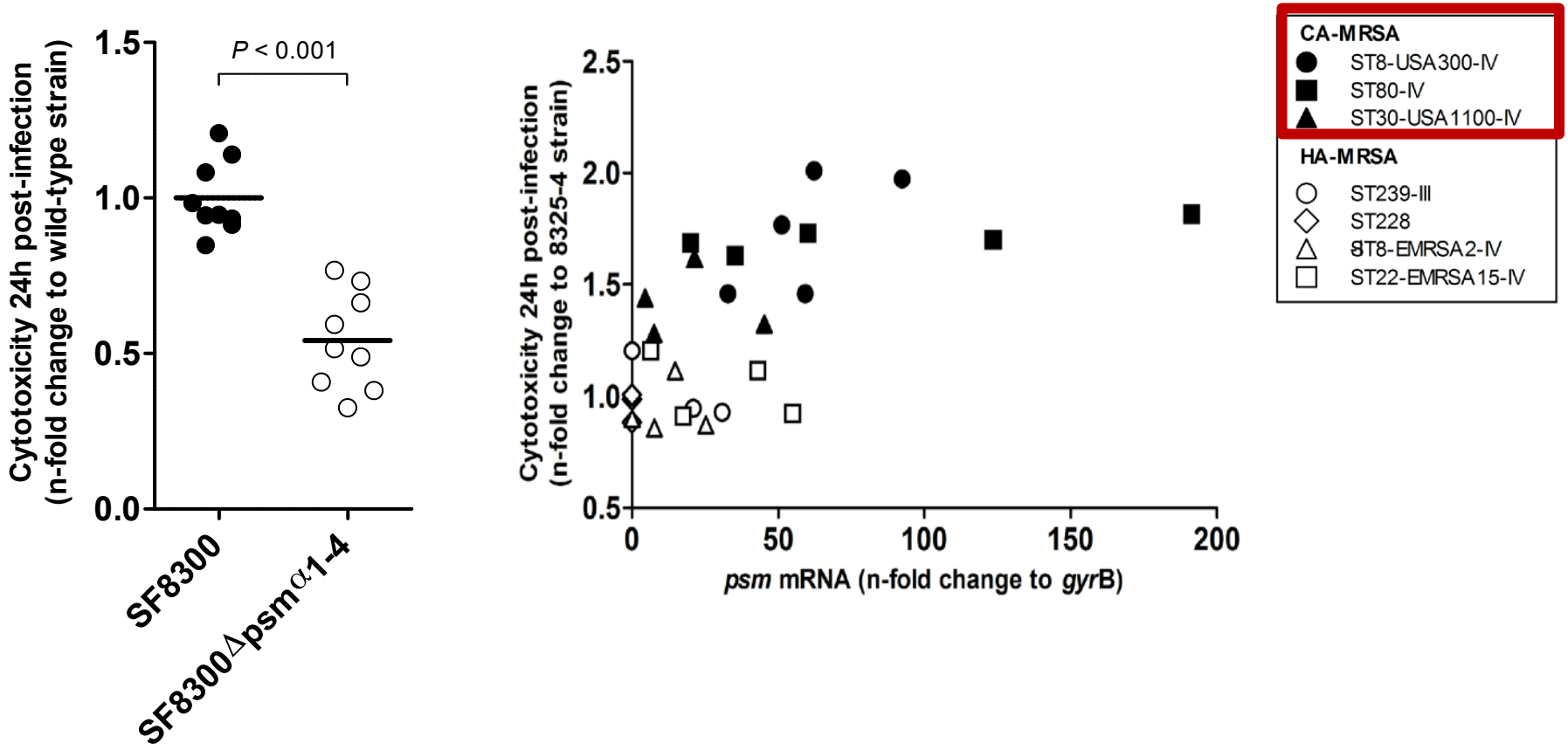




PVL = indirect impact on bone cells

Results

PSM expression associated with death of invaded osteoblasts

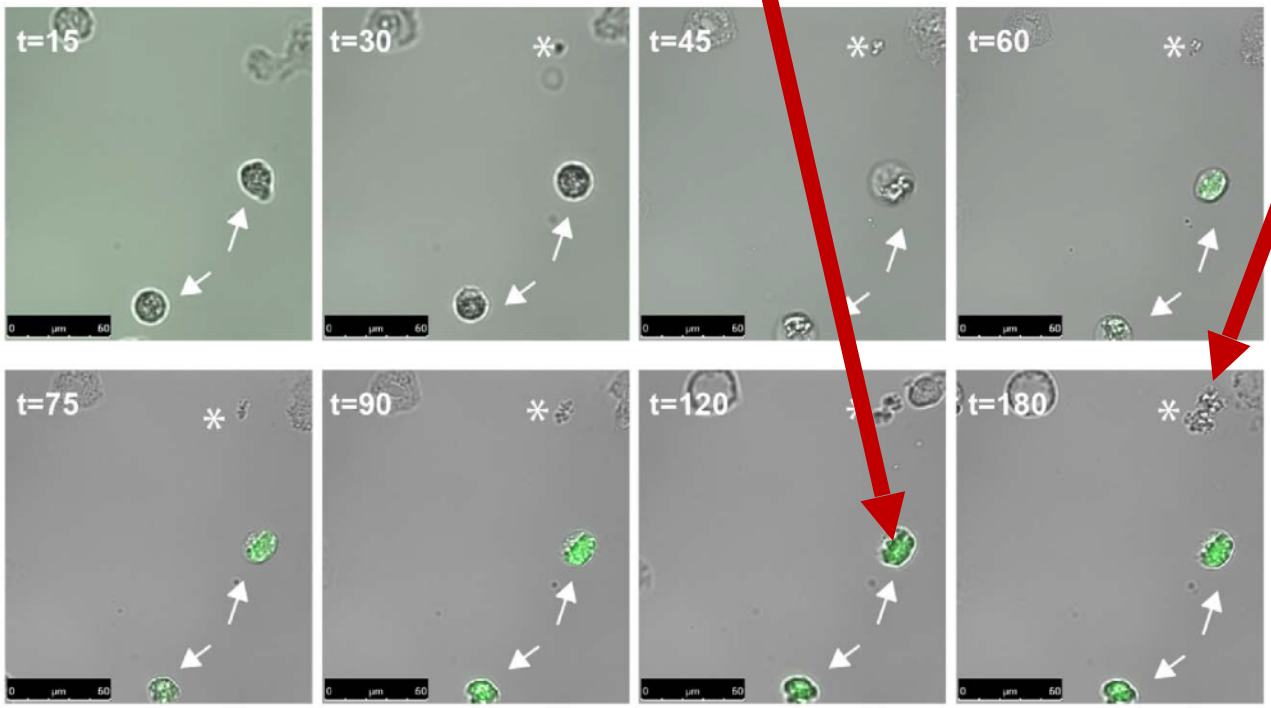


- **Alpha-type PSMs in strain USA300 SF8300 are involved in cytotoxicity**
- Level of expression of PSM (qRT-PCR) correlated to cytotoxic damages

Phenol soluble modulins

PSM are expressed after entrance in cells !

- Use Fluorescent marker for PSM expression
  - PSM expressed 1h post invasion
  - No or low expression by extracellular *S. aureus* extracellulaires



PSM = first intracellular toxins of *S. aureus*

Surewaard *et al*. PLoS Pathogens 2012

BJI due to CA-MRSA

Pathophysiological model

CA-MRSA

Extracellular Bacteria / PVL

Recruitement and lysis of PNN and macrophages release of cytotoxic intracellular content

”indirect damages”

Bone tissue destruction

”direct damages”

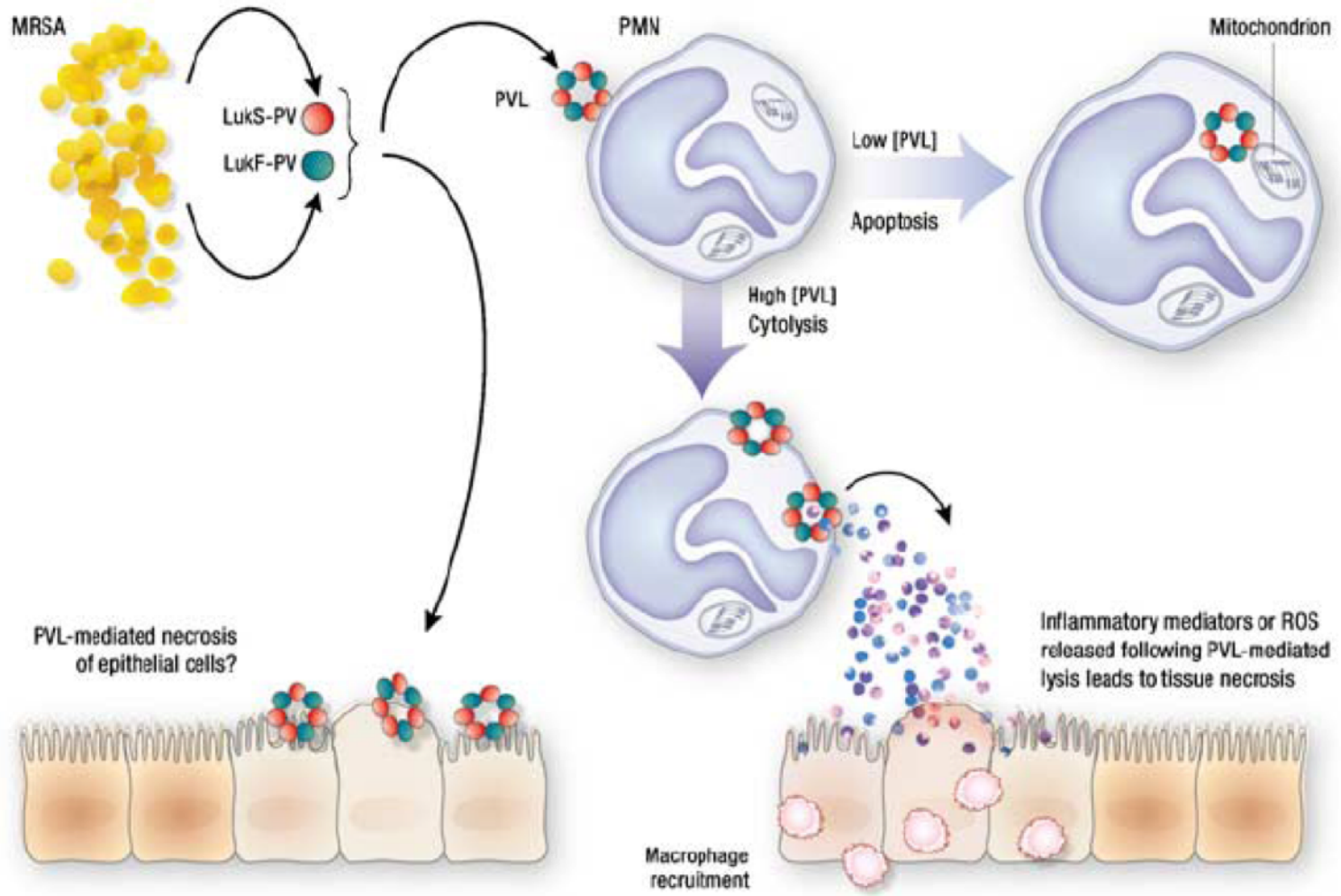
OB destruction

Invasion des OB

Intracellular PSM

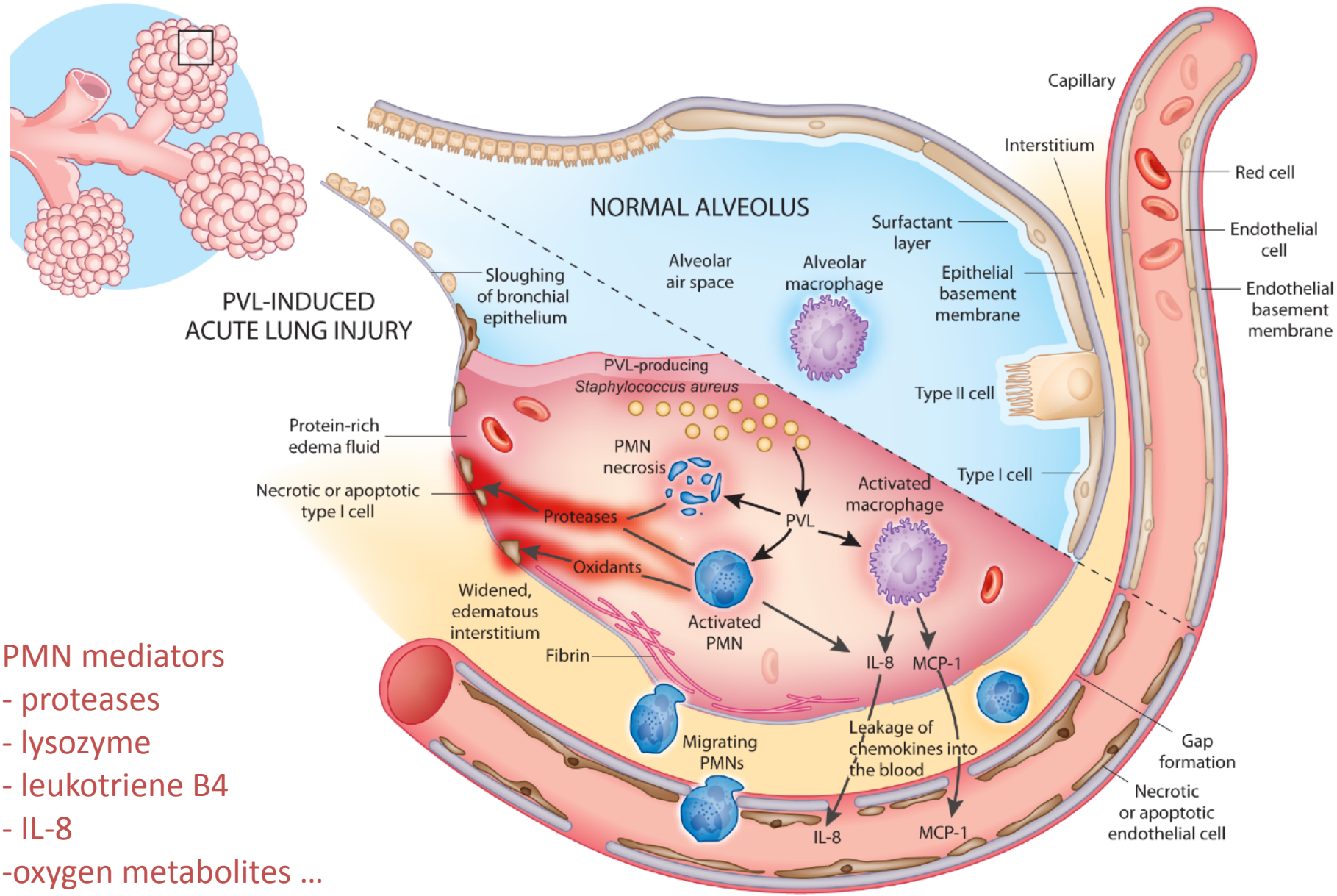
PVL : *in vivo* mechanism

MSSA +++



C5aR dependant: no expression of C5a on osteoblasts ???

Rabbit Model of *Staphylococcus aureus* pneumonia



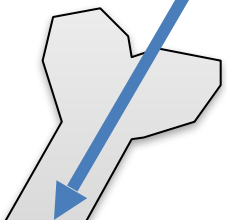
PMN mediators
- proteases
- lysozyme
- leukotriene B4
- IL-8
-oxygen metabolites …

Diep et al., Proc Natl Acad Sci U S A. 2010.

BJI due to CA-MRSA

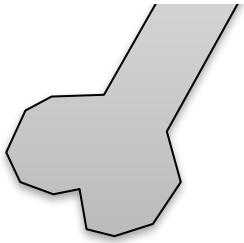
Pathophysiological model

PVL → Recruitement and lysis of PNN and macrophages release of cytotoxic intracellular content



”indirect damages”

Bone tissue destruction



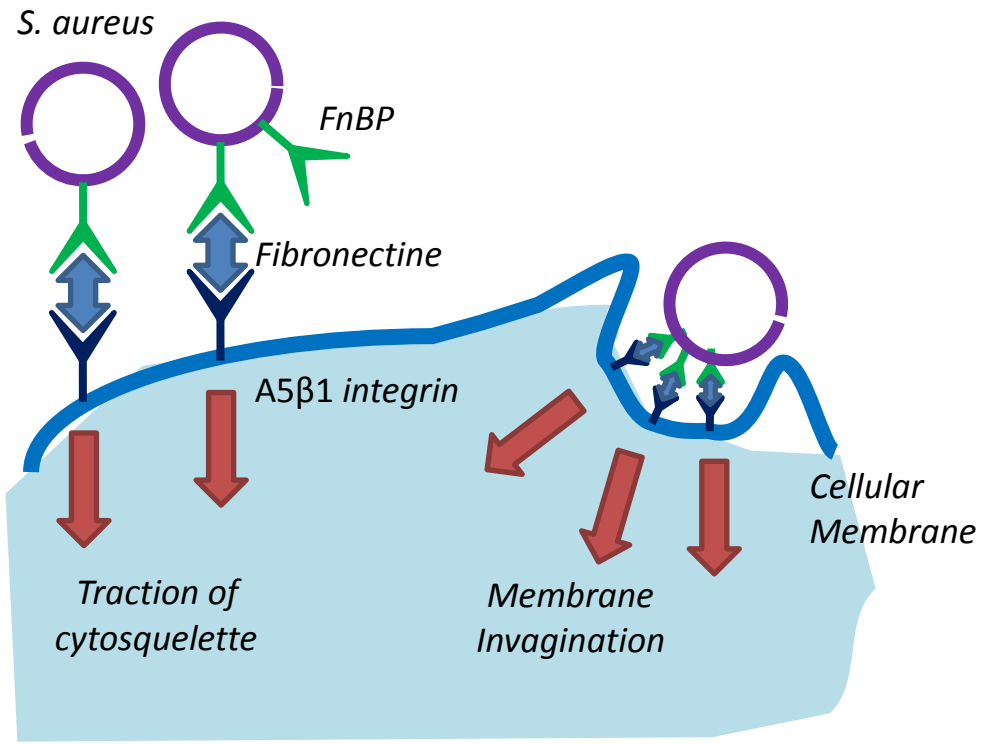
”direct damages”

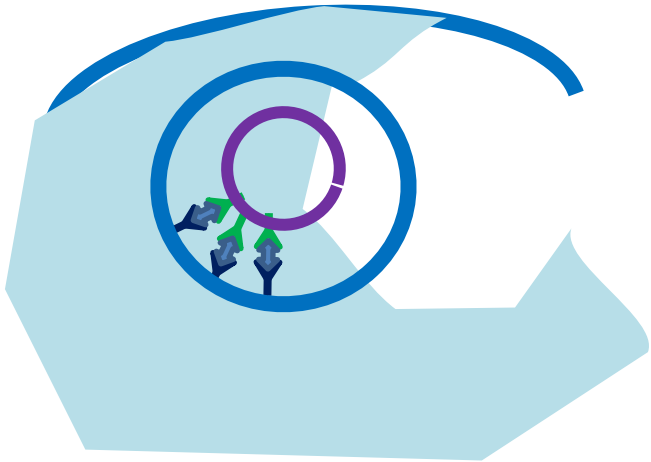
CA-MRSA

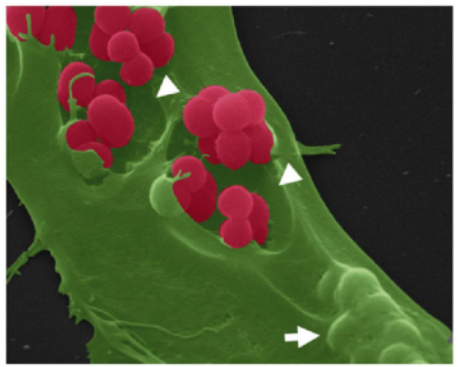
OB destruction

Invasion des OB → Intracellular PSM

Osteoblast invasion by *S. aureus*







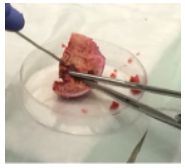
Electronic microscopy
Hoffmann *et al.*, Eur J Cell Biol 2011

Invasion of host cells is thought to result in a **bacterial sanctuary**

Osteoblast model

Osteoblasts

MC3T/MG63 cells
MOI:50



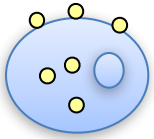
Human primary osteoblasts
MOI:250

Bacteria culture

24h 37°C

Infection

Invasion (2h)

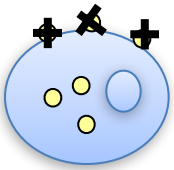


Cellular lysis

H2

Adhesion
Agar plate counting of extracellular bacteria

Lysostaphin



H24- H48

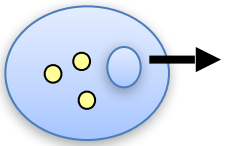
Cytotoxicity
Quantification of LDH release

Internalization
Agar plate counting of intracellular bacteria

H3

H48

Immune response
Quantification of cytokine concentrations in cellular supernatant (Luminex)

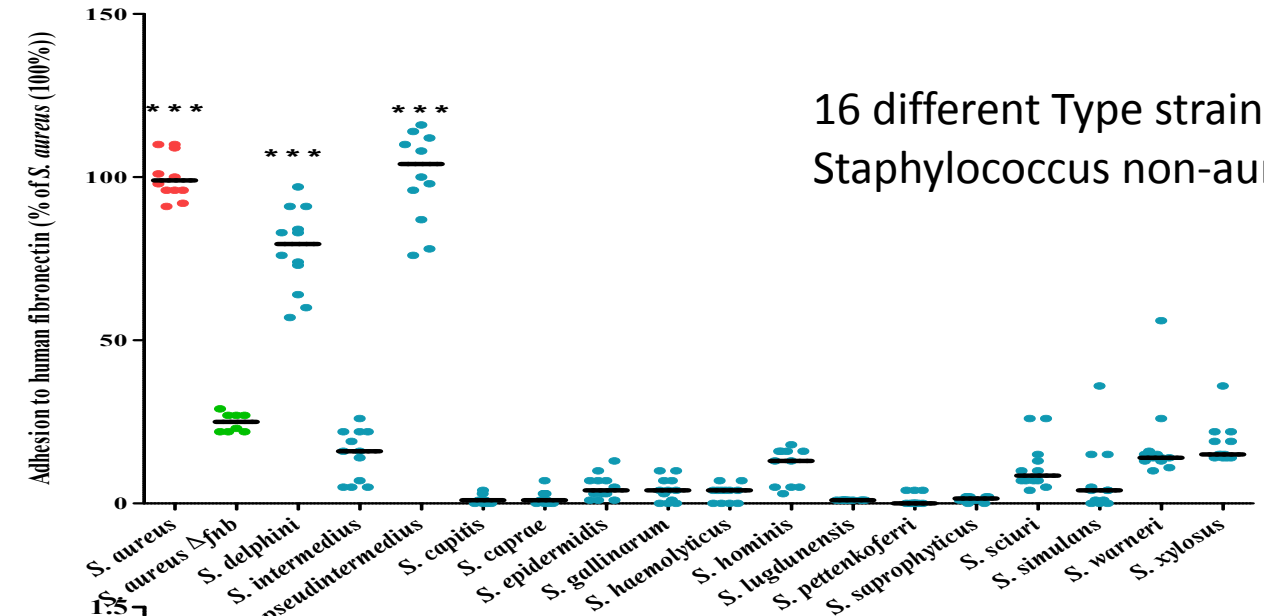


Cellular lysis

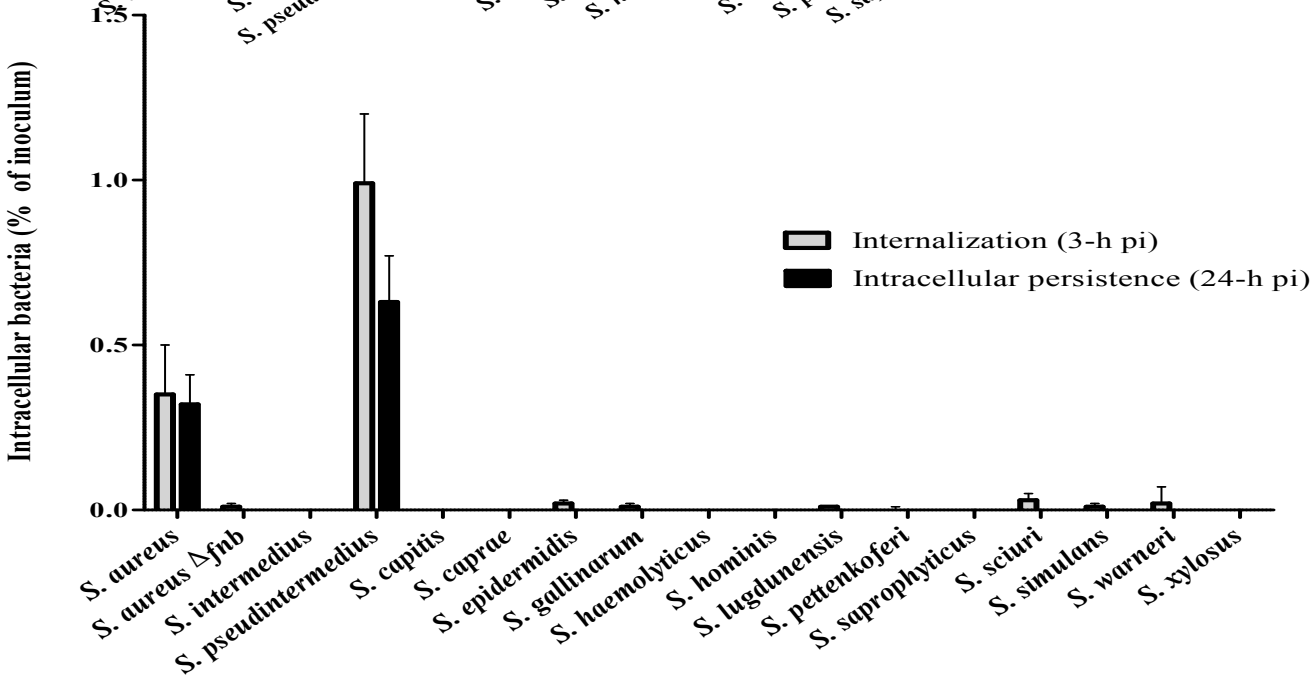
H24-H48-H72

Persistance
Agar plate counting of intracellular bacteria

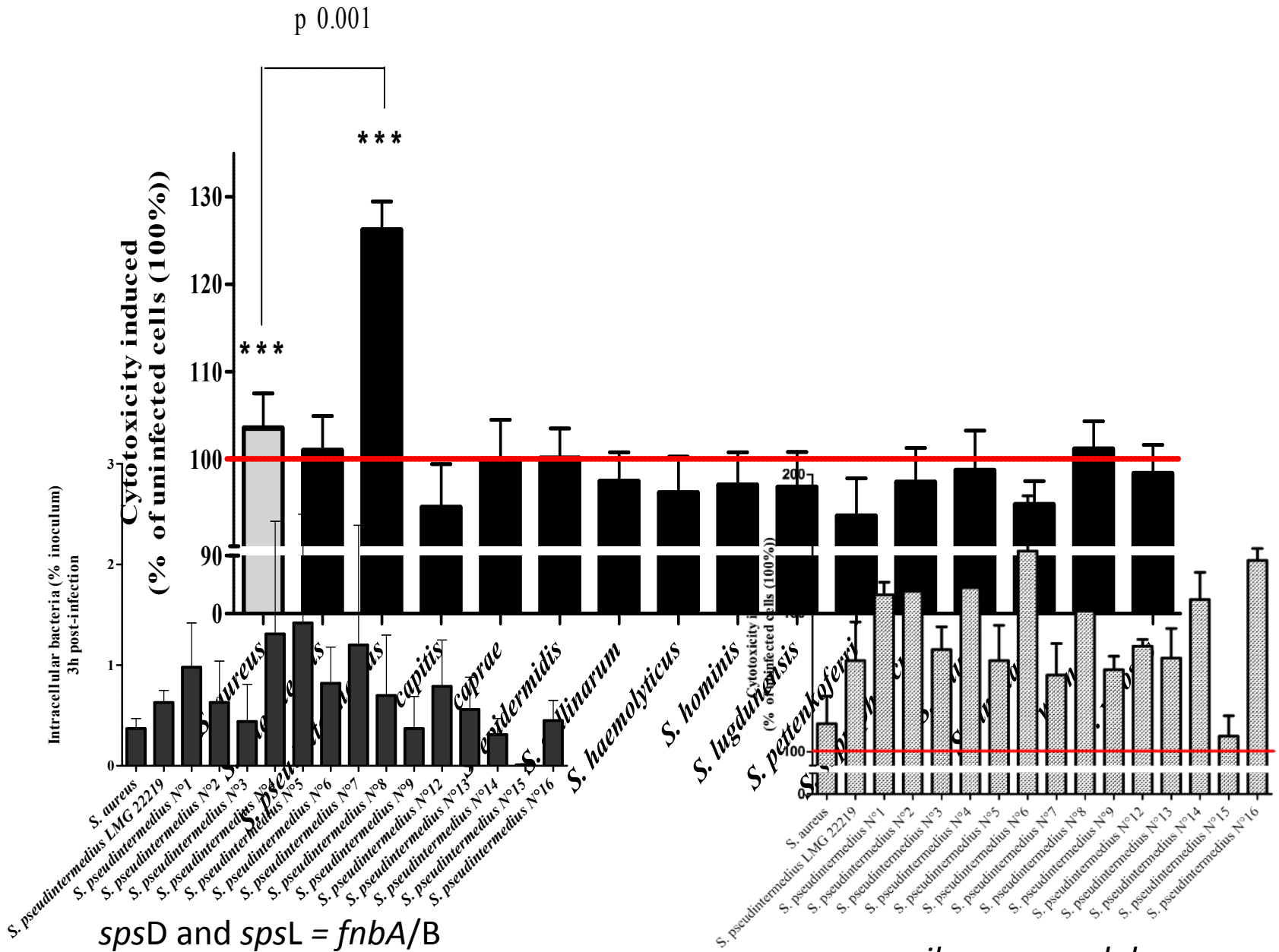
Osteoblast invasion by *S. non-aureus*



16 different Type strains belonging to 16 different Staphylococcus non-aureus species



Osteoblast invasion by *S.* non-*aureus*

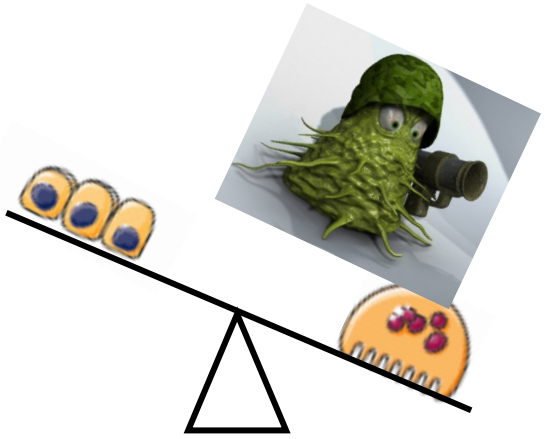


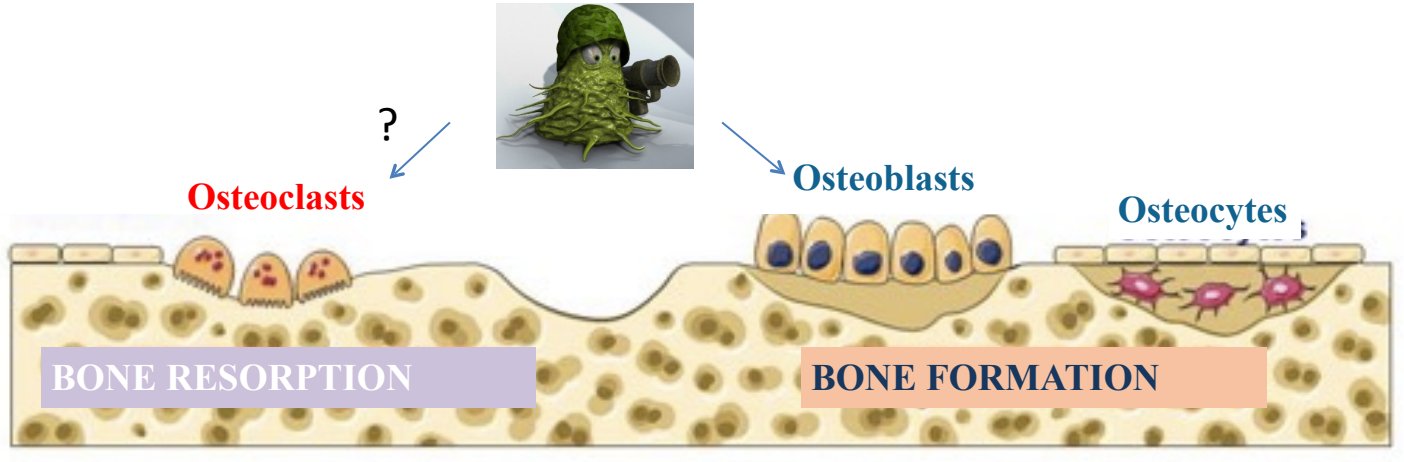
*sps*D and *sps*L = *fnbA*/B

psm epsilon = psm alpha

S. aureus invasion
and osteoclasts

Direct and indirect impacts on bone resorption



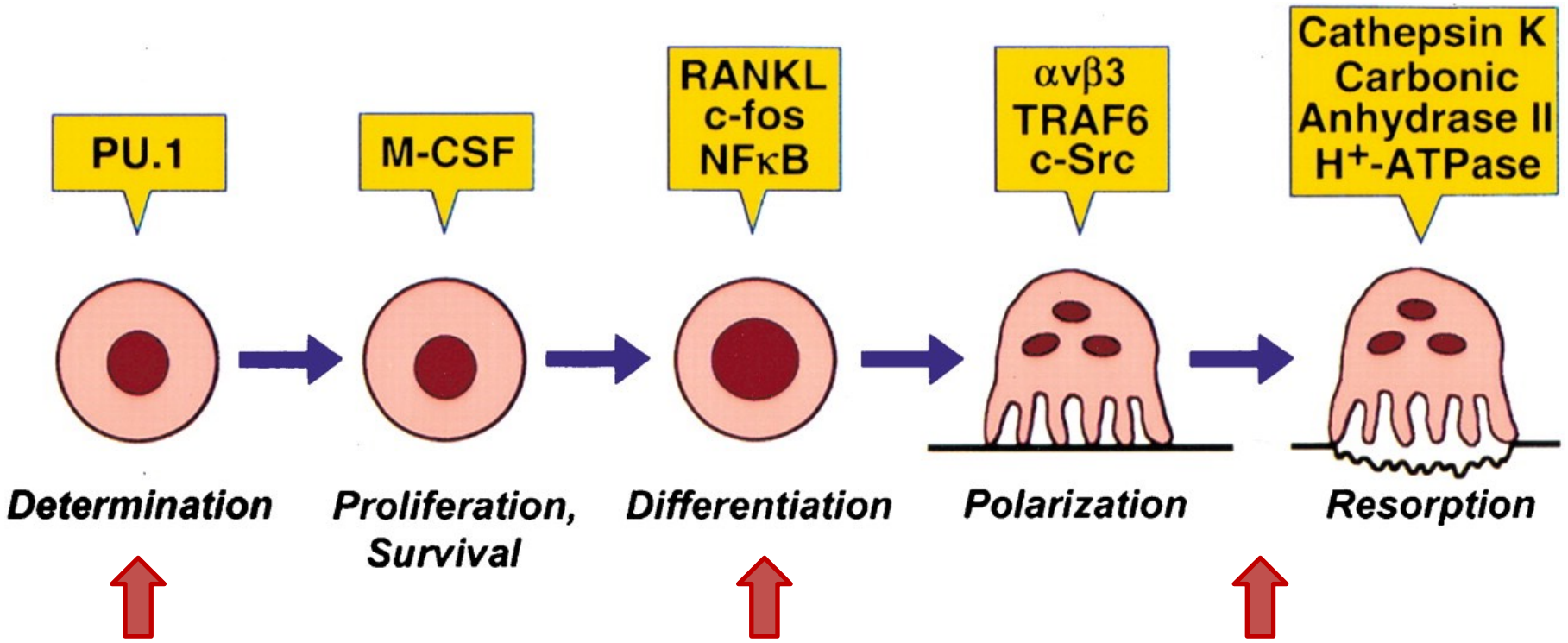


Aim :

- To study the direct impact of *S. aureus* on osteoclasts :
  - Phenotypic aspect (osteoclastogenesis)
  - Functional aspect (bone resorption)

Strategy of study

To test the *S. aureus* impact on different phase of osteoclastic differentiation



Myelo-monocytic precursors

Differentiation
TRAP +

Fusion
Large and multinucleated cells

Tartrate-resistant acid Phosphatase

Material and methods

Bone marrow

blood

Precursors

INVASION

2h

Gentamicine 200 µg/mL

- Reference strain **8325-4**
- **DU5883**, Delta-FnBP strain (essential protein for *S. aureus* internalization by osteoblasts)

Différenciation in media with M-CSF and RANK-L

Gentamicine (40µg/ml)

Osteoclastogenesis ?

Différenciation in media with M-CSF and RANK-L

INVASION

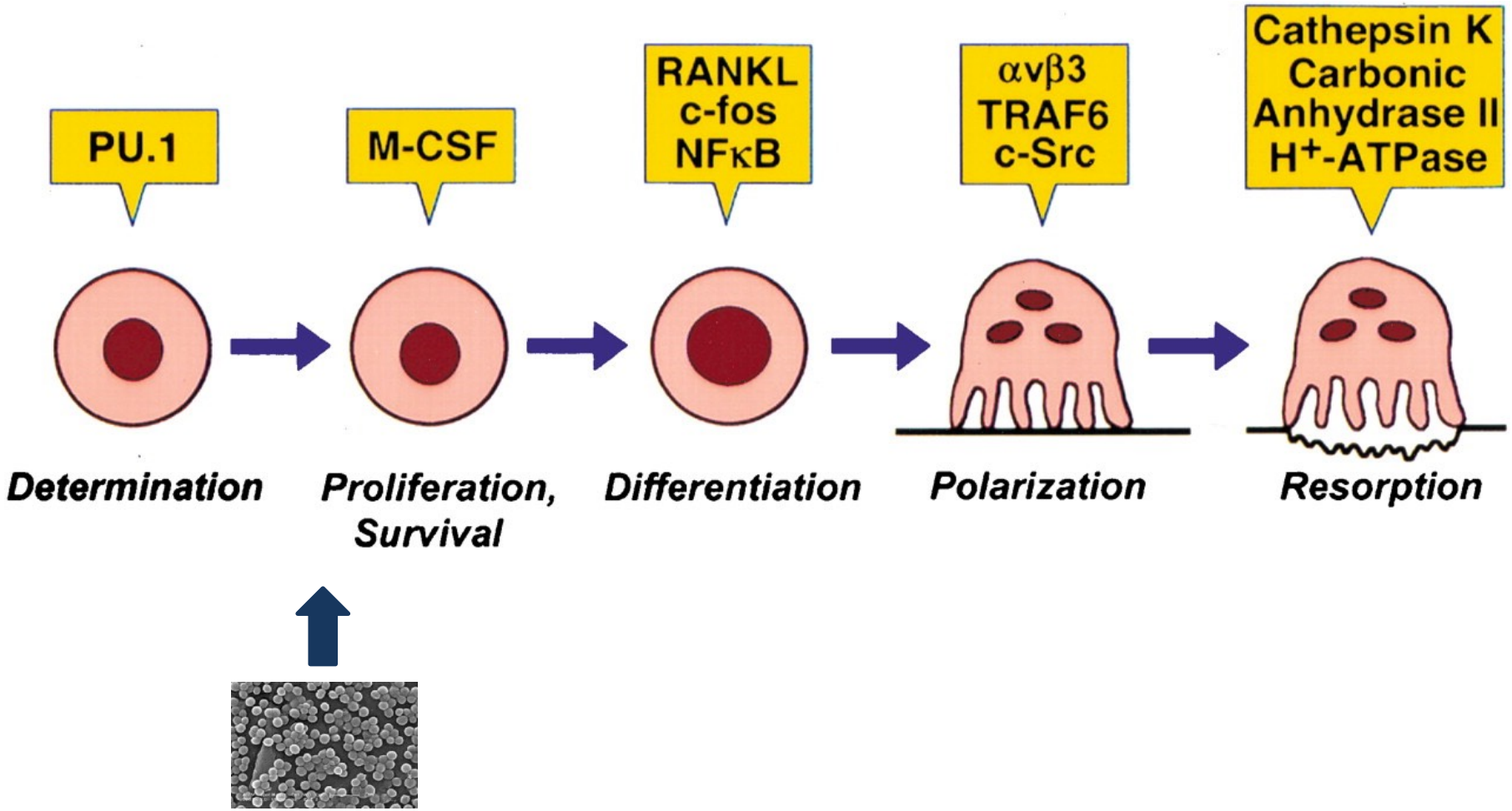
2h

Gentamicine 200 µg/mL

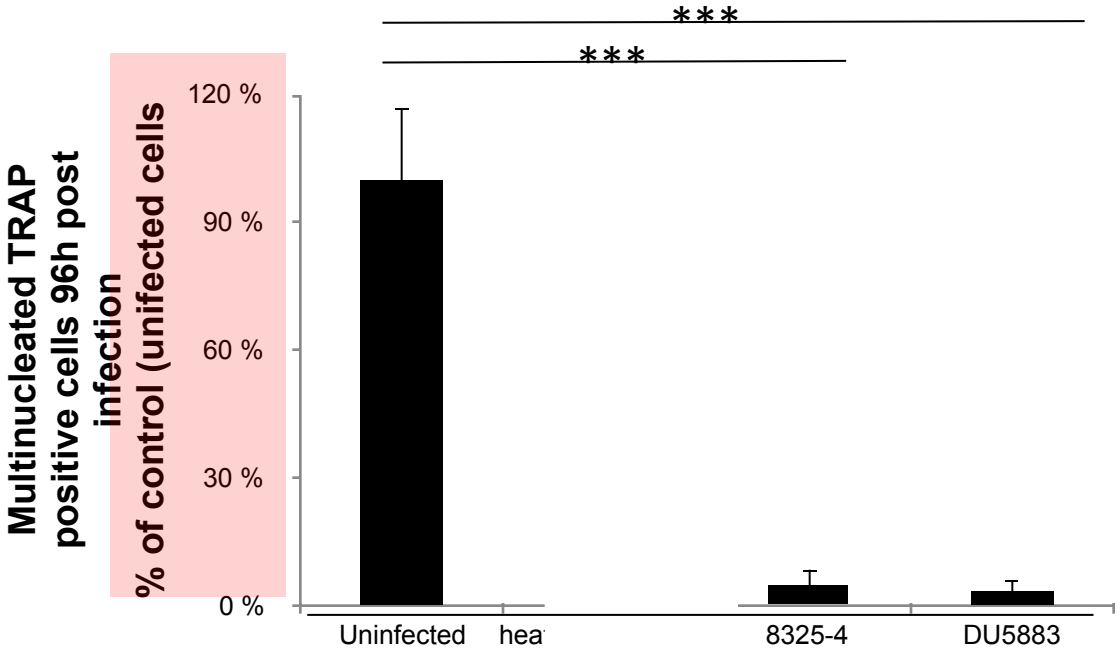
Evaluation of bone resorption on bone matrix

Interaction Osteoclasts – *S. aureus*

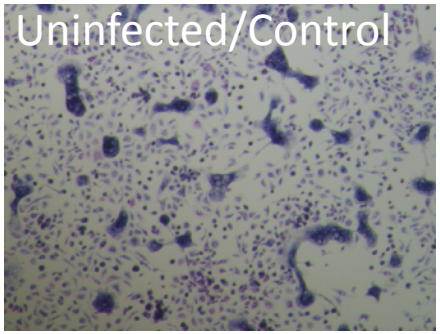
Infection of osteoclastic precursors

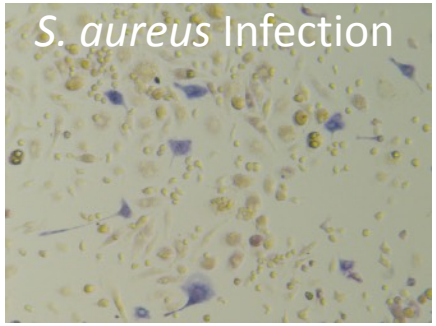


Impact of live *S. aureus* infection on osteoclastogenesis



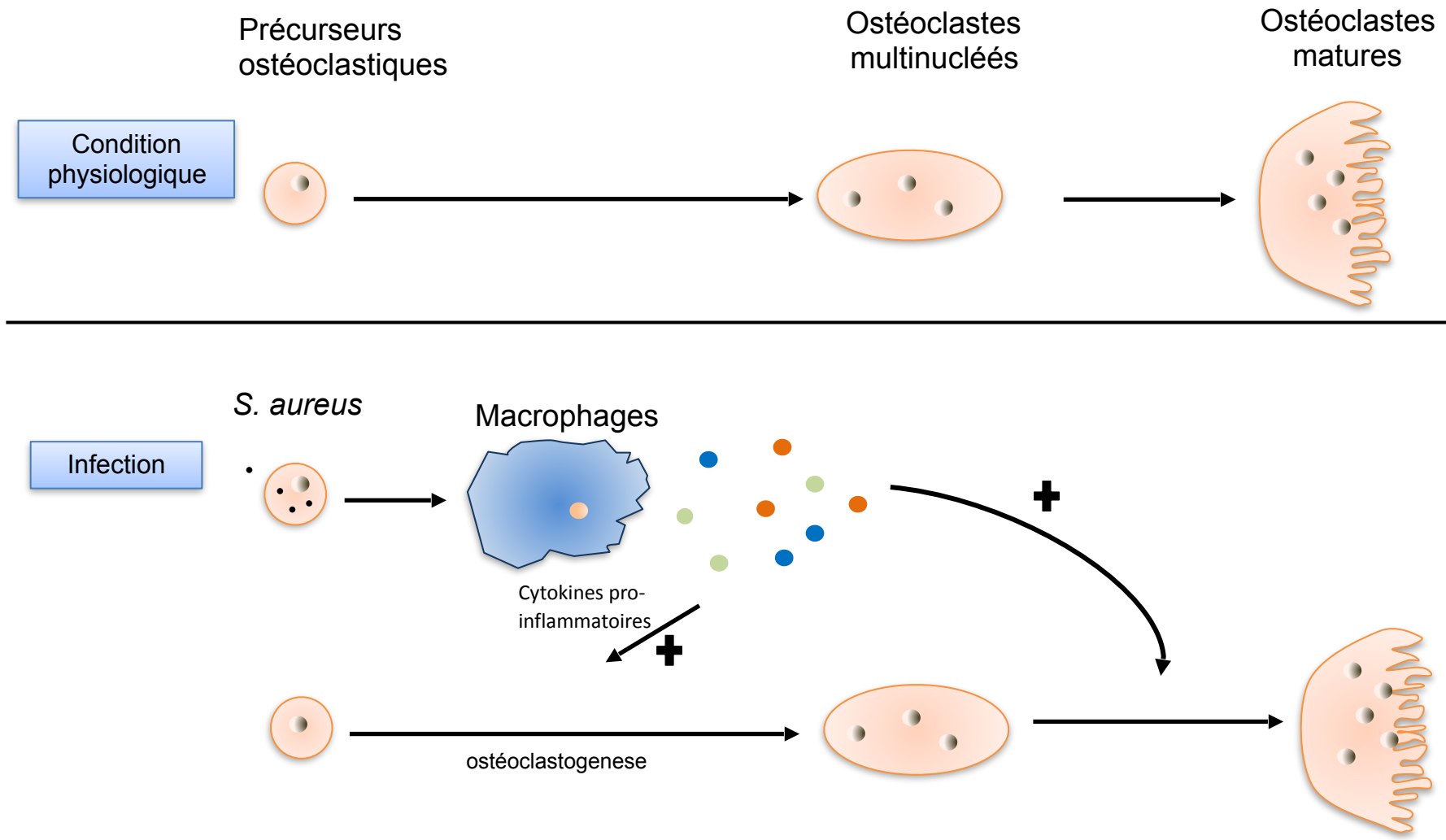
Inhibition of osteoclastogenesis after live *S. aureus* internalization





- Cytometry typing = **macrophage**
- **Pro-inflammatory chemokine release**
- Supernatent = **boost resorption by mature uninfected OC**

Interaction Ostéoclastes – *S. aureus*



Material and methods

Bone marrow

blood

Precursors

INVASION

2h

Gentamicine 200 µg/mL

- Reference strain **8325-4**
- **DU5883**, Delta-FnBP strain (essential protein for *S. aureus* internalization by osteoblasts)

Différenciation in media with M-CSF and RANK-L

Gentamicine (40µg/ml)

Osteoclastogenesis ?

Différenciation in media with M-CSF and RANK-L

INVASION

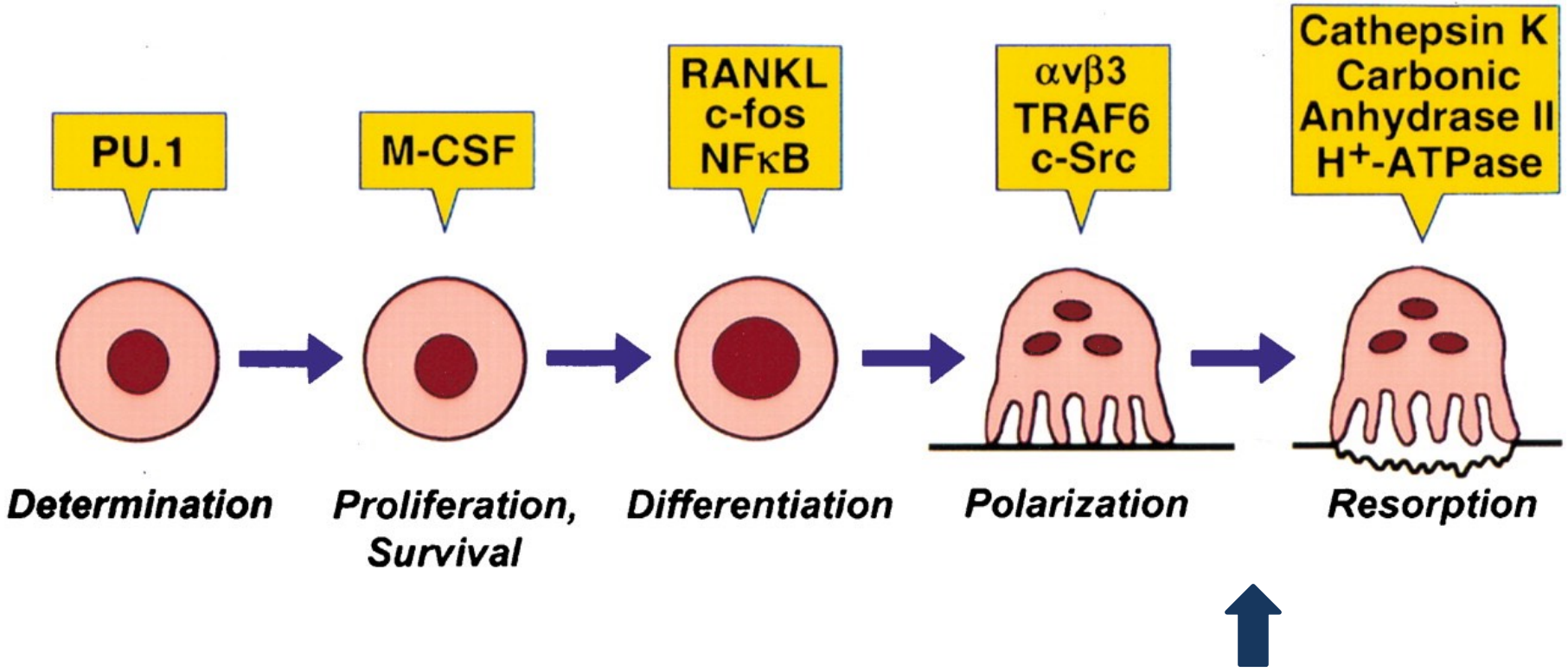
2h

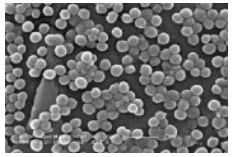
Gentamicine 200 µg/mL

Evaluation of bone resorption on bone matrix

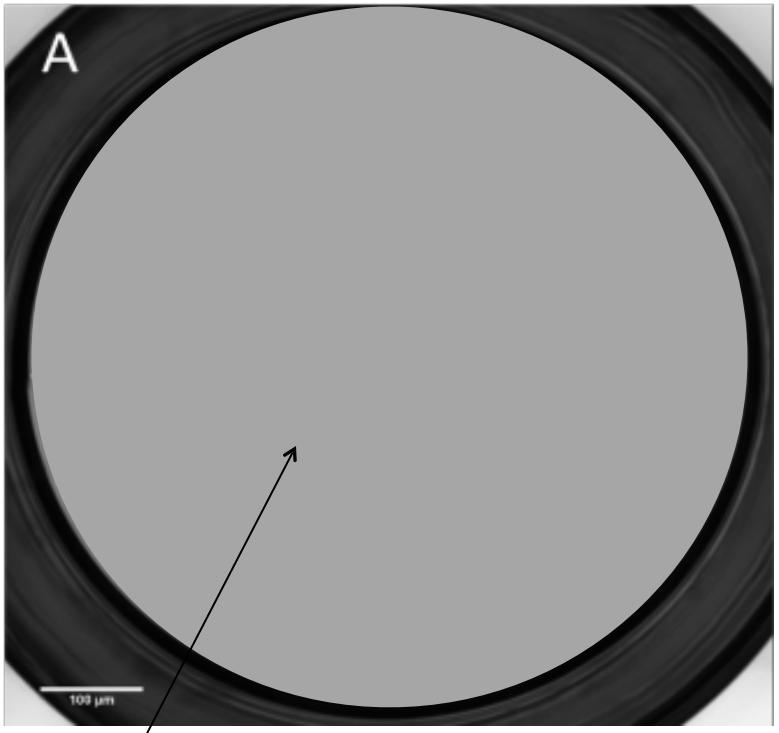
Interaction Osteoclasts – *S. aureus*

Infection of mature osteoclasts



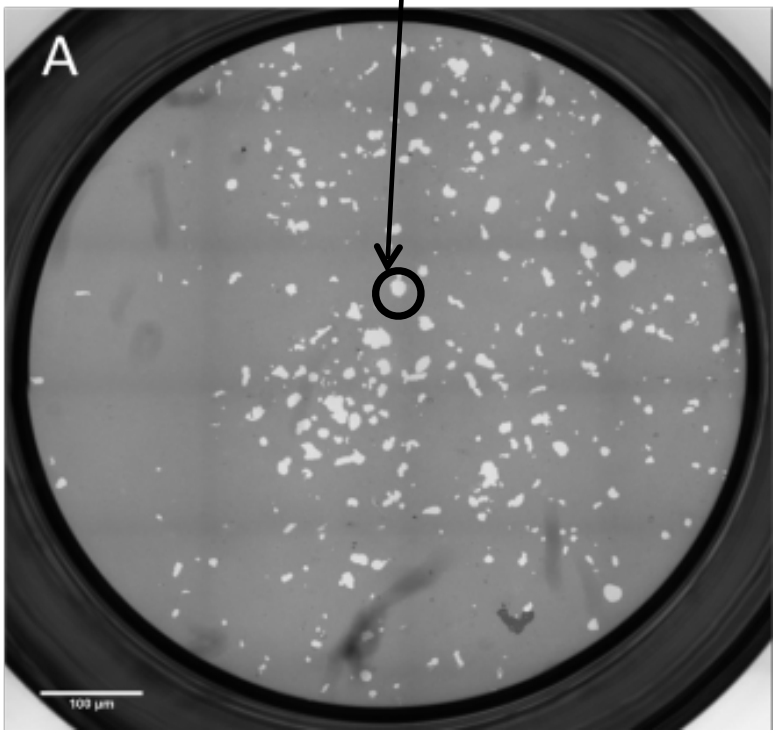


Interaction osteoclasts – *S. aureus*



Mineral matrix mimicking bone

Resorption

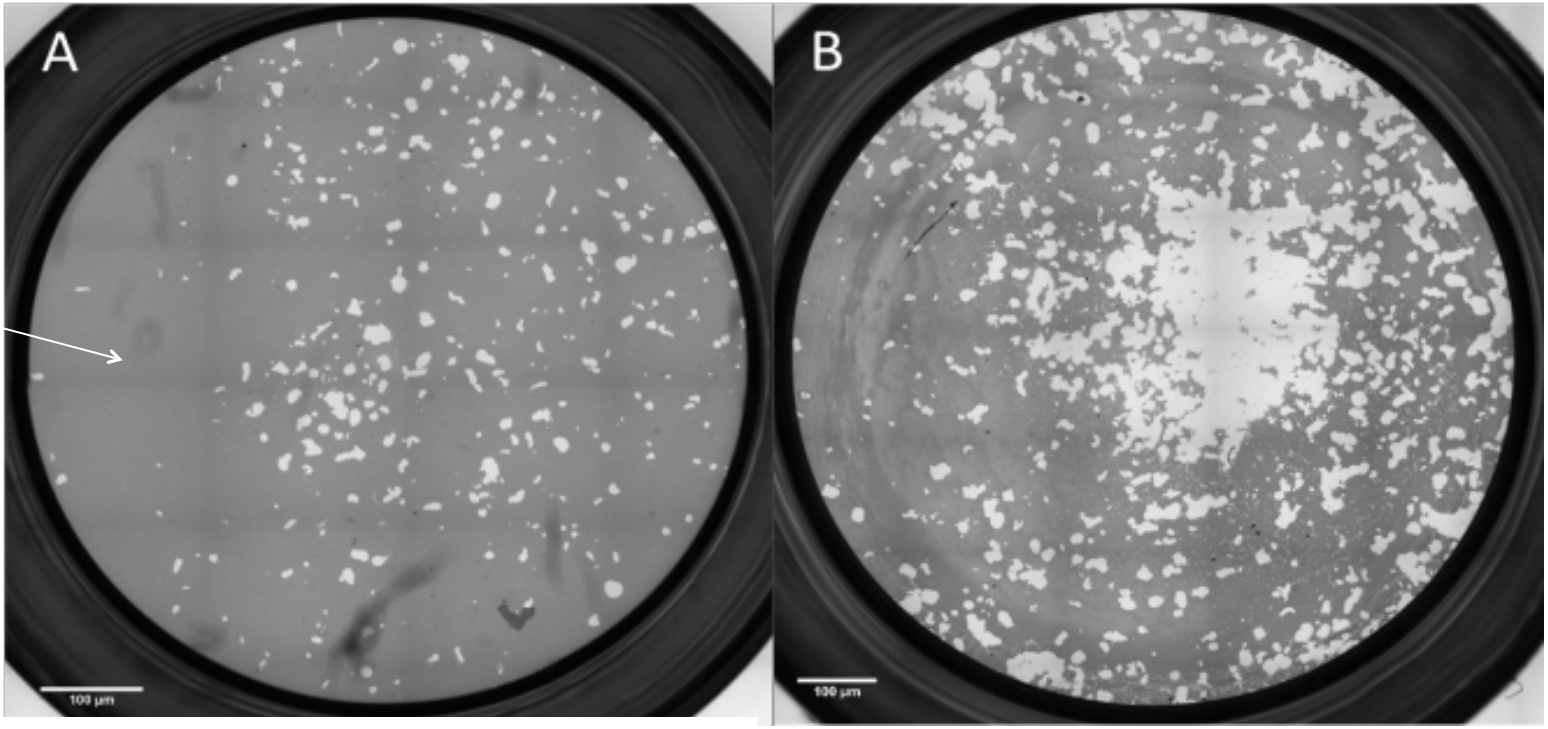


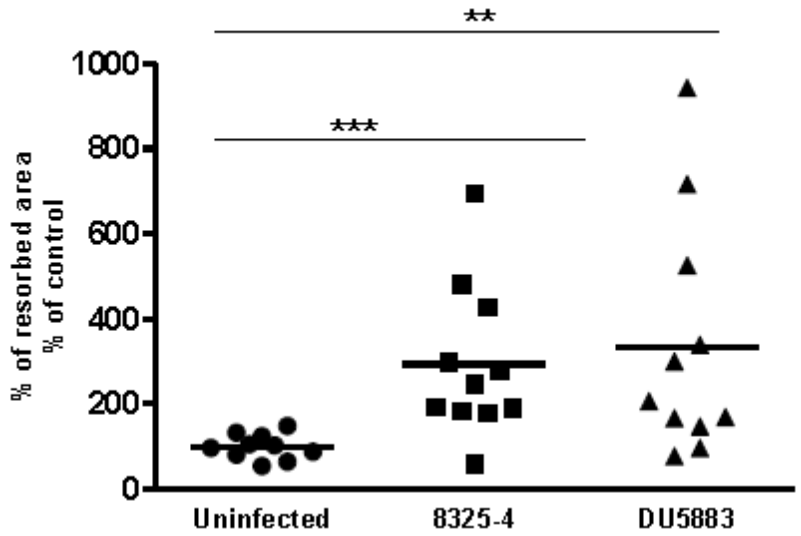
Interaction Osteoclasts – *S. aureus*

unfected mature osteoclast - Control

S. aureus-infected osteoclast

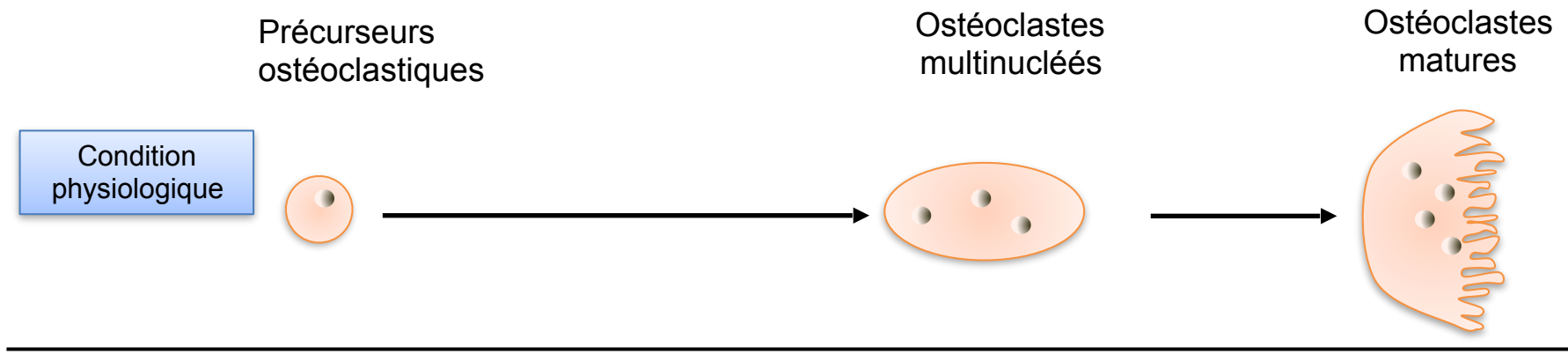
Matrice minérale mimant la matrice osseuse

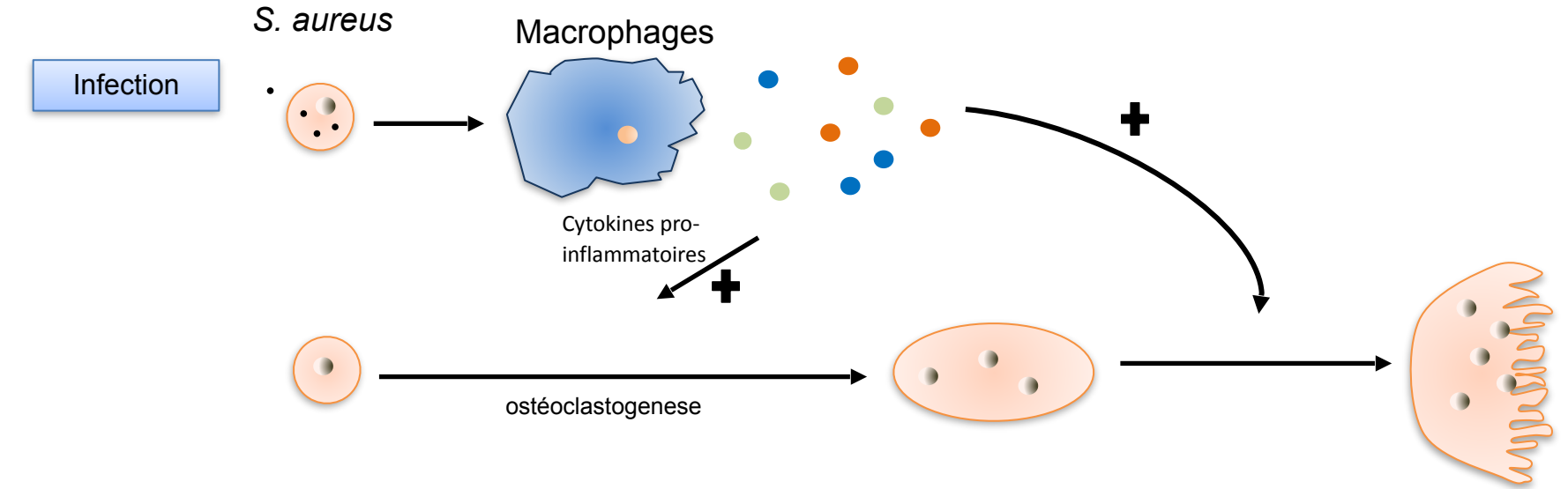




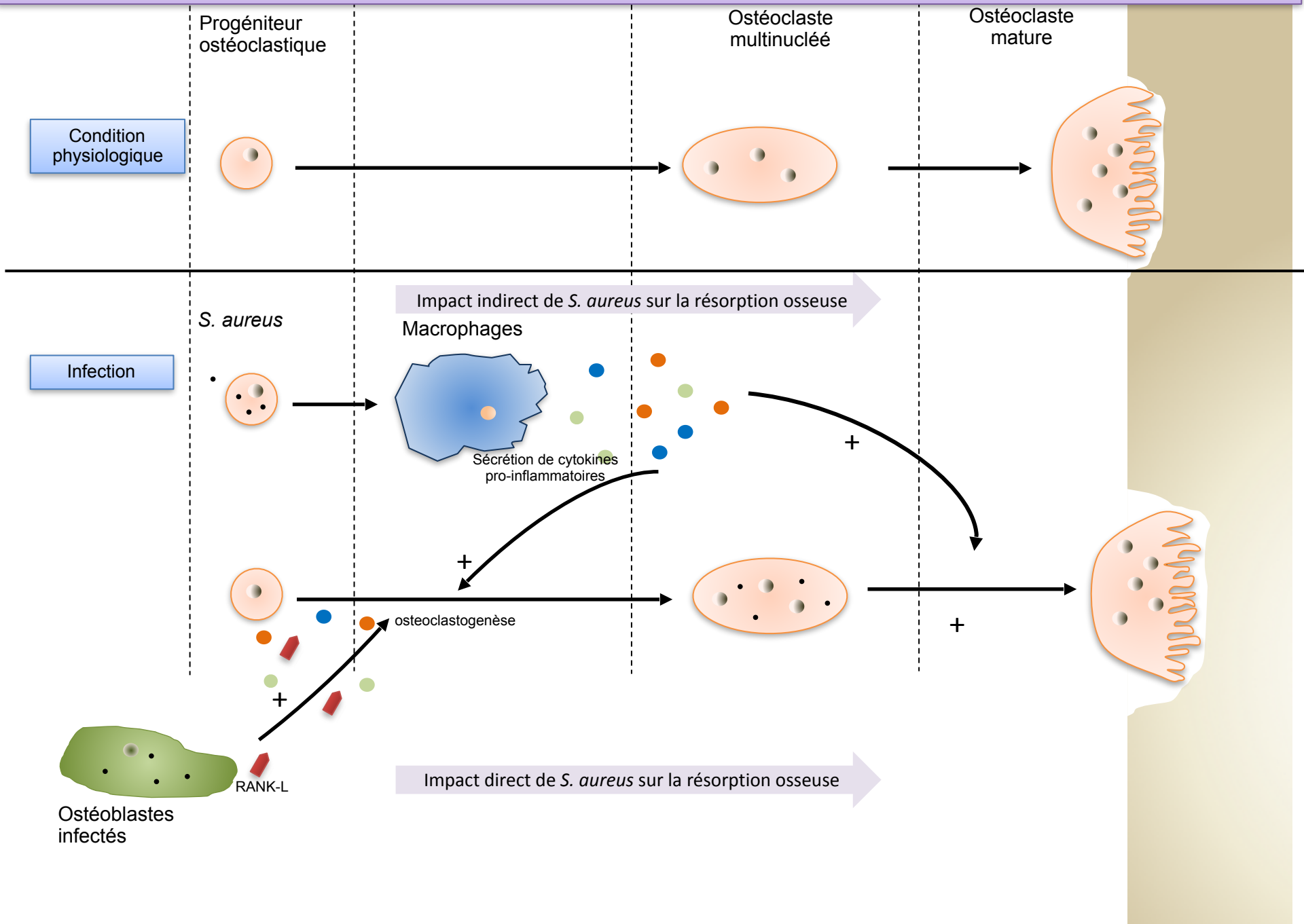
Intracellular S. aureus increases the capacity of resorption of mature osteoclasts

Interaction osteoclasts – *S. aureus*

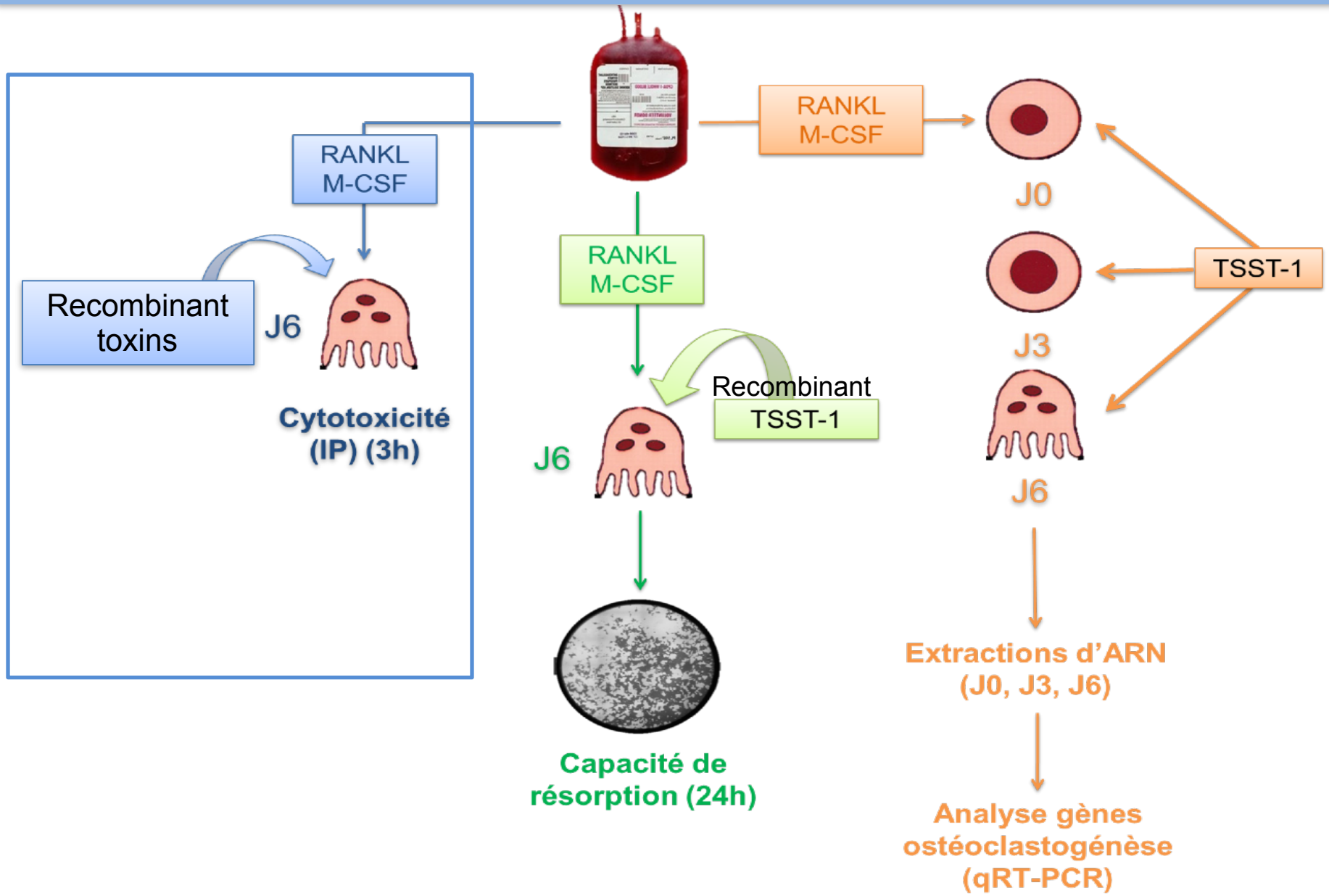




Interaction osteoclasts – *S. aureus*



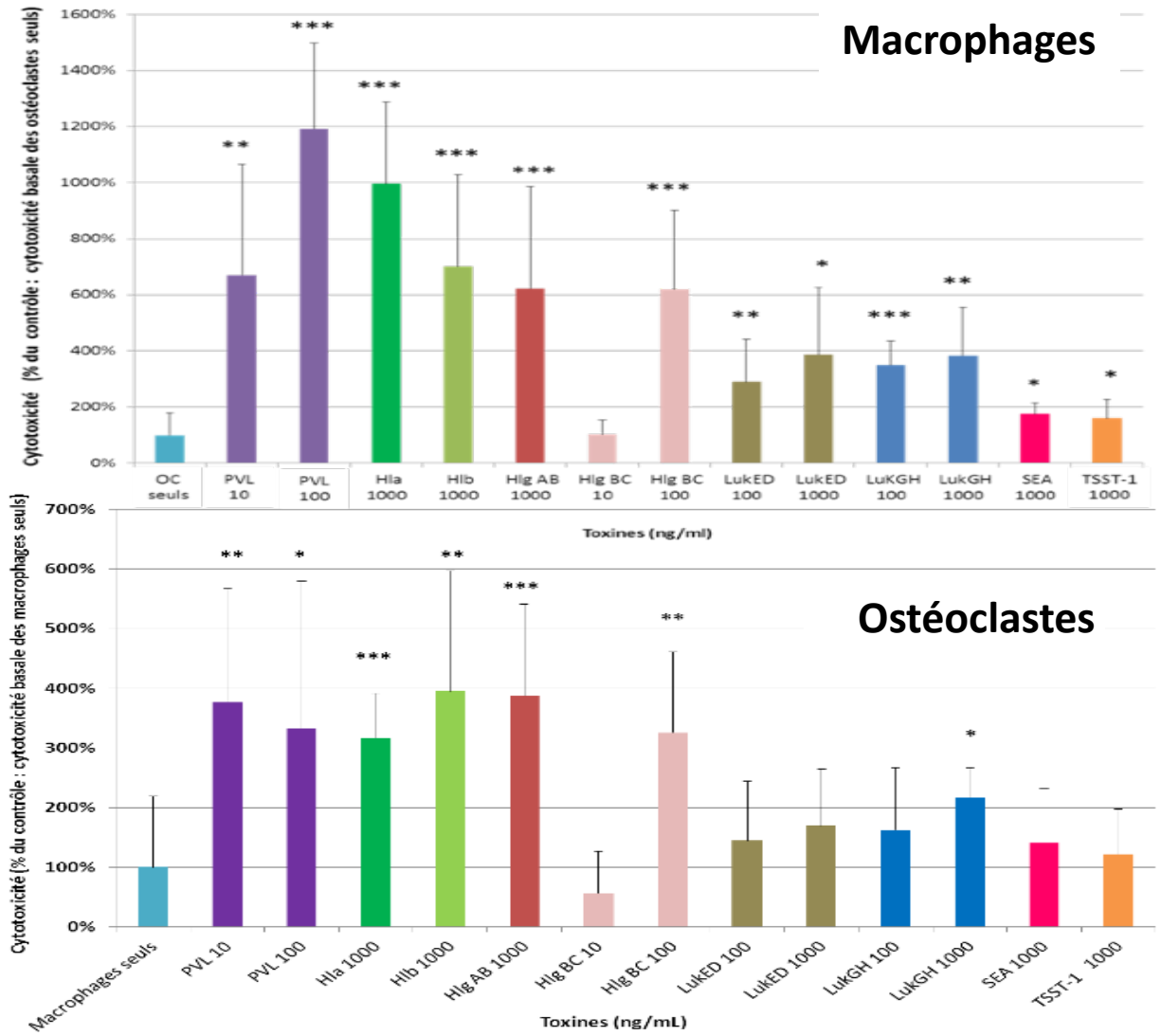
Direct impact of recombinant toxins on osteoclasts



Cytotoxic effect of staphylococcal toxins

"pore forming" toxins

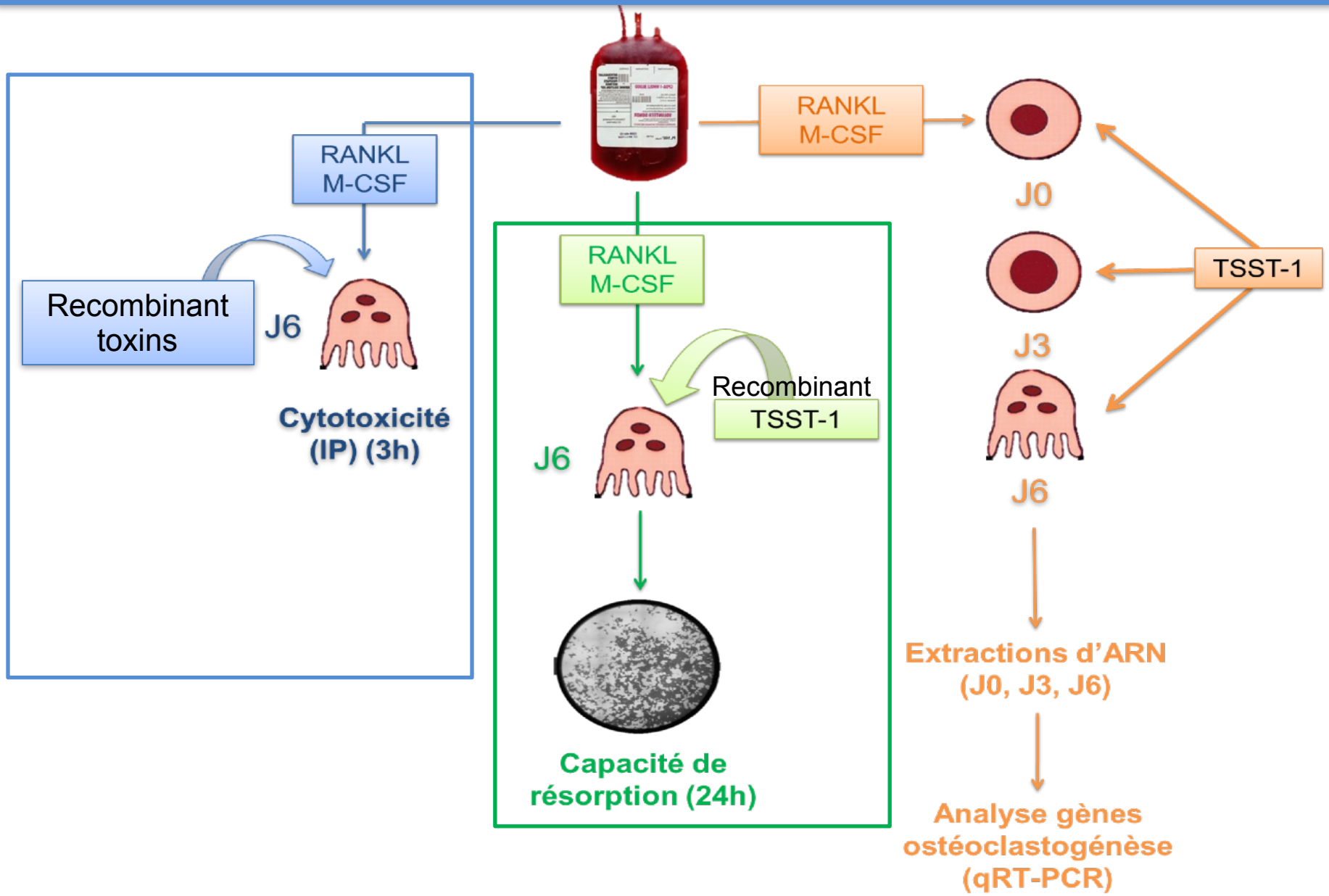
Superantigen toxin



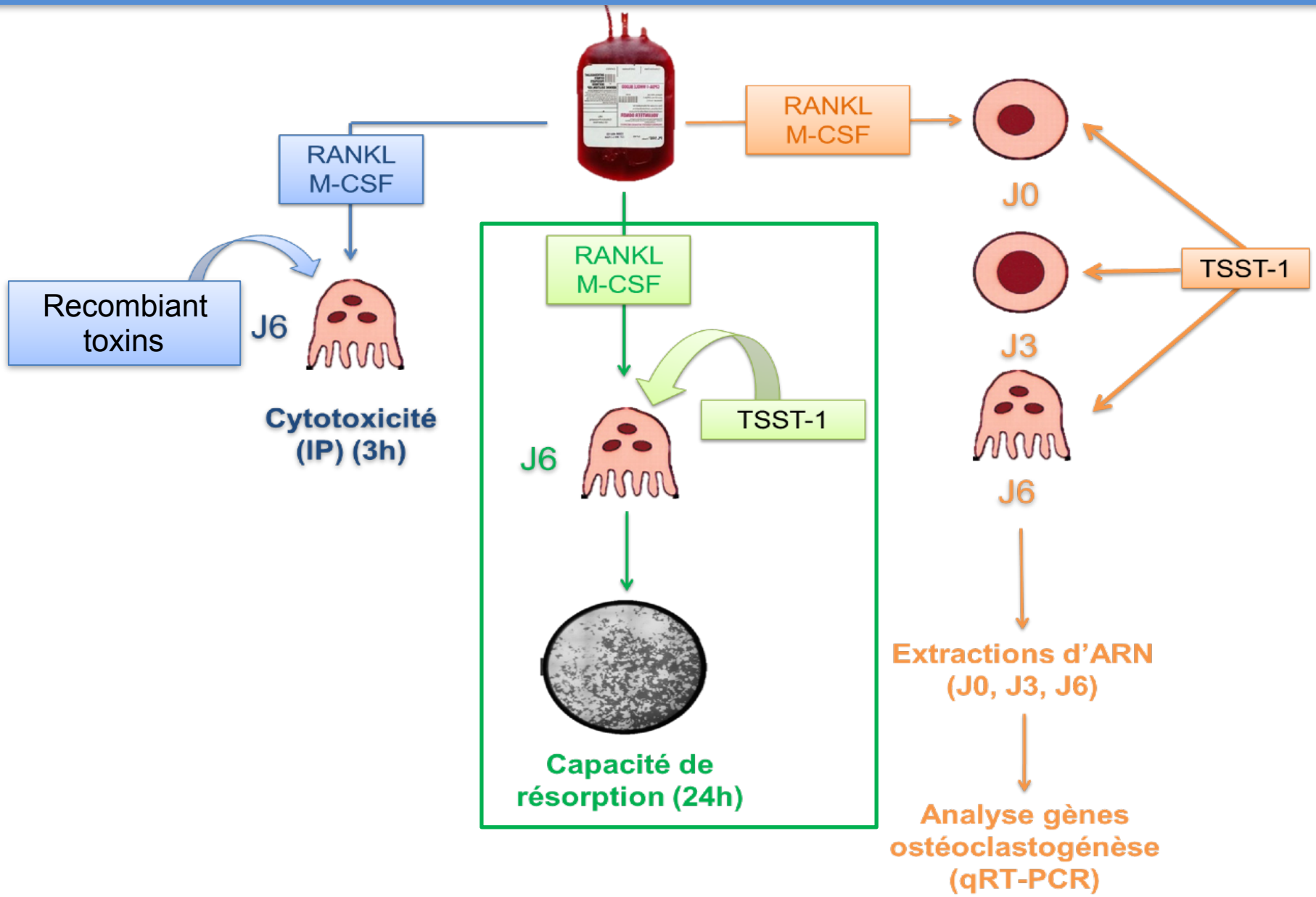
MAC vs OC: same profile of susceptibility

3 replicates in 3 different donors

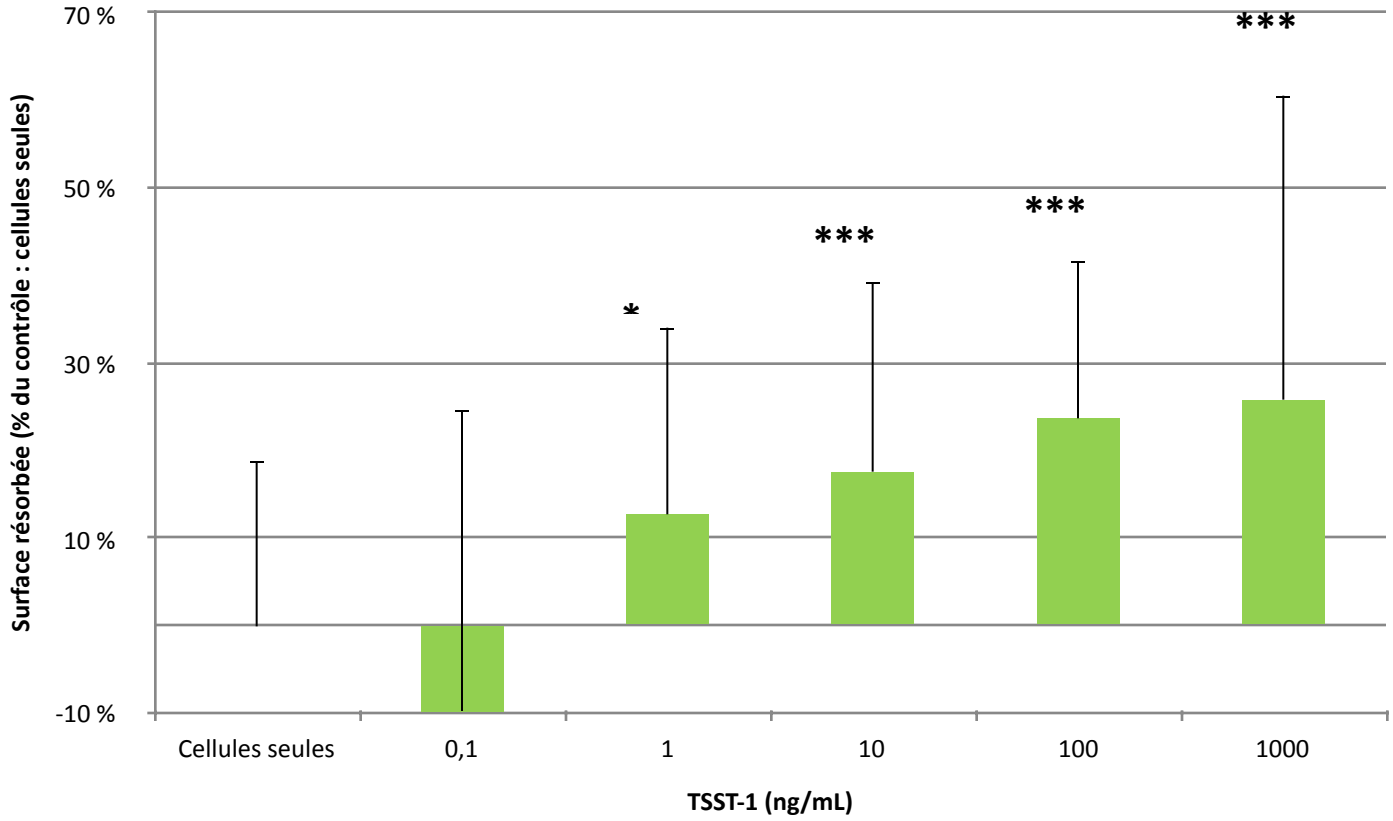
Direct impact of recombinant toxins on osteoclasts



Direct impact of recombinant toxins on osteoclasts



Impact of recombinant toxins on osteoclasts



→ TSST-1 **increase the capacity of resorption of mature osteoclasts**

3 expériences en triplicat sur 3 donneurs

From acute BJI to chonic BJI

Bettina Loeffler showed us in vitro data and data from animal about SCV

To date, **no study** comparing isolates recovered from **the same patient** at time of acute BJI and at time of chronicization have been performed

Aim of the study

To date, **no study** comparing isolates recovered from **the same patient** at time of acute BJI and at time of chronicization have been performed

To determine if bacterial mechanisms involved in persisting BJIs are present at acute infection or are the consequence of ***in vivo*** adaptation.

S.aureus strains

3 couples of MSSA strains isolated from patients suffering from recurrent or persisting prosthetic joint infection (PJI) at ***initial diagnosis*** of PJI and at the ***time of relapse.***

Clinical data

Patient no	Sexe, age (year)	Site of infection	Duration of symptoms (days)	Surgical treatment	Duration of antibiotherapy (days)	Time to failure or relapse (days)
1	H,26	Tibia osteosynthesis material	12	Material Removed	82	82
2	H,80	Total knee arthroplasty	3	Irrigation and debridment	191	201
3	F,82	Total hip arthroplasty	3	Irrigation and debridment	98	134

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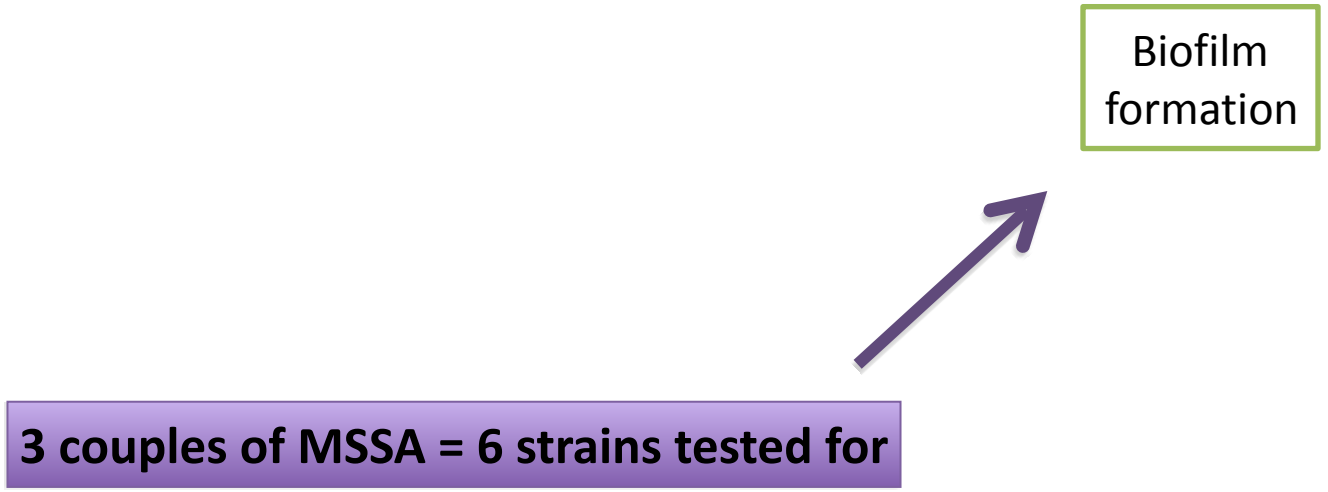
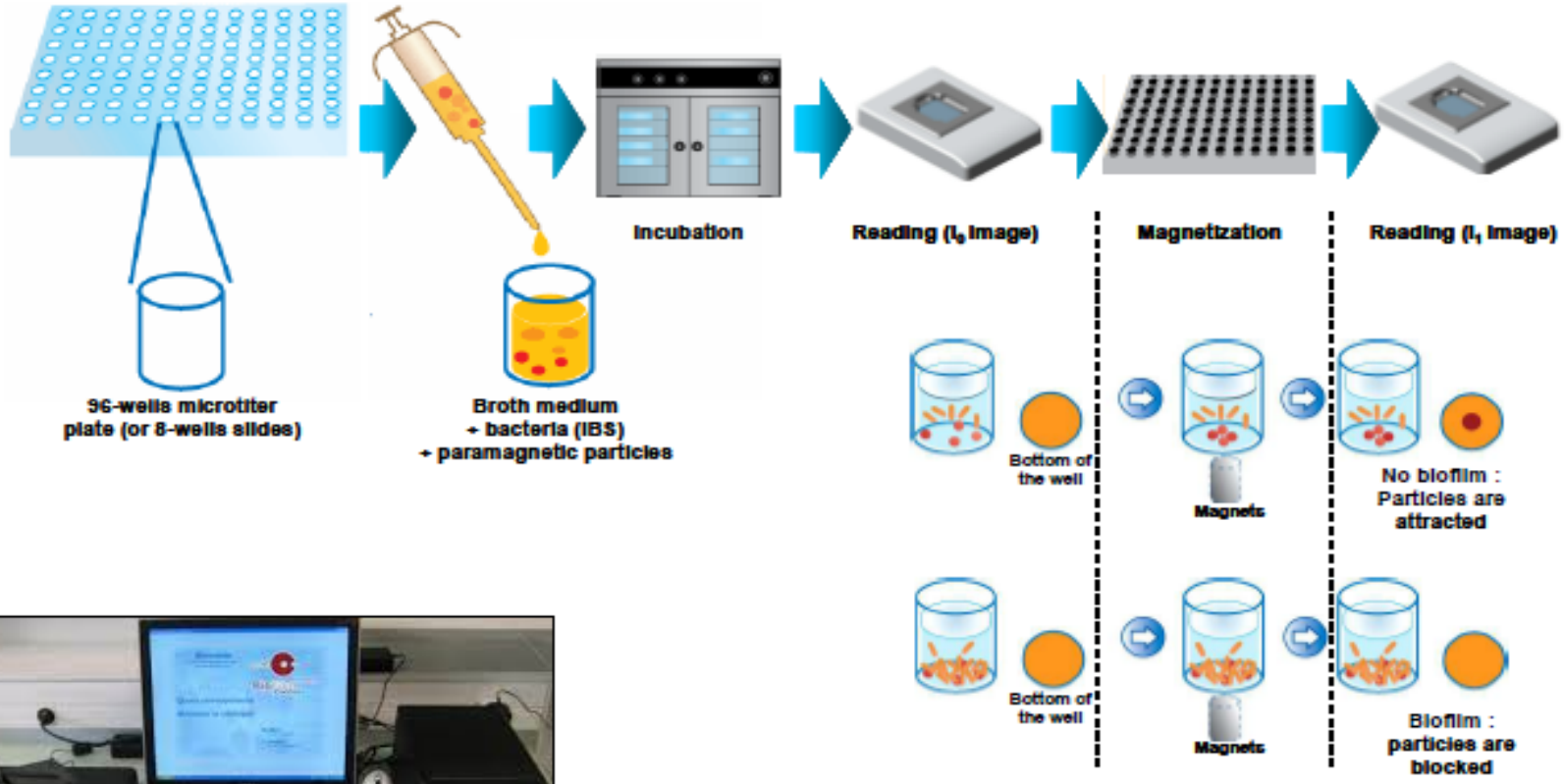
Same antibiotic susceptibility profile

Material and methods



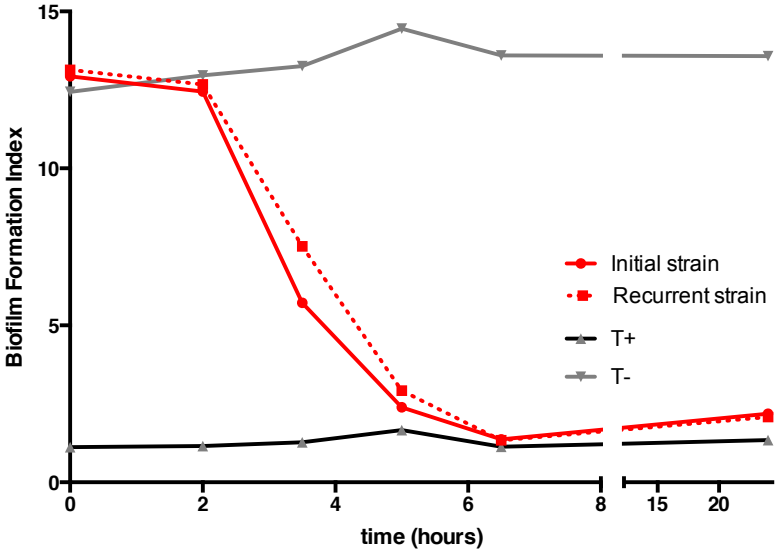
Biofilm formation

Biofilm ringtest® method: Evaluation of ***early*** biofilm formation



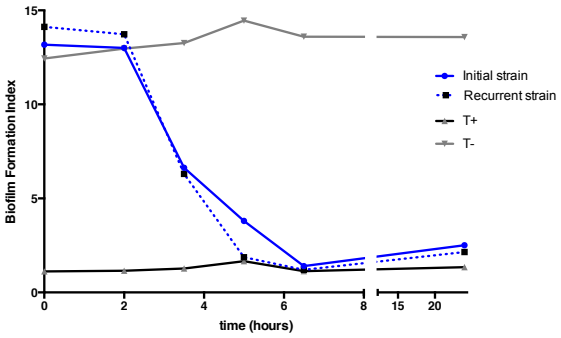
Biofilm formation

All patients

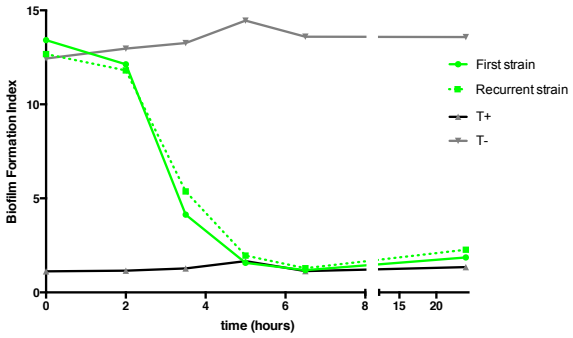


2 experiments in duplicate

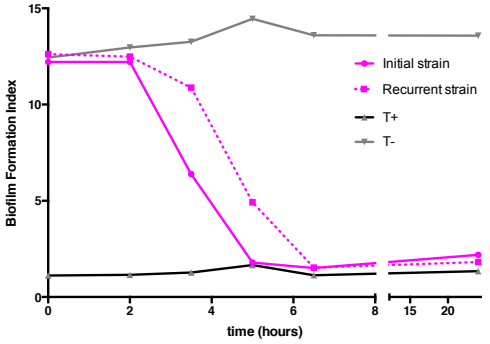
Patient 1



Patient 2

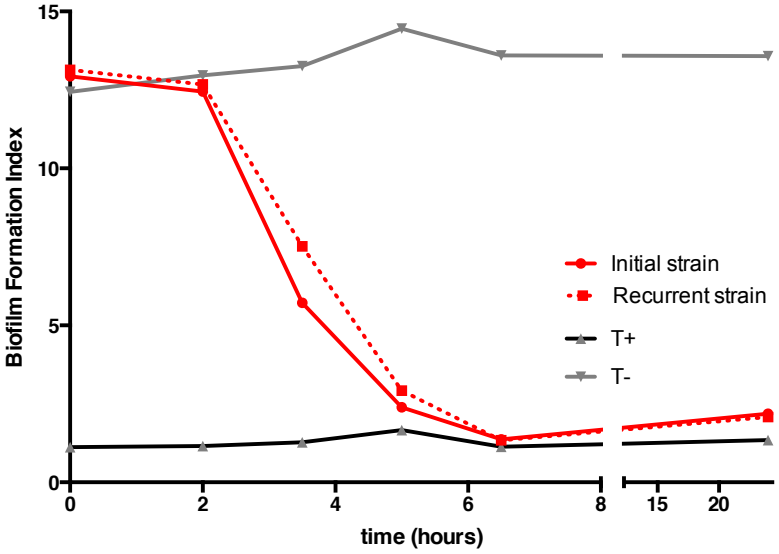


Patient 3



Biofilm formation

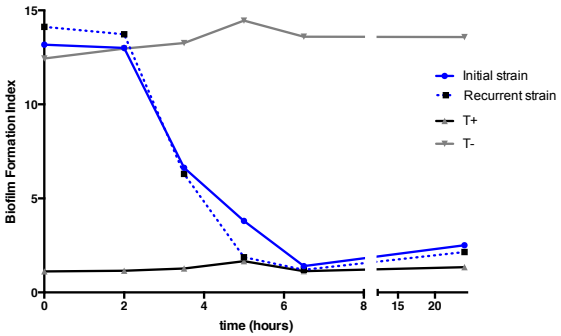
All patients



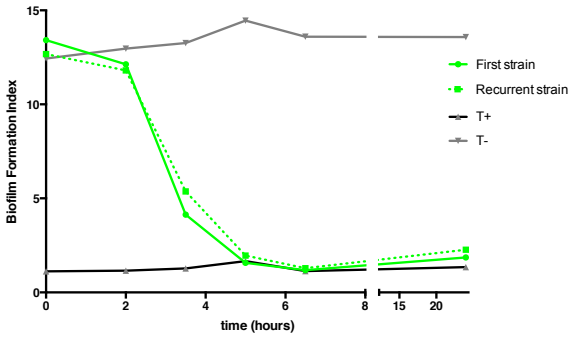
Same capacity of formation of precoce biofilm

2 experiments in duplicate

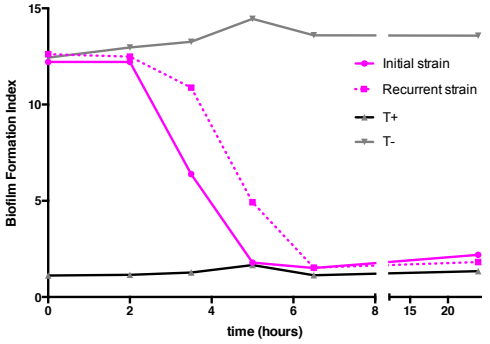
Patient 1



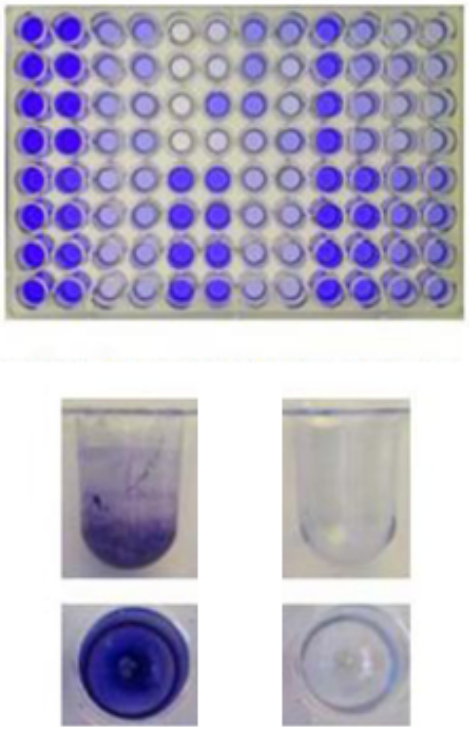
Patient 2

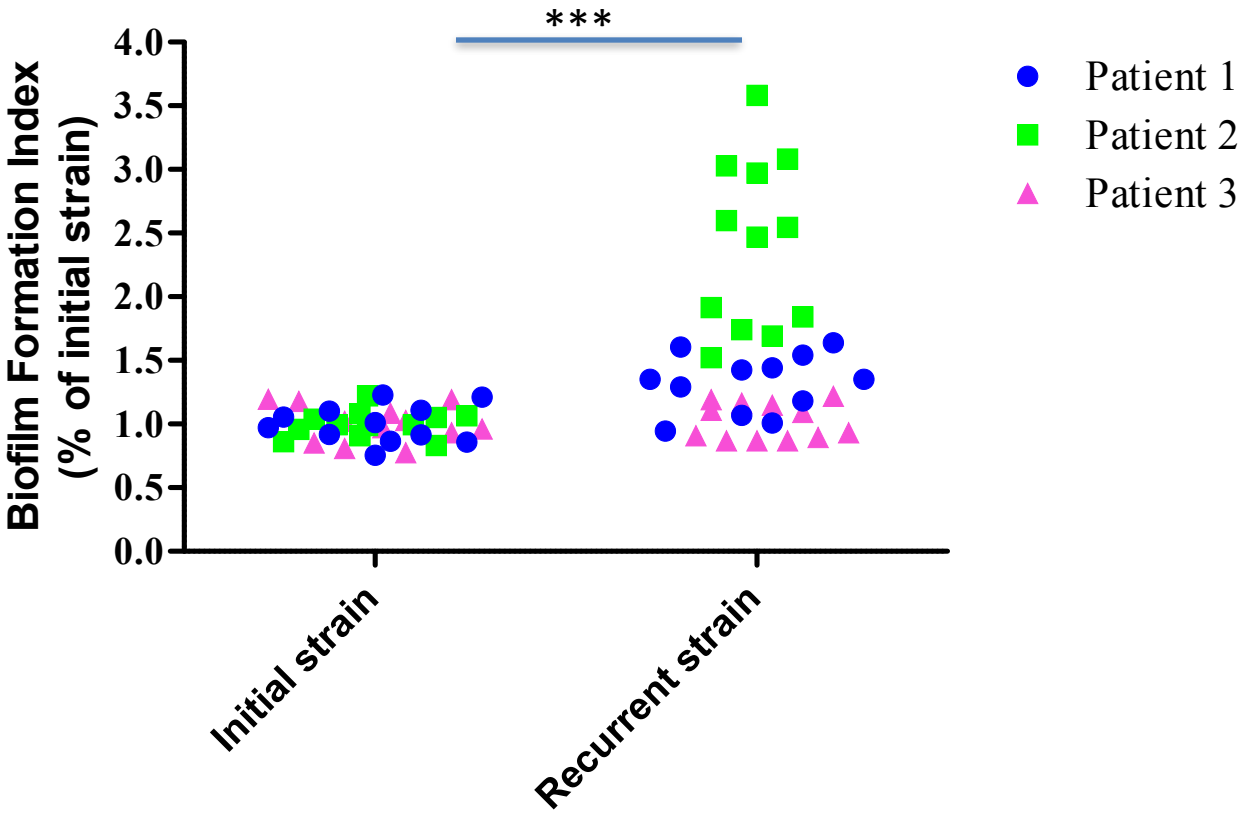


Patient 3



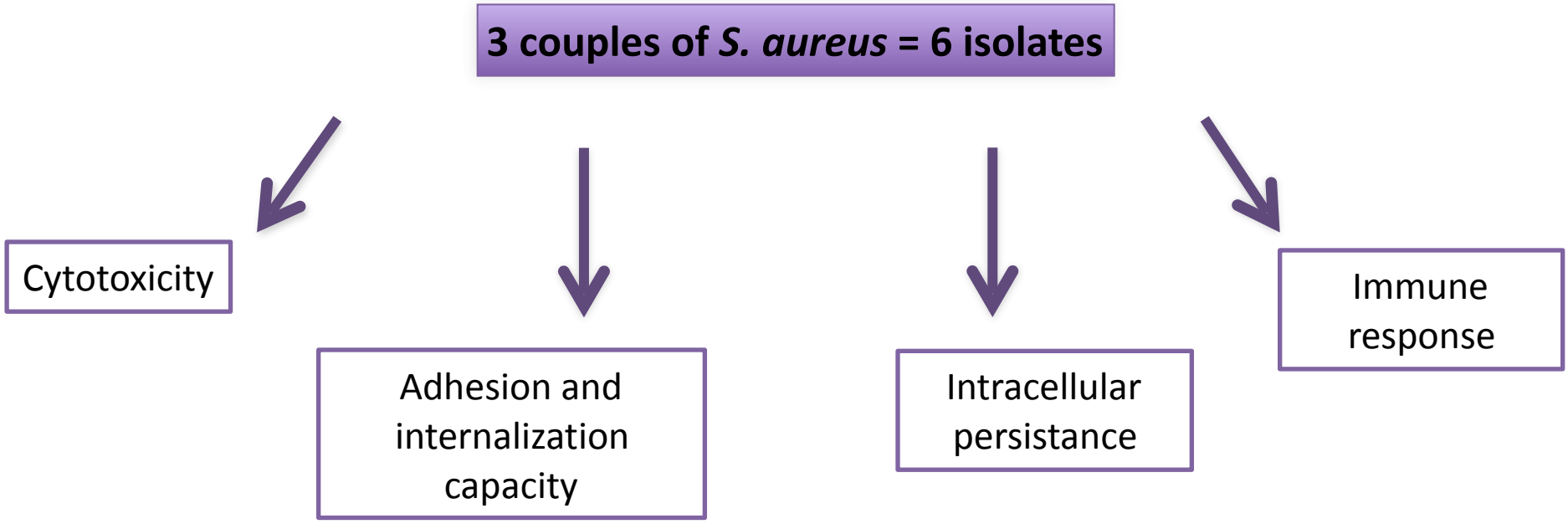
Biofilm formation





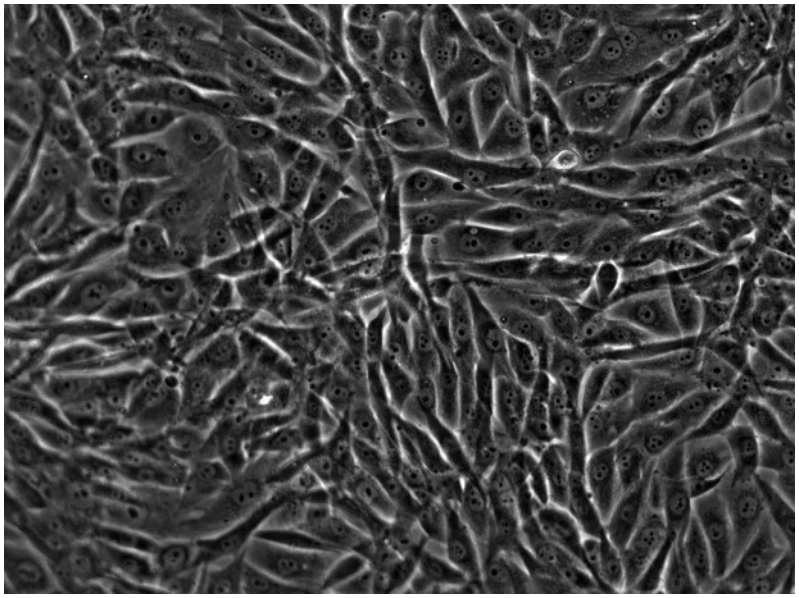
Persisting strains form more mature biofilm than initial ones.

Materials et methods

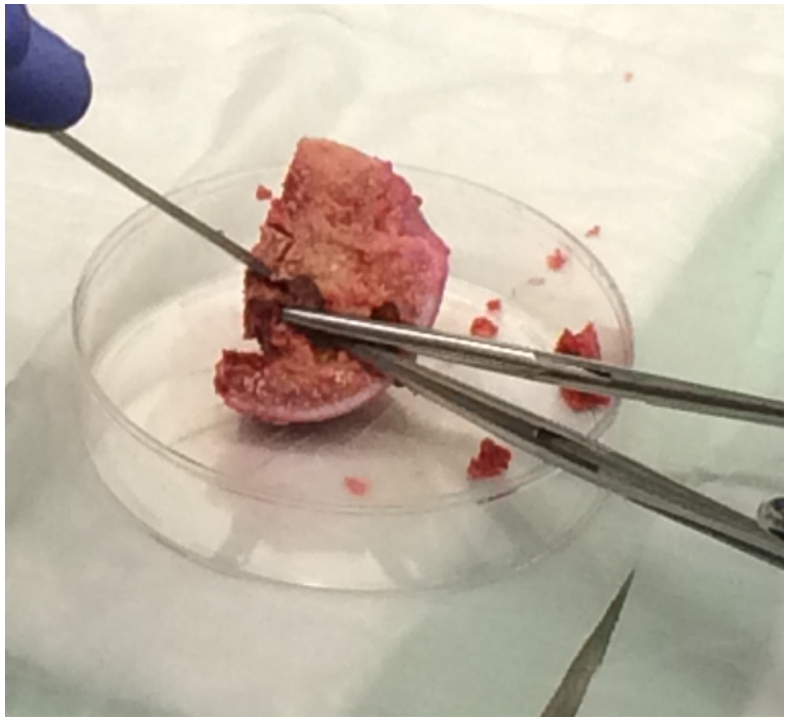


***in vitro* infection model**

Cell culture

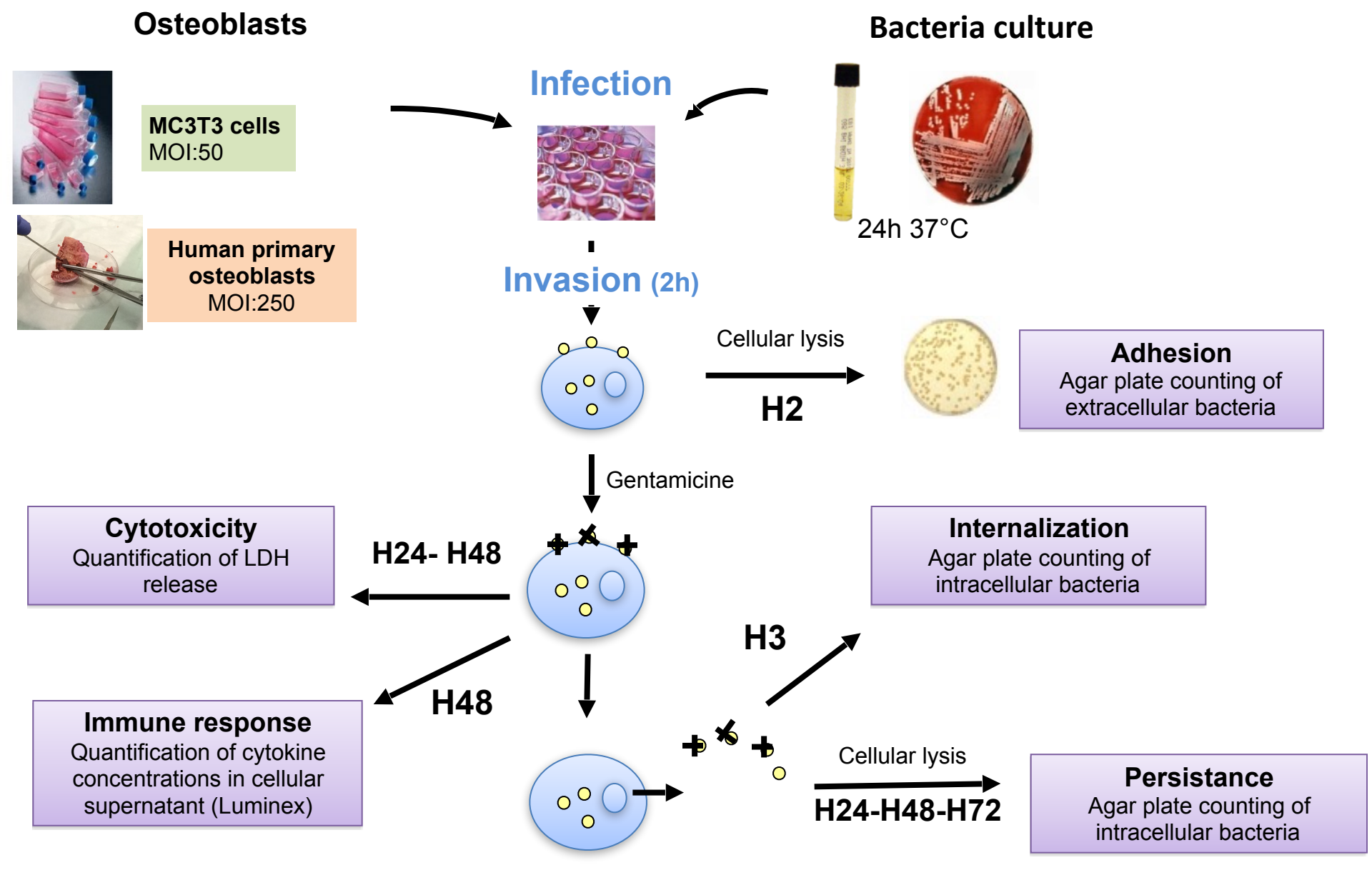


MC-3T3

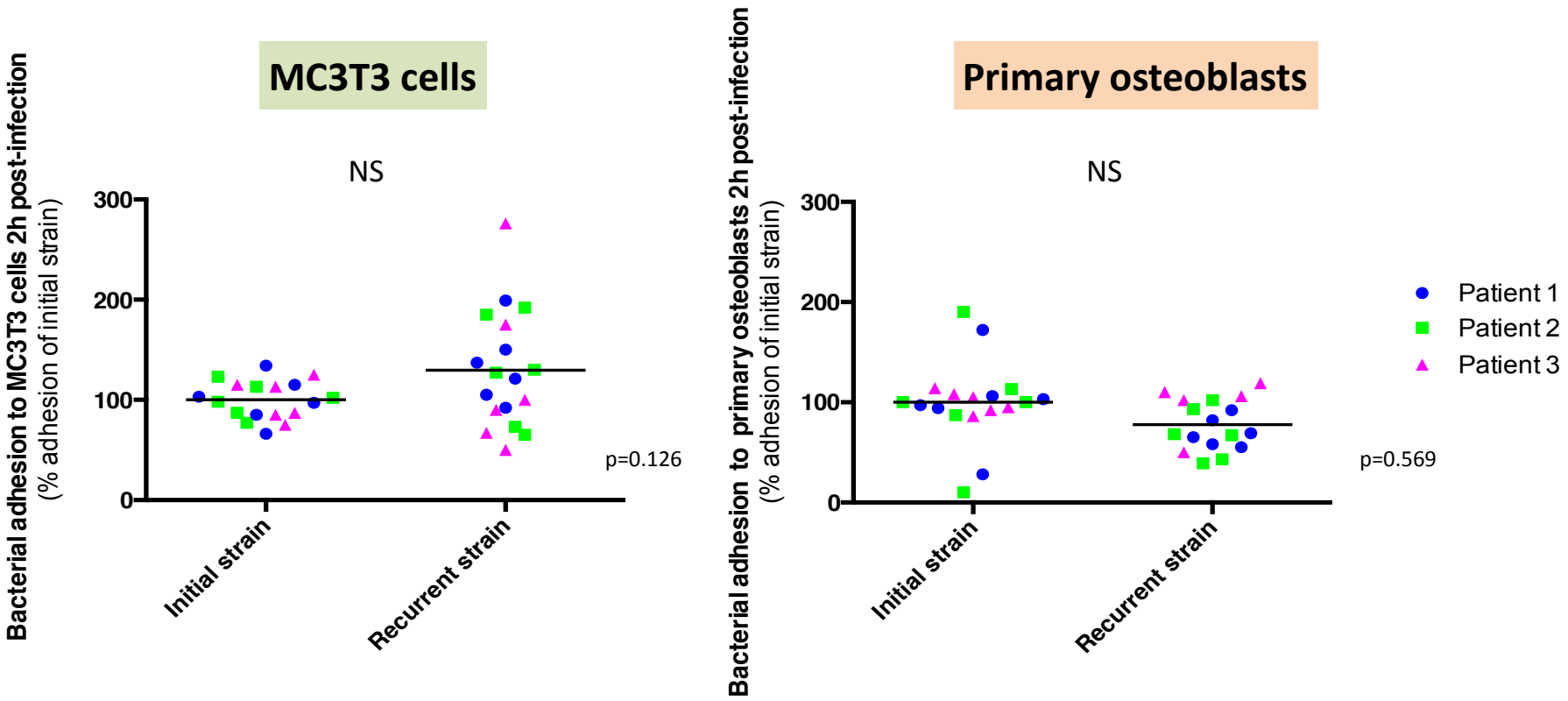


Human primary osteoblasts isolated from non-infected patients and sampled during routine hip surgery

In vitro: infection model of osteoblasts



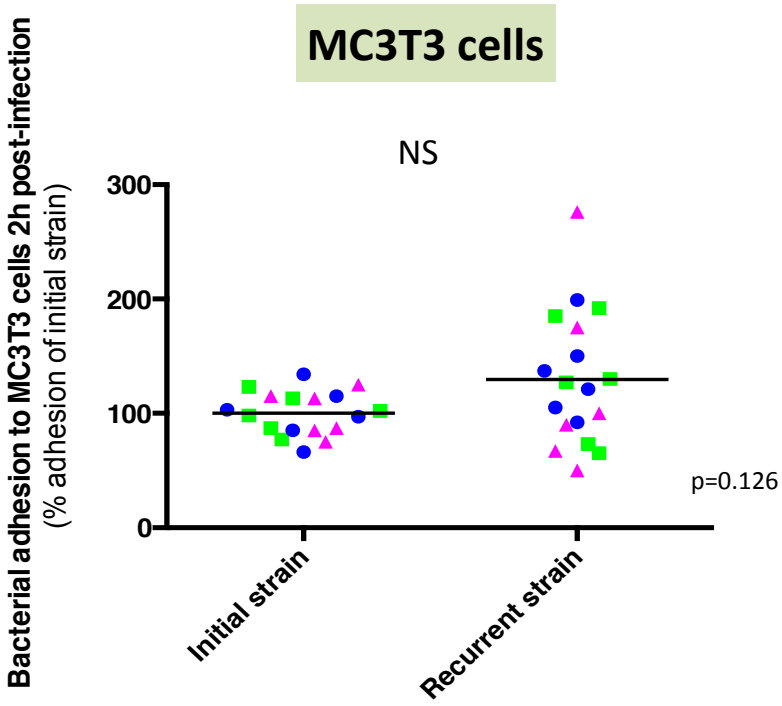
Adhesion to osteoblasts

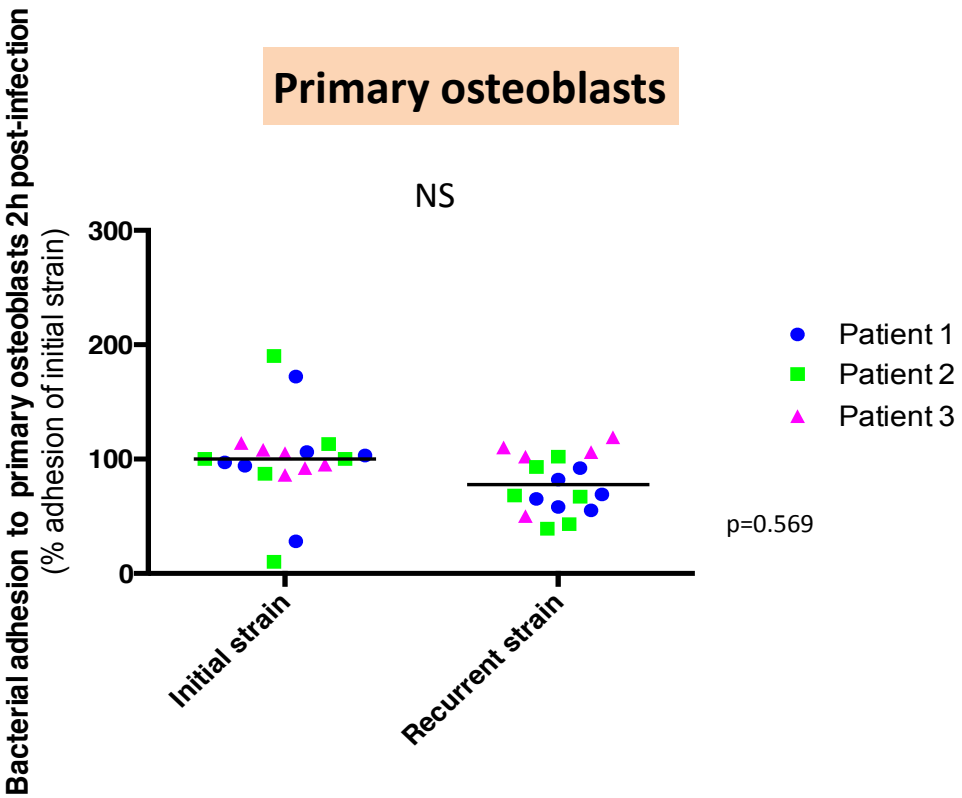


	Adhesion to osteoblasts, mean (%) +/- SD		
	Initial strain	**Recurrent strain**	p
MC3T3 cells	100 +/- 19	115 +/- 45	0.126
Primary cells	100 +/- 40	78 +/- 25	0.569

3 experiments in duplicate. Statistical analysis: Mann- Whitney test (*) confirmed by multivariate analysis controling with patients (Ŧ)

Adhesion to osteoblasts

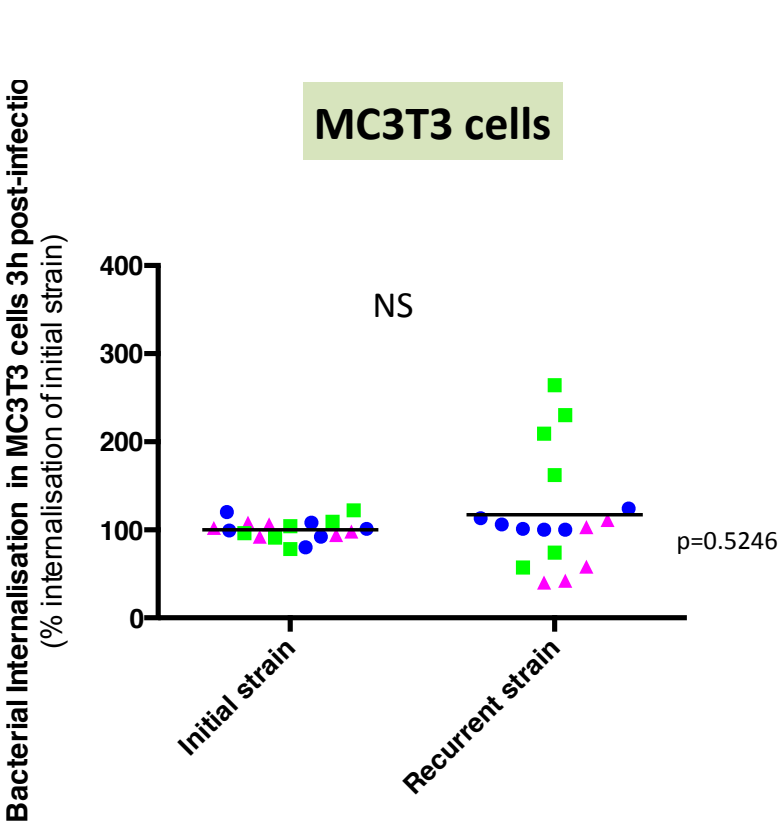


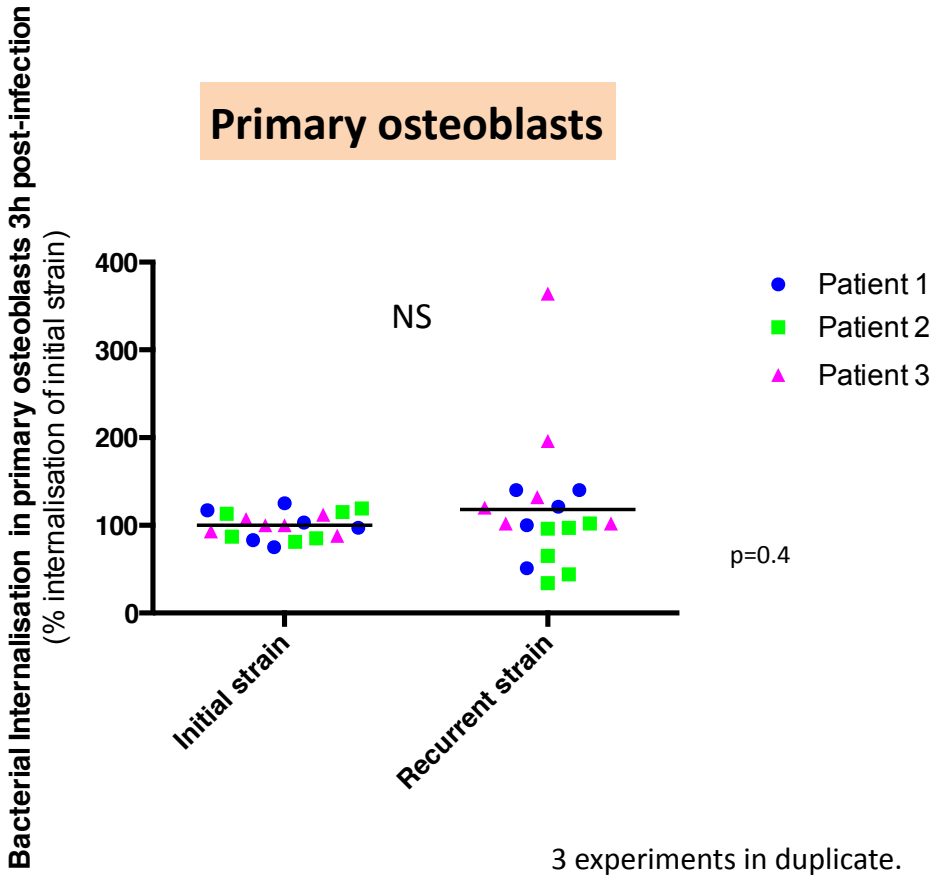


Same capacity of adhesion to osteoblasts for initial and recurrent strains

3 experiments in duplicate. Statistical analysis: Mann- Whitney test (*) confirmed by multivariate analysis controling with patients (Ŧ)

Capacity of internalization in osteoblasts



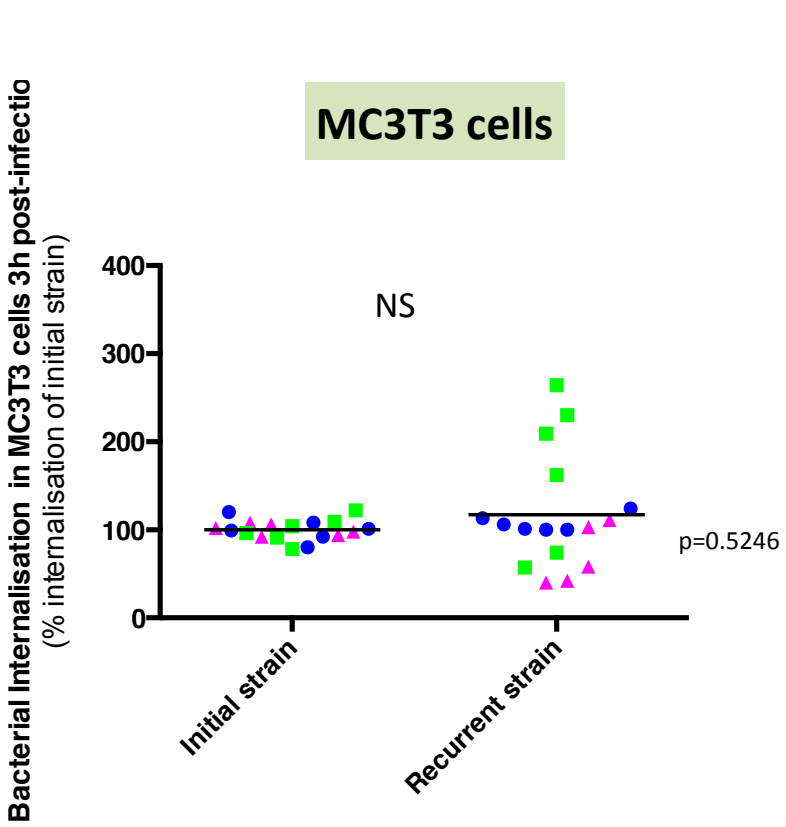


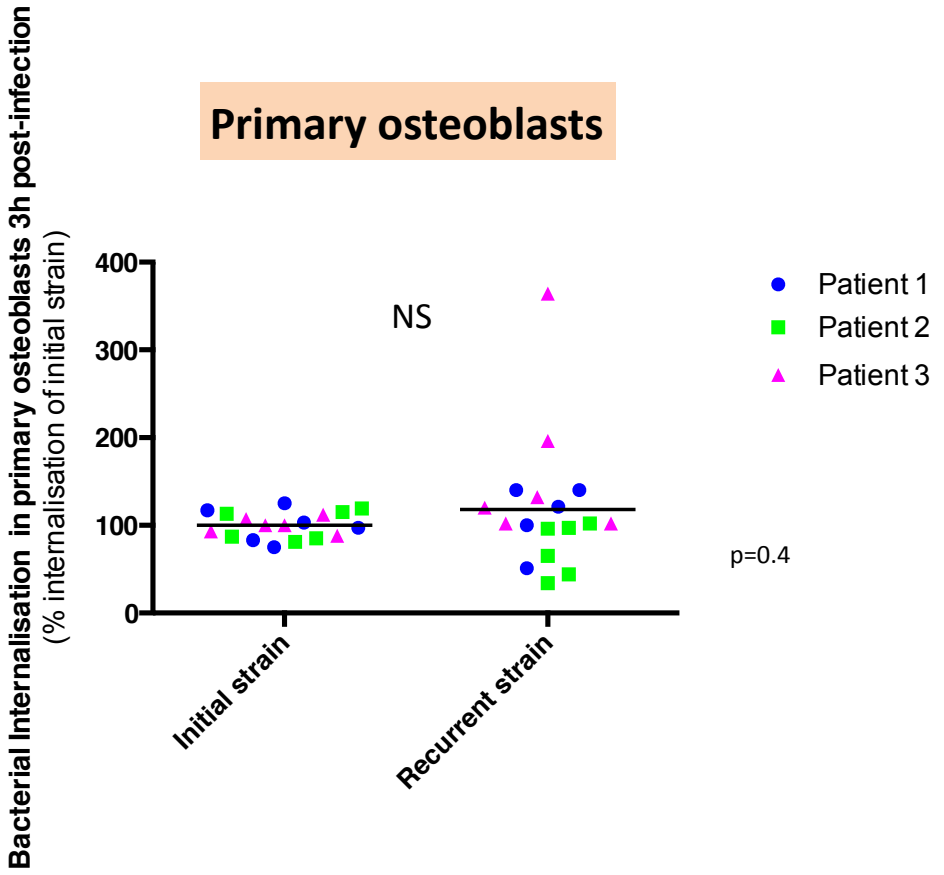
3 experiments in duplicate.

	Internalisation in osteoblasts, mean (%) +/- SD		
	Initial strain	**Recurrent strain**	p
MC3T3 cells	100 +/- 12	117 +/- 64	0.525
Primary cells	100 +/- 15	118 +/- 75	0.4

Statistical analysis: Mann- Whitney test

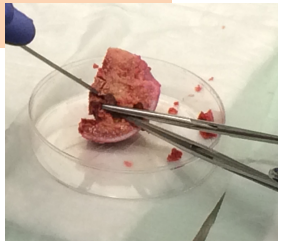
Capacity of internalization in osteoblasts

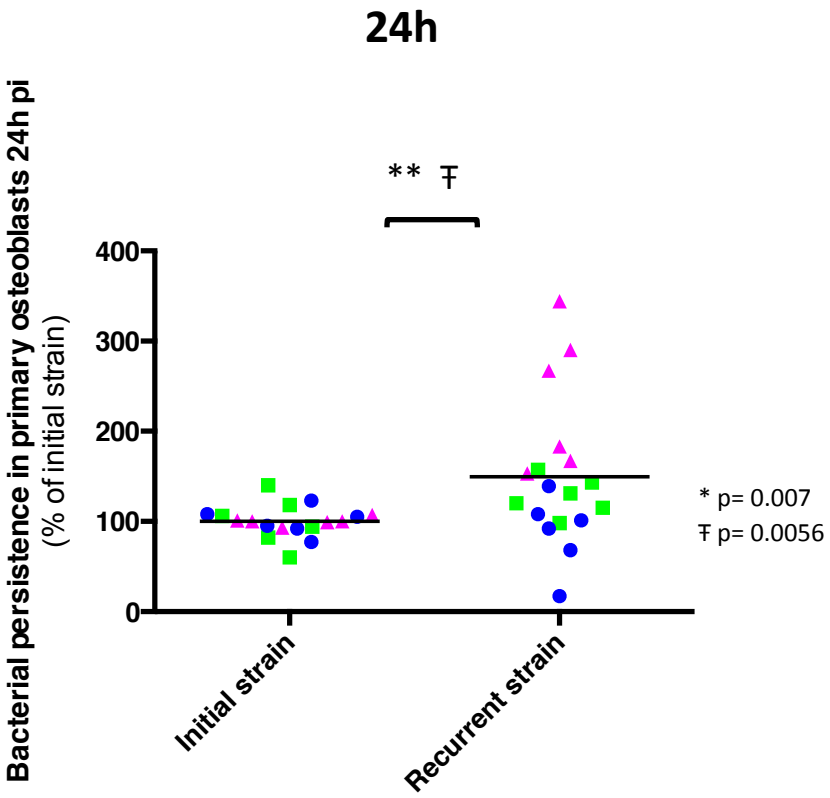


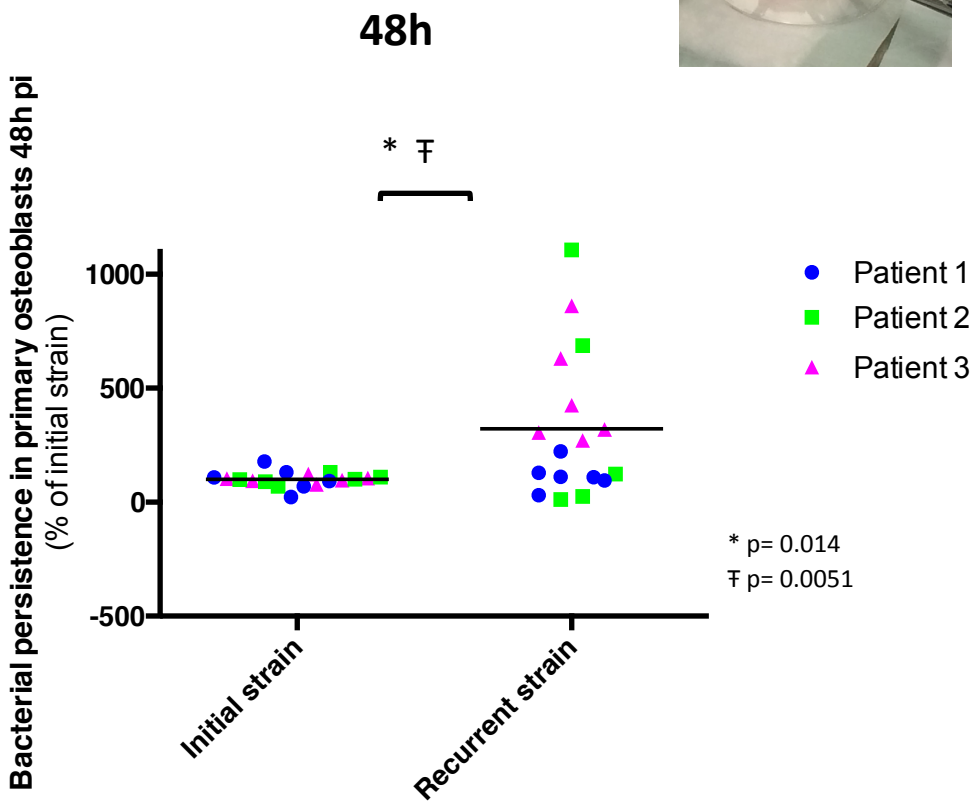


Same capacity of internalization in osteoblasts for initial and recurrent strains

Persistence in primary osteoblasts







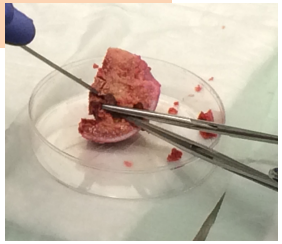
Bacterial persistence in primary osteoblasts, mean (%) +/- SD

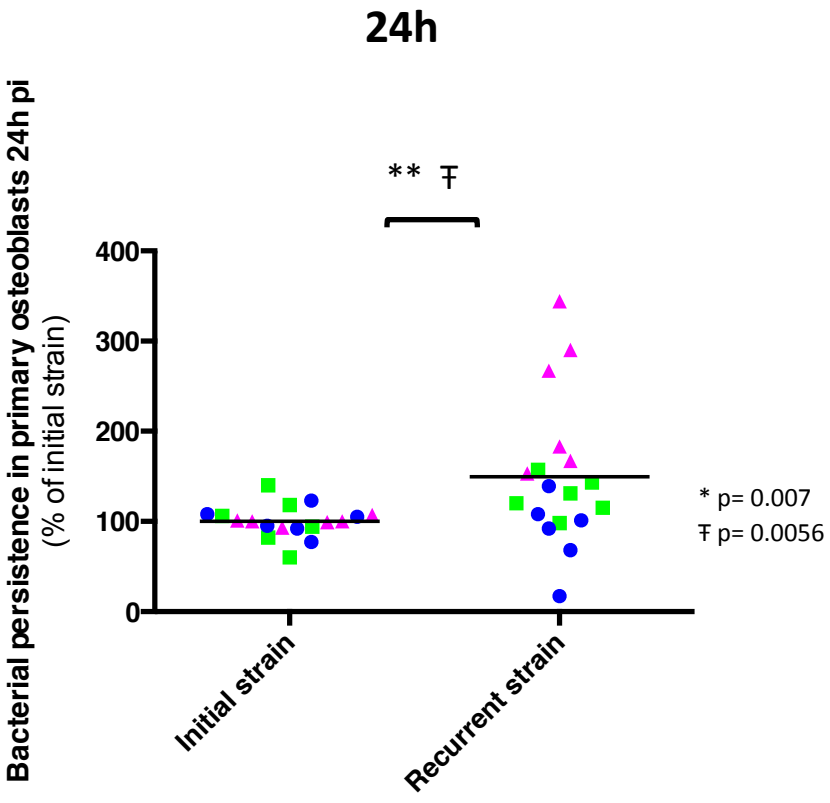
	Initial strain	Recurrent strain	p
24h pi	100 +/- 17	125 +/- 80	0.007
48h pi	100 +/- 32	322 +/- 320	**0.014**

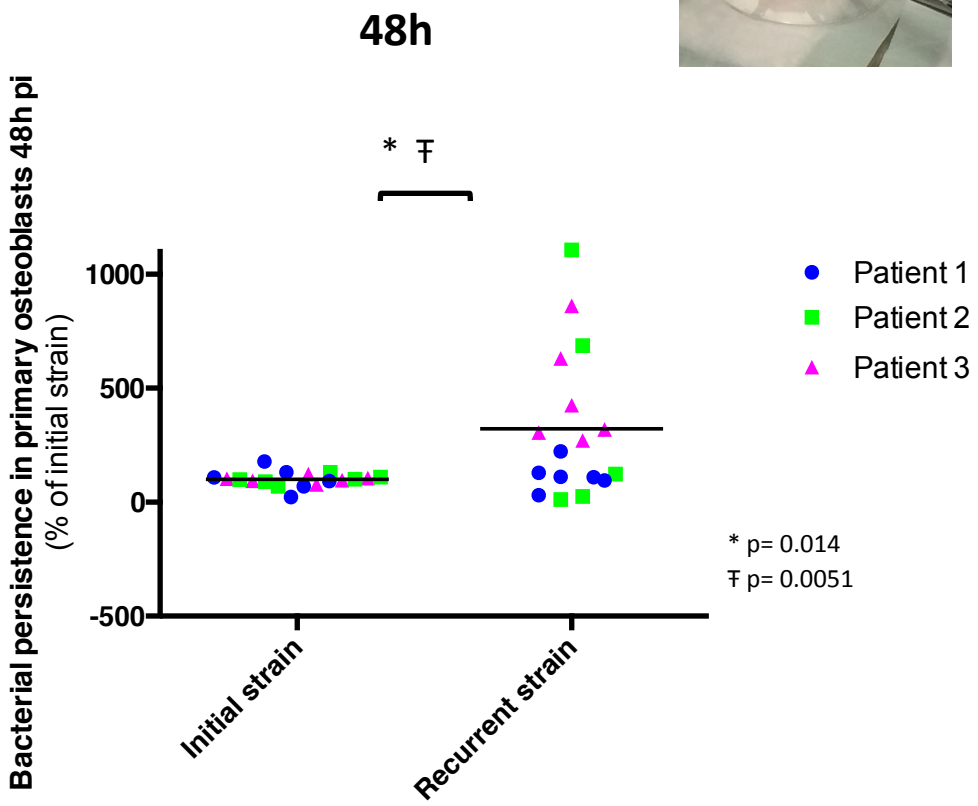
3 experiments in duplicate.

Statistical analysis: Mann- Whitney test (*) confirmed by multivariate analysis controling with patients (∓)

Persistence in primary osteoblasts

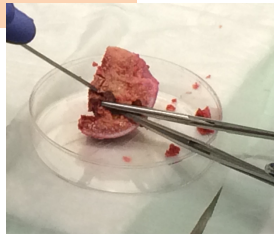


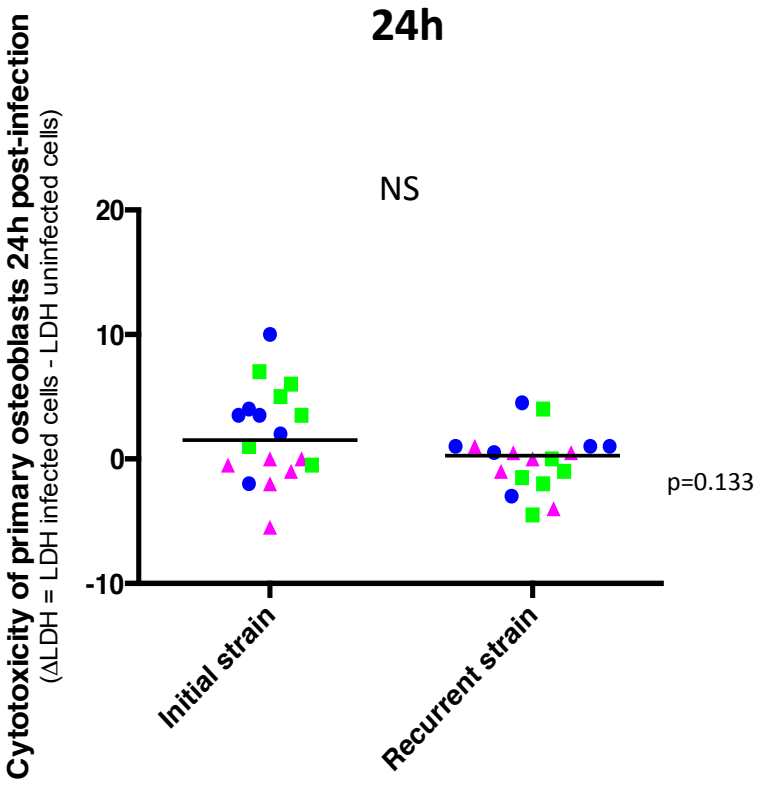


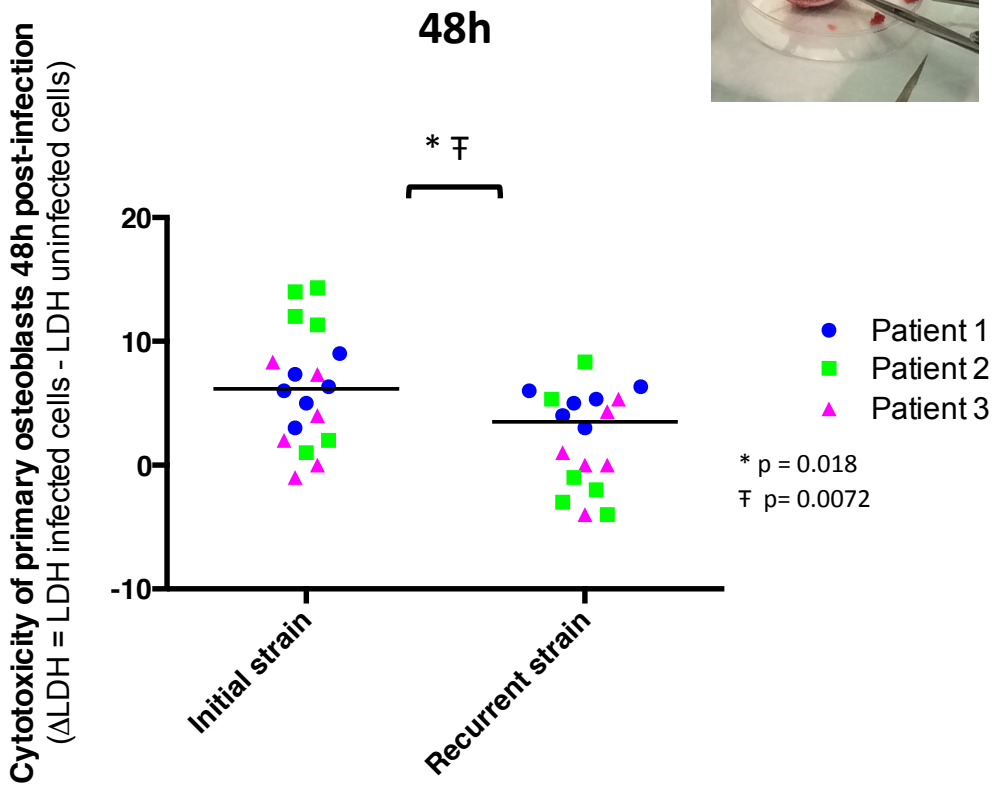


Recurrent strains have a higher capacity of persistence in primary osteoblasts than initial strains at 24h and 48h

Cytotoxicity: primary human osteoblasts





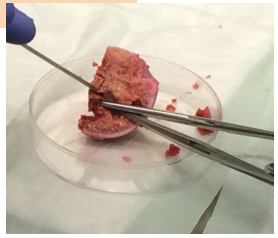


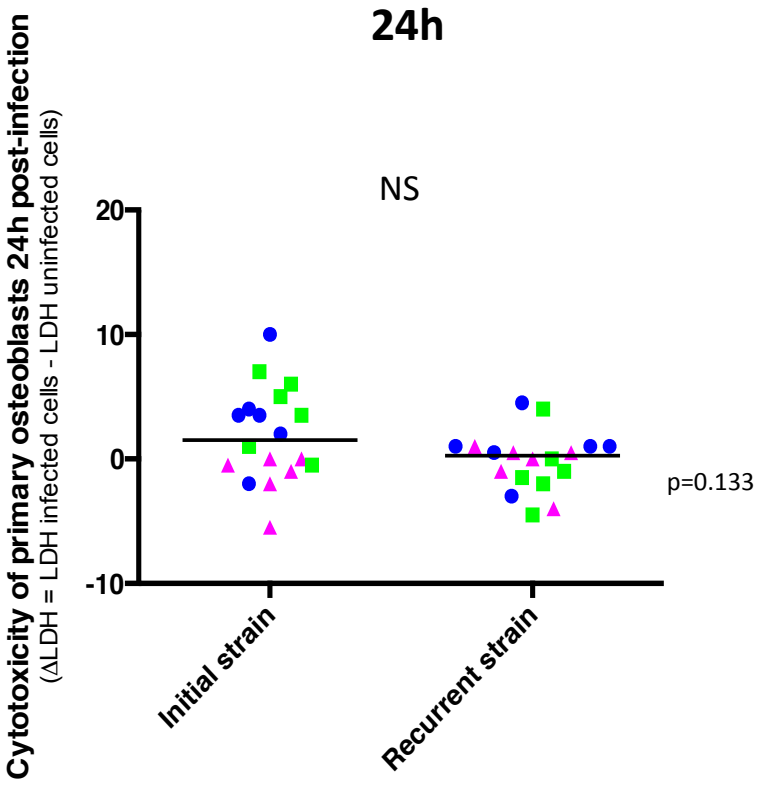
Cytotoxicity of MC3T3 cells (ΔLDH), mean (%) +/- SD			
	Initial strain	**Recurrent strain**	p
24h pi	1.89 +/- 3.78	- 0.17 +/- 2.35	0.133
48h pi	8 +/- 4.02	3.17 +/- 3.47	0.018

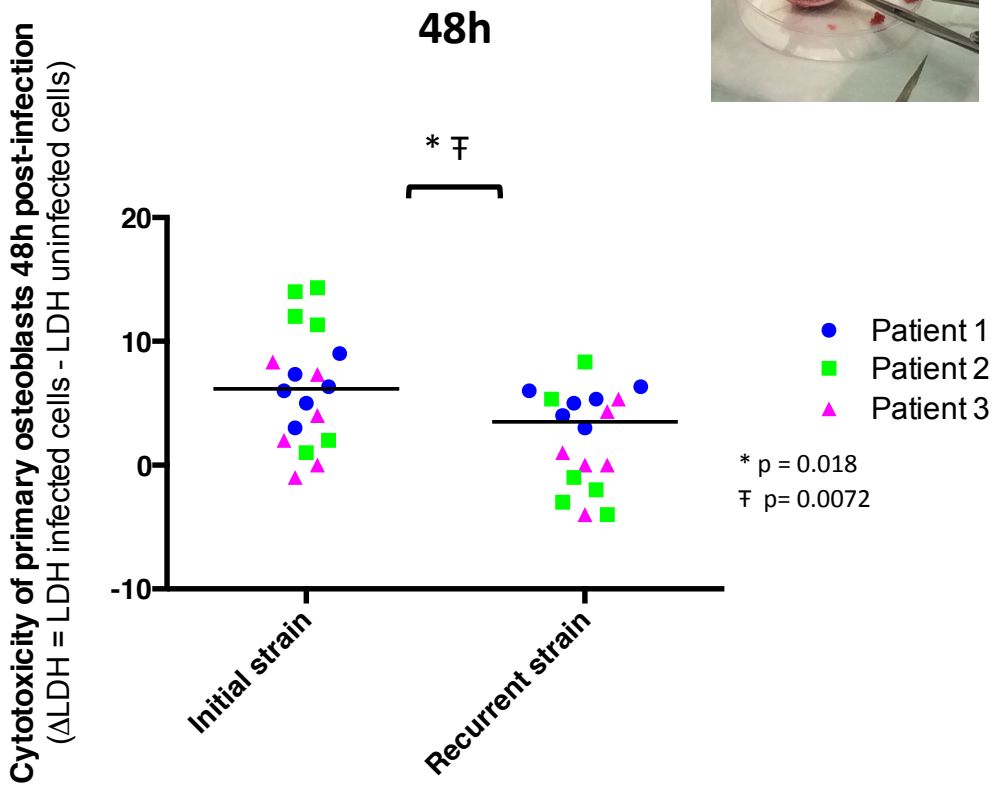
3 experiments in duplicate.

Statistical analysis: Mann- Whitney test (*) confirmed by multivariate analysis controling with patients (Ŧ)

Cytotoxicity: primary human osteoblasts



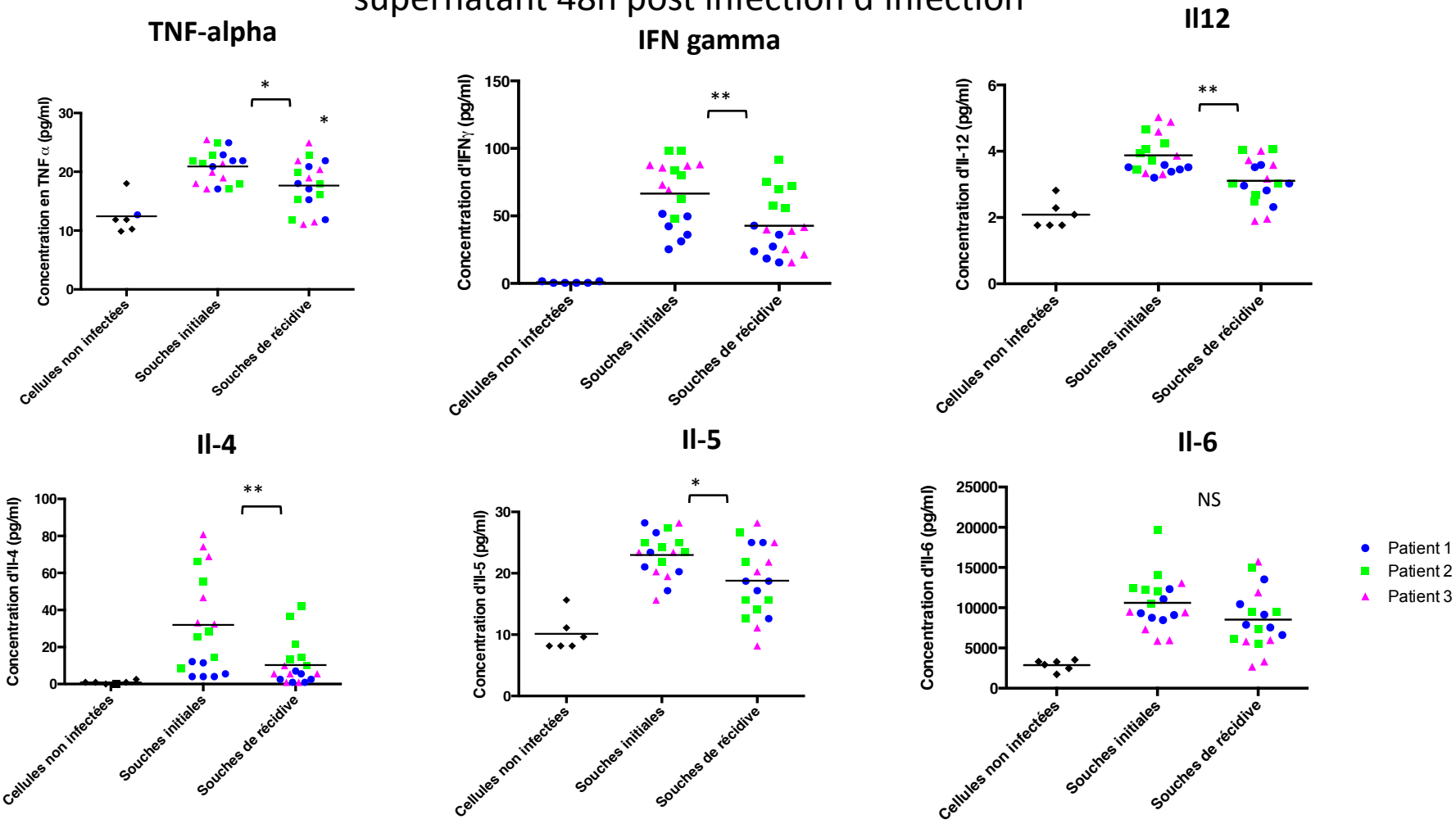




Recurrent strains are less cytotoxic than initial strains in primary osteoblasts

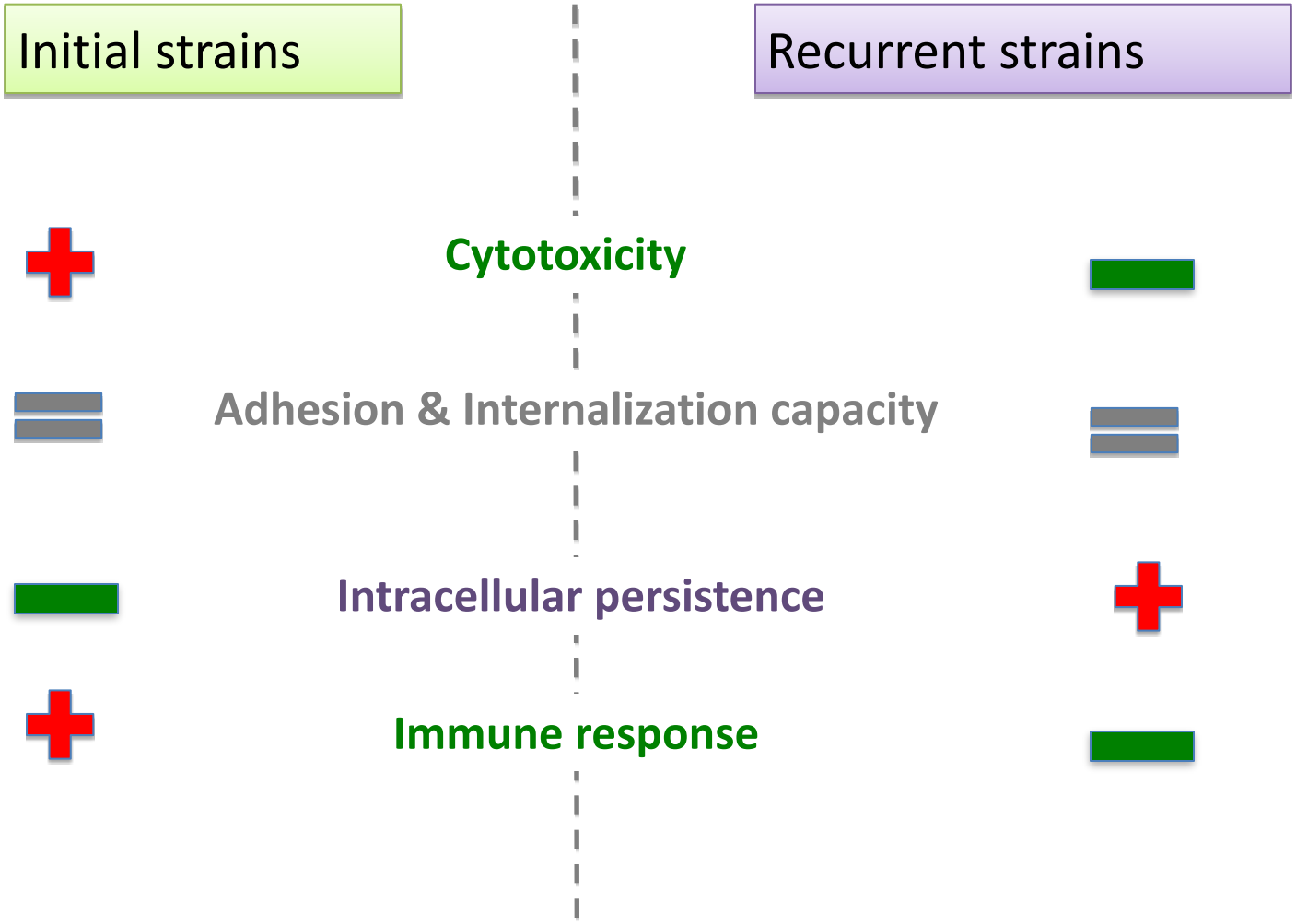
Immune response

Quantification of cytokines concentrations in cell culture supernatant of human primary supernatant 48h post infection d'infection

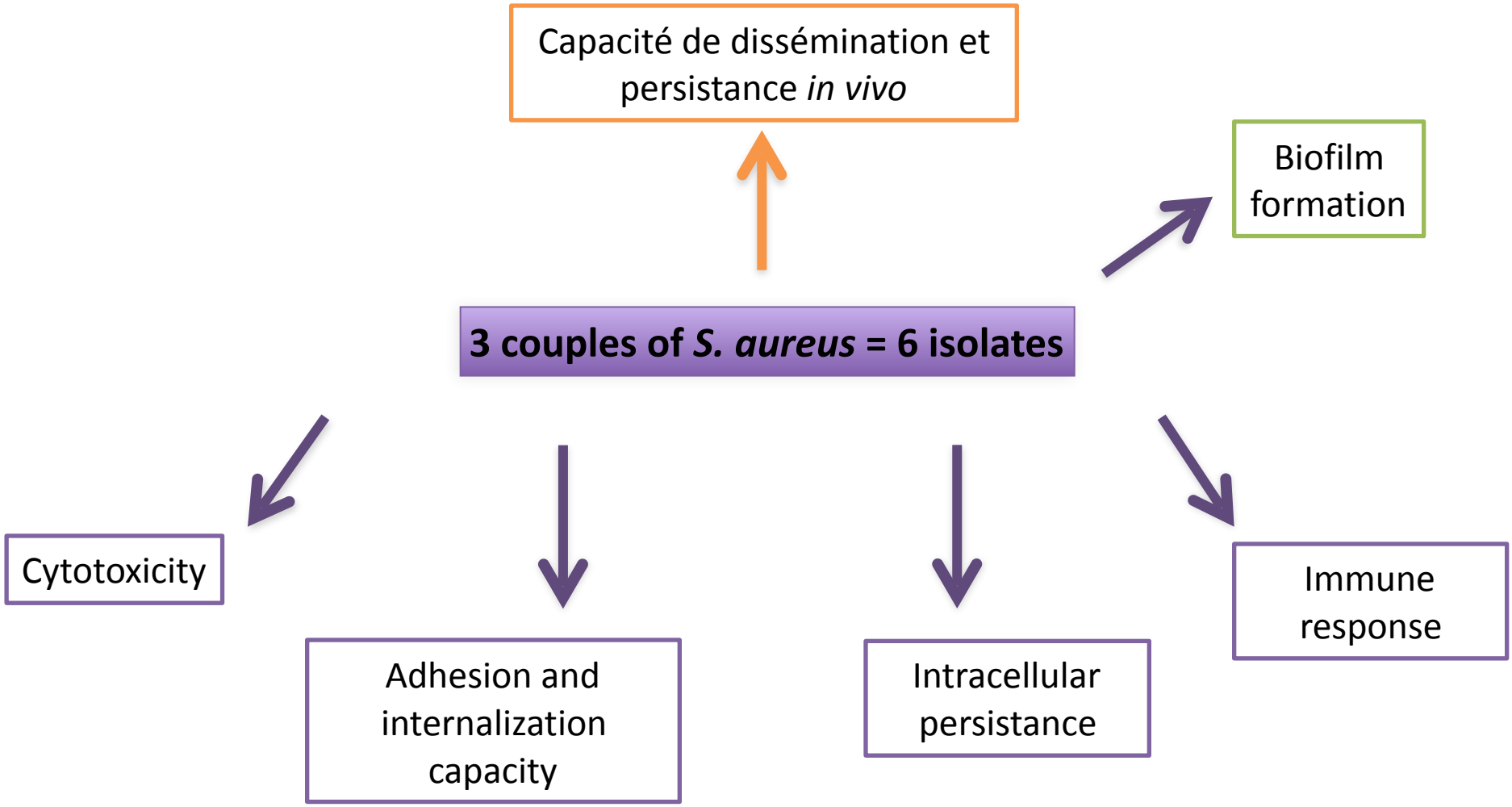


Osteoblasts infected by recurrent isolates secrete **less inflammatory cytokines** that those infected by initial ones

To sum up data from in vitro infection model



Materials et methods

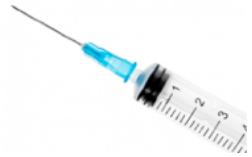


***in vitro* infection model**

In vivo: intra-peritoneal infection model

Evaluation of bacterial dissemination and persistance

48 CD-1 swiss mice



Injection IP 5.10^6
S. aureus

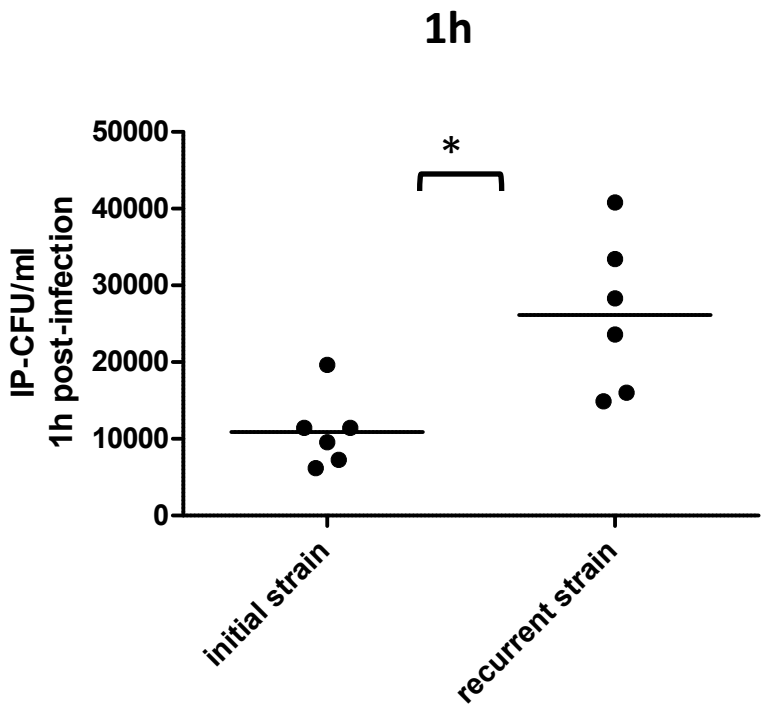
24 mice infected with initial isolates

24 mice infected with recurrent isolates

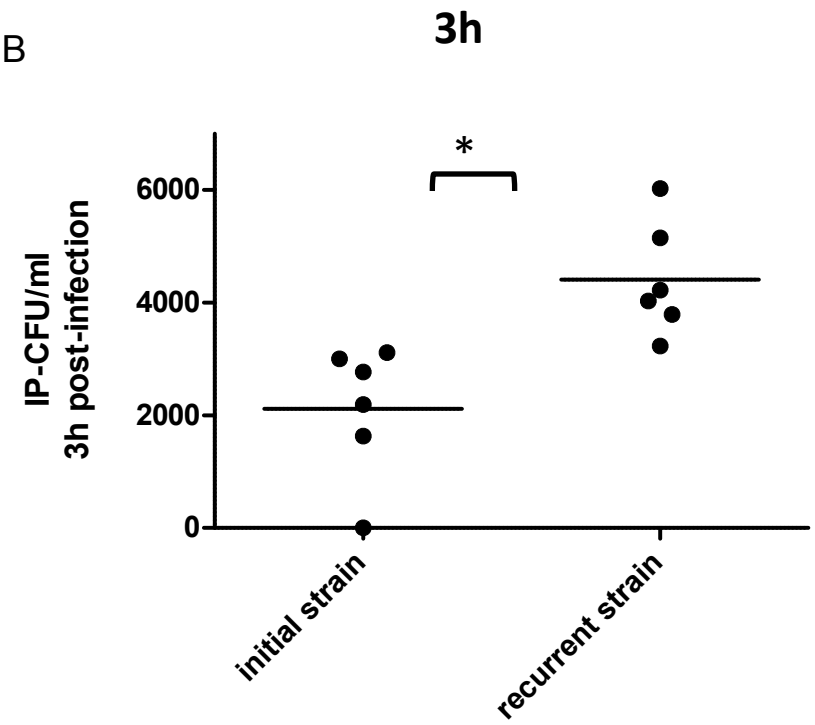
Collection of bacteria by intra-cardiac punction and intra-peritoneal punction **H1, H3, H24 et H48**

Agar plate counting

In vivo: intra-peritoneal infection model



B



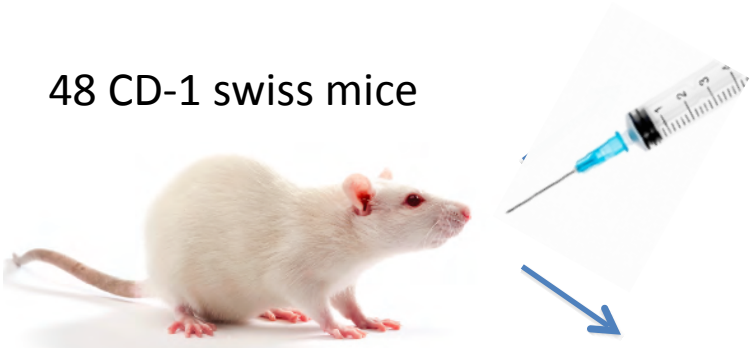
Recurrent isolates **persist longer in peritoneal cavity** than initial ones
-> less recognized by immune cells (because less virulent)

Test de Mann- Whitney

In vivo: lung infection model

Evaluation of mortality and bacterial dissemination and persistance

48 CD-1 swiss mice



Nasal Injection
5.10^8 *S. aureus*

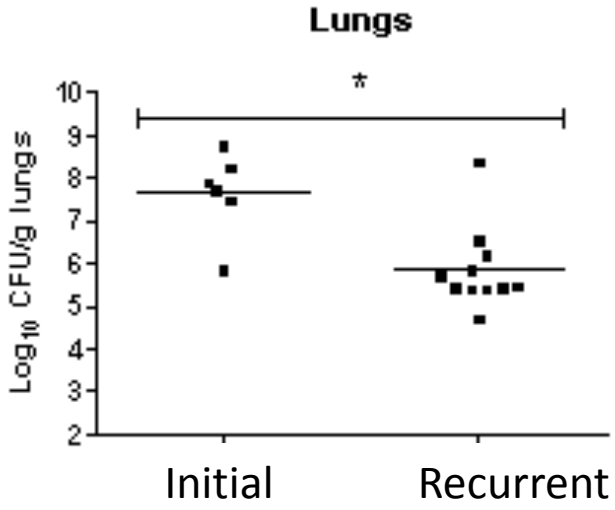
24 mice infected with initial isolates

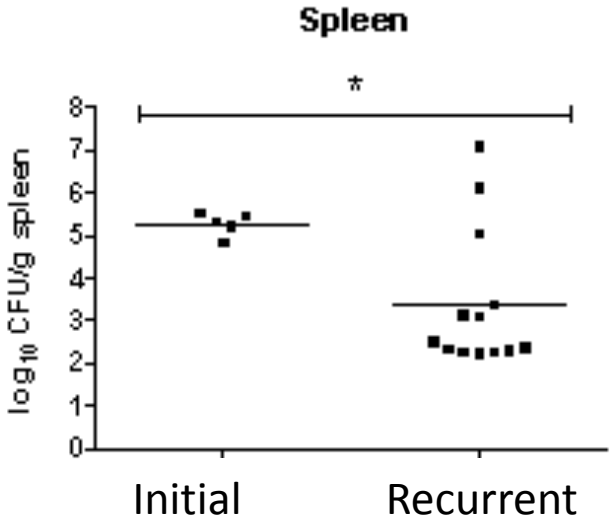
24 mice infected with recurrent isolates

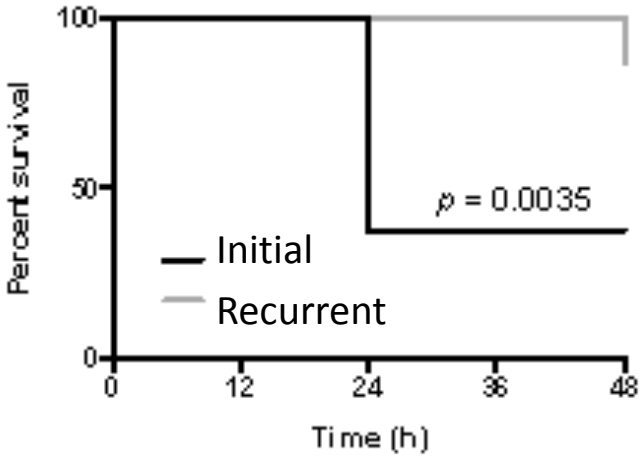
Quantification of bacteria in lung and spleen **H48 post infection**

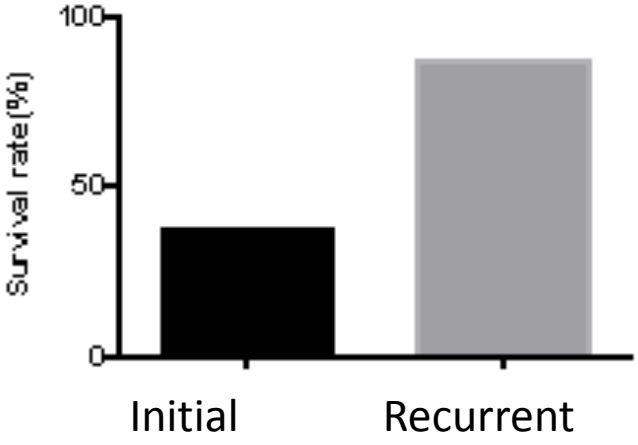
Agar plate counting

In vivo: lung infection model

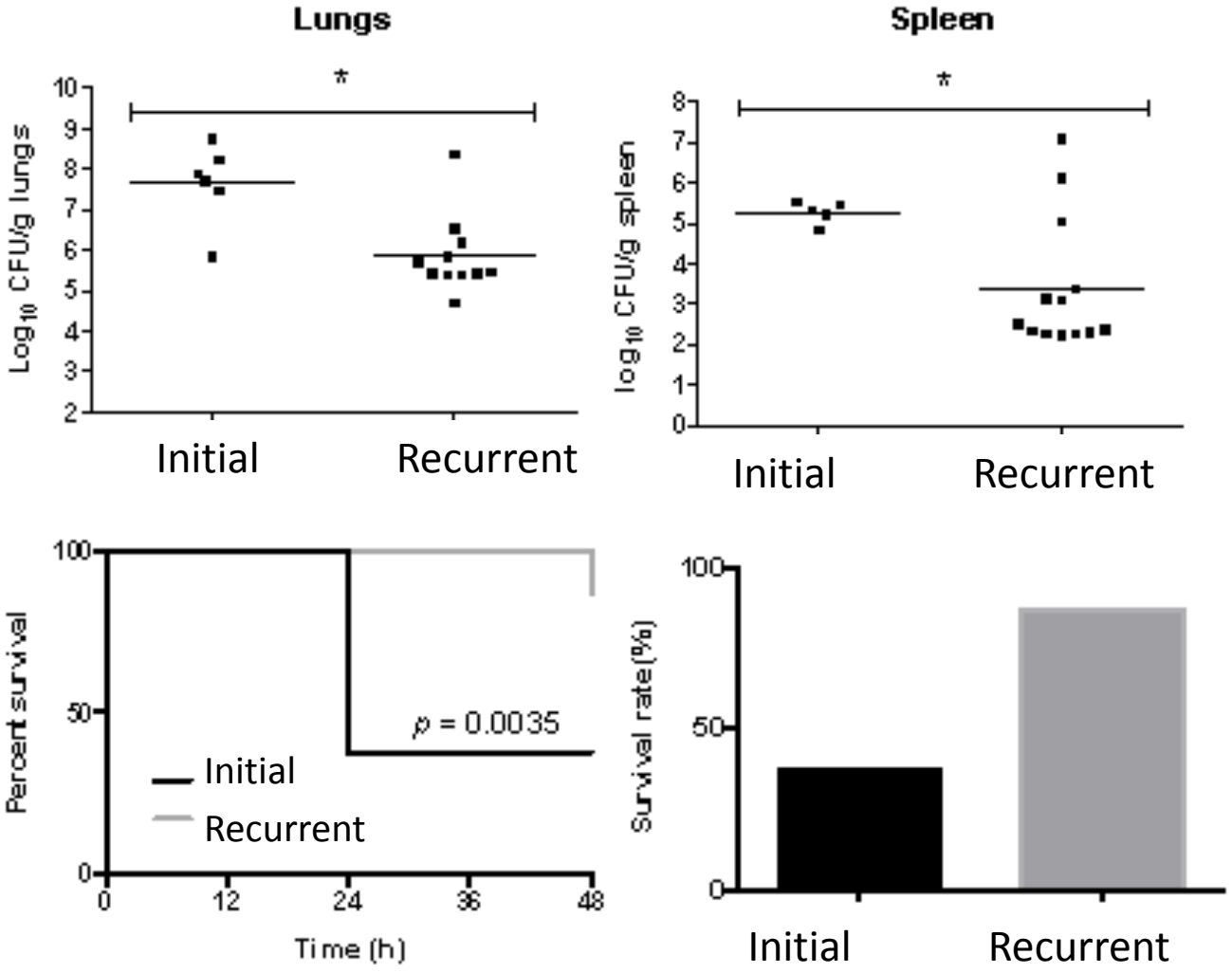








In vivo: lung infection model

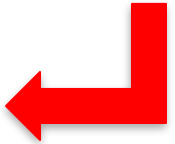


Lower mortality observed with recurrent isolates

Genomic data

Patient	Isolate	Chromosome size (bp)	Plasmid size (bp)	% GC content chromosome	% GC content plasmid	Overall alignment rate (initial genome covered by recurrent isolate reads)	Total nb of SNPs (recurrent *vs* initial)	Nb of SNPS in coding regions
1	initial	2726238	20632	32,89	28,38	98.88	5	0
	recurrent	2726193	20633	32,89	28,38			
2	initial	2749852	17307	32,82	28,38	99,07	5	0
	recurrent	2749888	17307	32,82	28,38			
3	initial	2679642	20720	32,86	28,37	99,14	4	3
	recurrent	2678500	no plasmid	32,86	-			

No genetic convergenceentr when comapring SNP in the three pairs
No gene with specific function involved



If it is not DNA ….. Could it be RNA ? RNAseq underway …
If its not RNA … Could it be epigenetic modification ? …

Modèles *in vivo*

Séquençage des génomes complet

Patient	Isolate	Chromosome size (bp)	Plasmid size (bp)	% GC content chromosome	% GC content plasmid	Overall alignment rate (initial genome covered by recurrent isolate reads)	Total nb of SNPs (recurrent *vs* initial)	Nb of SNPS in coding regions
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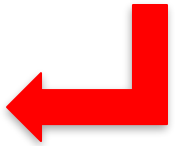
	recurrent	2749888	17307	32,82	28,38			
3	initial	2679642	20720	32,86	28,37	99,14	4	3
	recurrent	2678500	no plasmid	32,86	-			

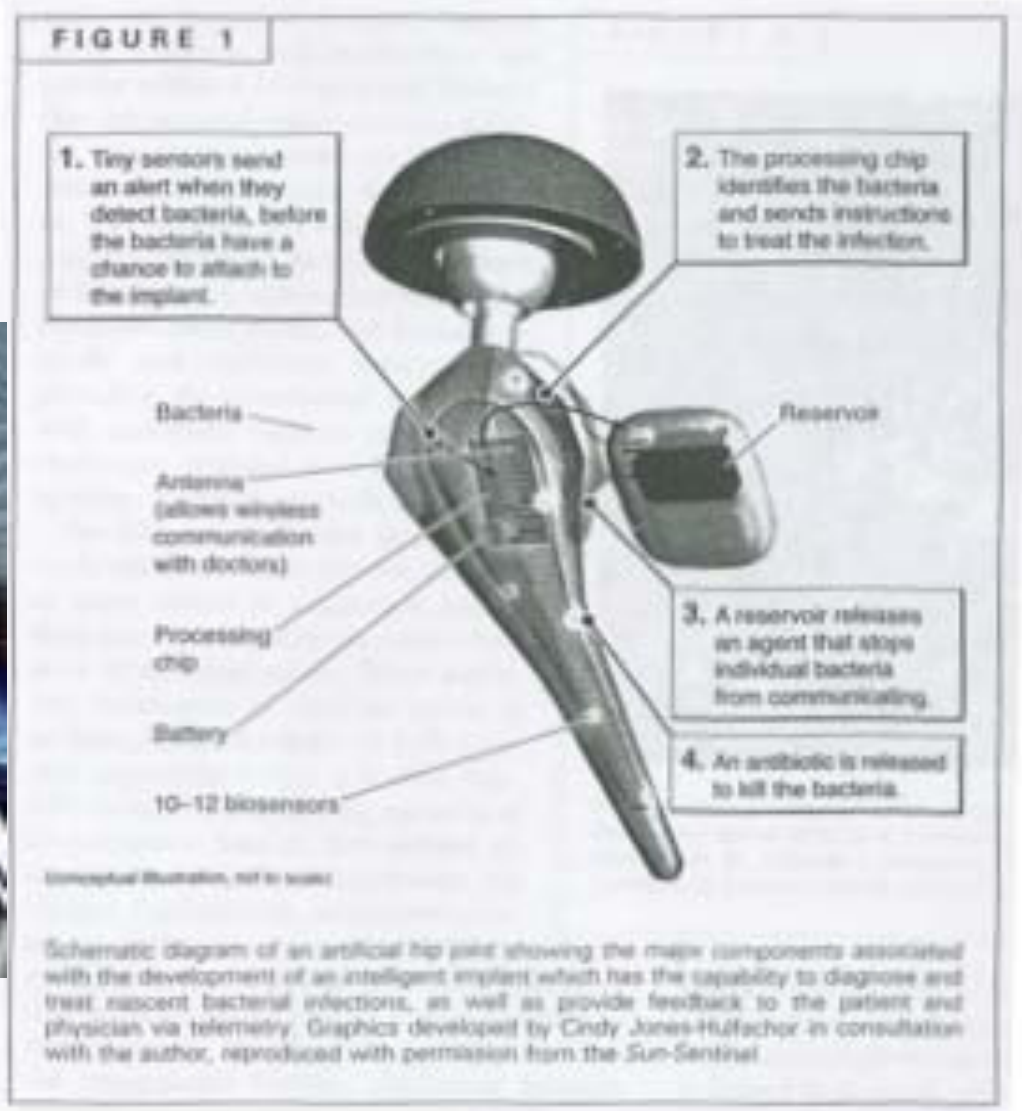
Pas de convergence génétique entre les trois couples
Pas de gènes de fonction connue



Si ce n'est pas l'ADN… c'est l'ARN ??? Technique RNAseq en cours

It is crucial to explore BJI …

it is and it will be a hot topic of interest in the future



It is crucial to explore BJI …

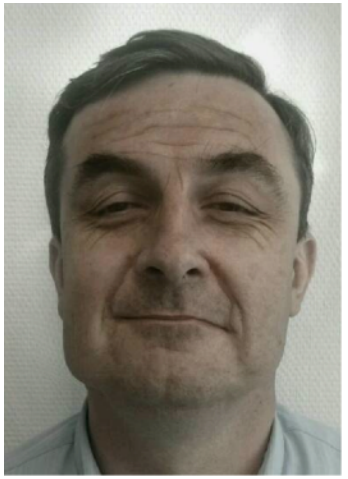
it is and it will be a hot topic of interest in the future

2016

It is crucial to explore BJI …

it is and it will be a hot topic of interest in the future

2016

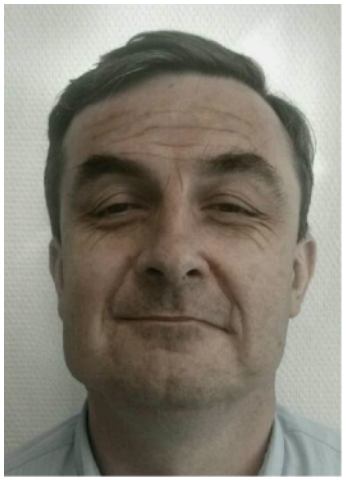


2035

It is crucial to explore BJI …

it is and it will be a hot topic of interest in the future

2016



2035

STAPHYLOCOQUES
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Hospices Civils de Lyon

ciri Centre International de Recherche en Infectiologie

CRIOAc LYON

Hospices Civils de Lyon

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Yousef MAALI
Sacha FLAMMIER
Virginie TAFANI
William MOUTON
Régis VILLET
Yann DUMONT
…

Pr. Francois VANDENESCH
Pr. Jérome ETIENNE
Pr. Gérard LINA
Dr. Anne TRISTAN
Dr. Michèle BES
Dr. Olivier DAUWALDER
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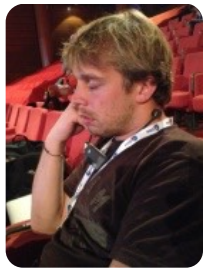
Pr. Tristan FERRY



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Anne-Gaelle RANC
Dr.Sylvestre TIGAUD
Dr. Chantal ROURE
Dr. Hélène SALORD
toutes les techniciennes du Nord !!!

Pr Sébastien LUSTIG

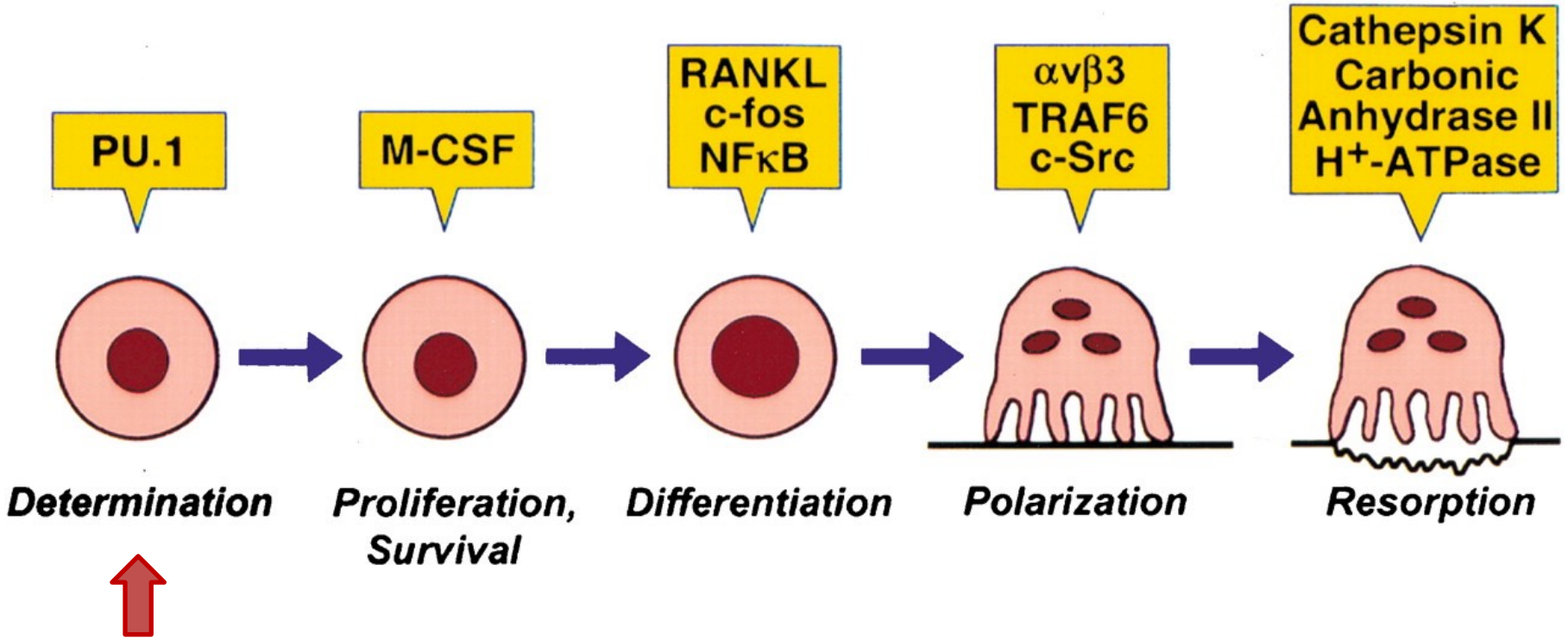
Patricia SIMOES

Guilherme Loss
Ana Tereza Vasconcelos

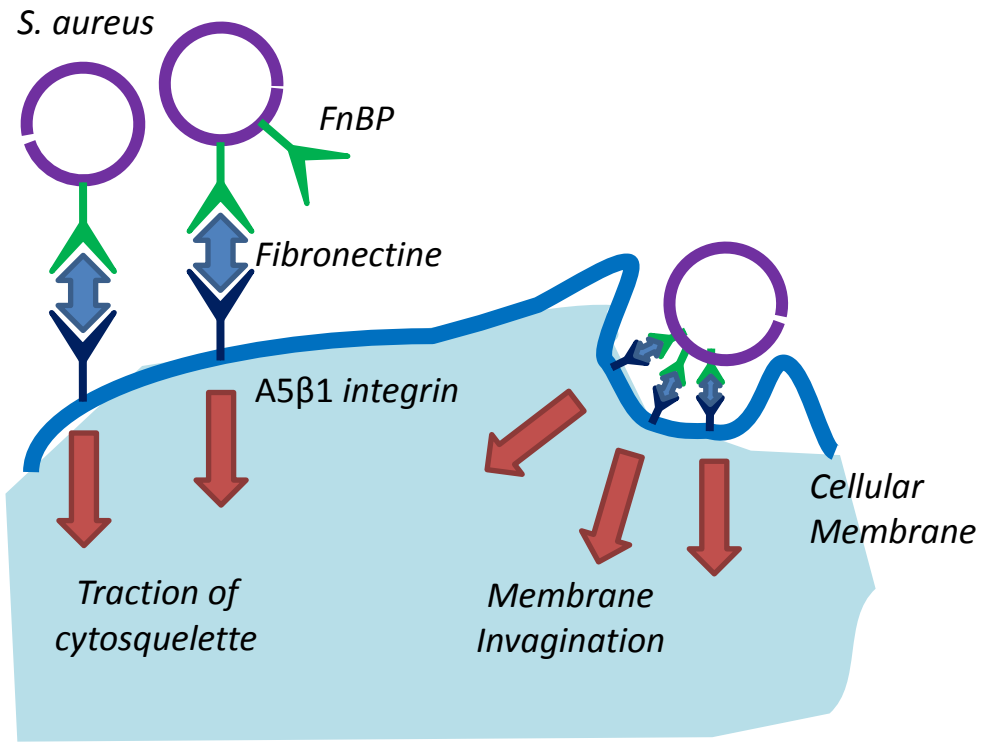
Laboratório Nacional de Computação Científica

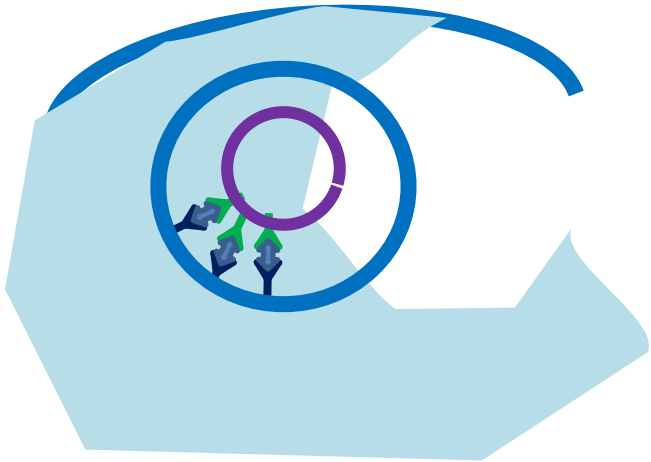
Results

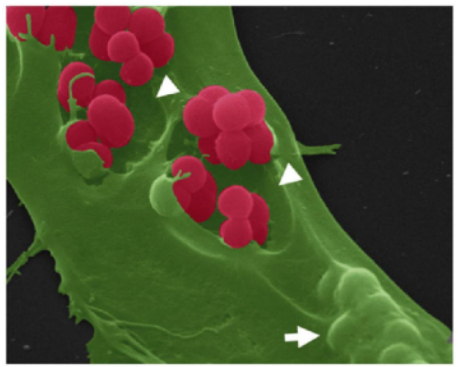
Part I : Impact of *S. aureus* on osteoclastic precursors



Osteoblast invasion by *S. aureus*



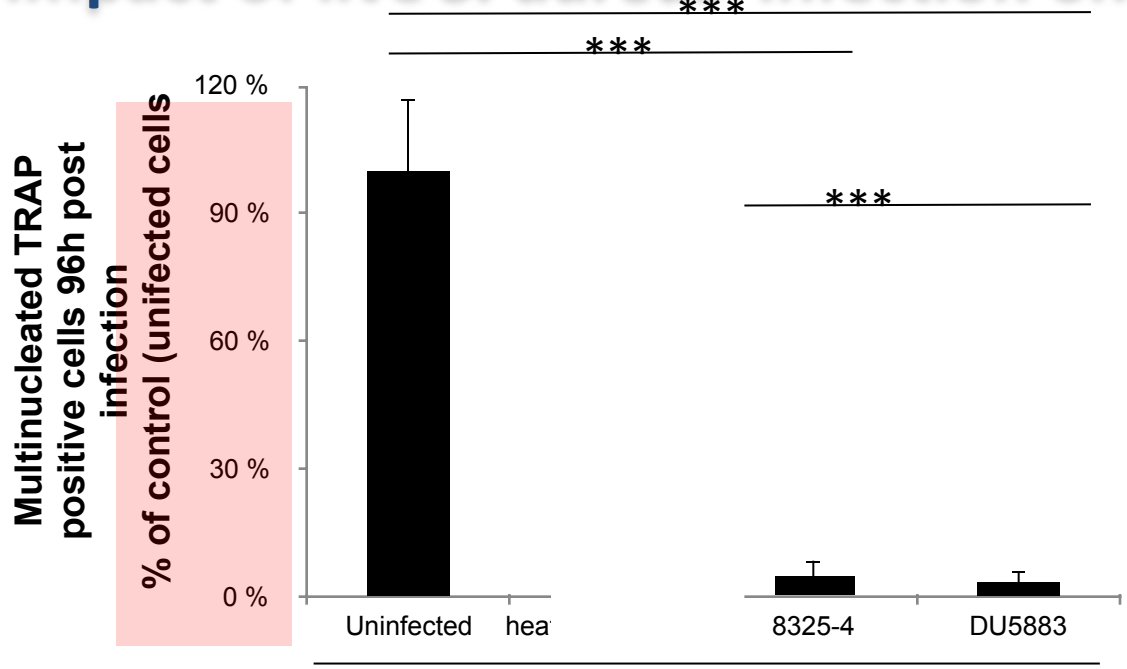




Electronic microscopy
Hoffmann *et al.*, Eur J Cell Biol 2011

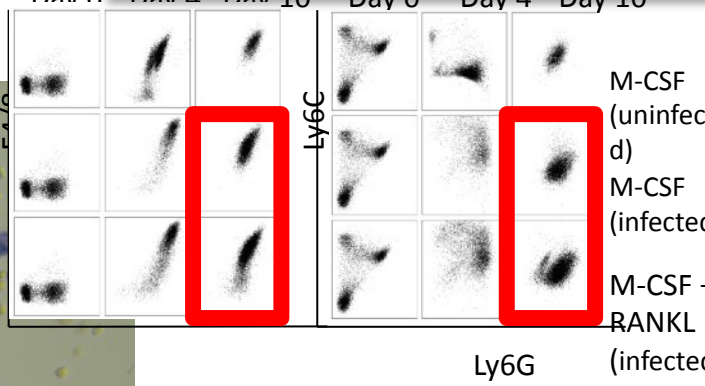
Invasion of host cells is thought to result in a **bacterial sanctuary**

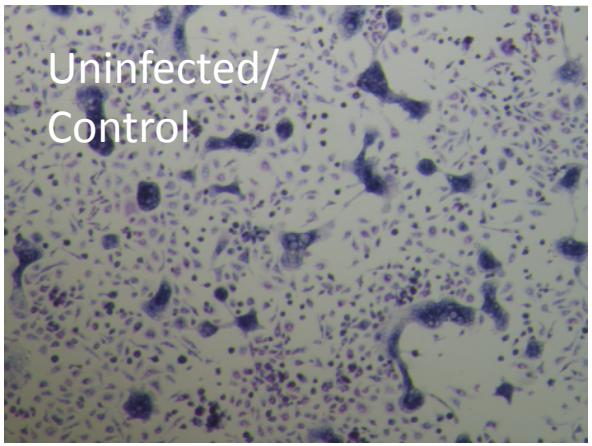
Impact of live *S. aureus* infection on osteoclastogenesis

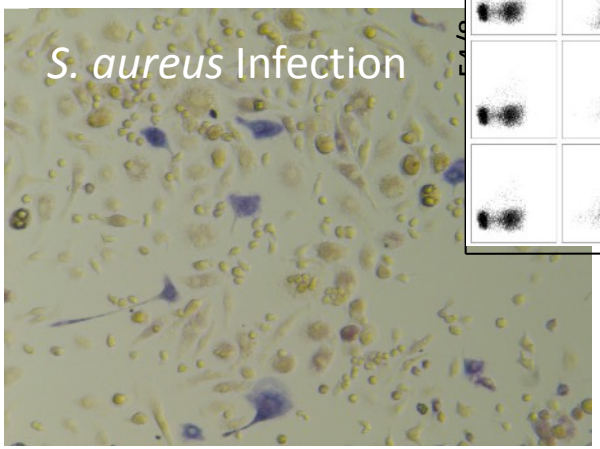


Inhibition of osteoclastogenesis after live *S. aureus* internalization

cytotyping



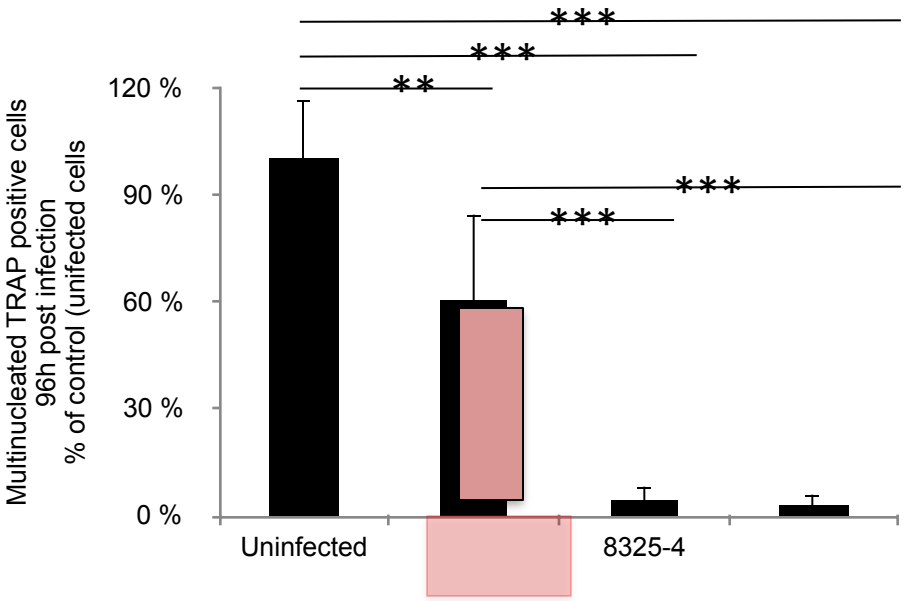




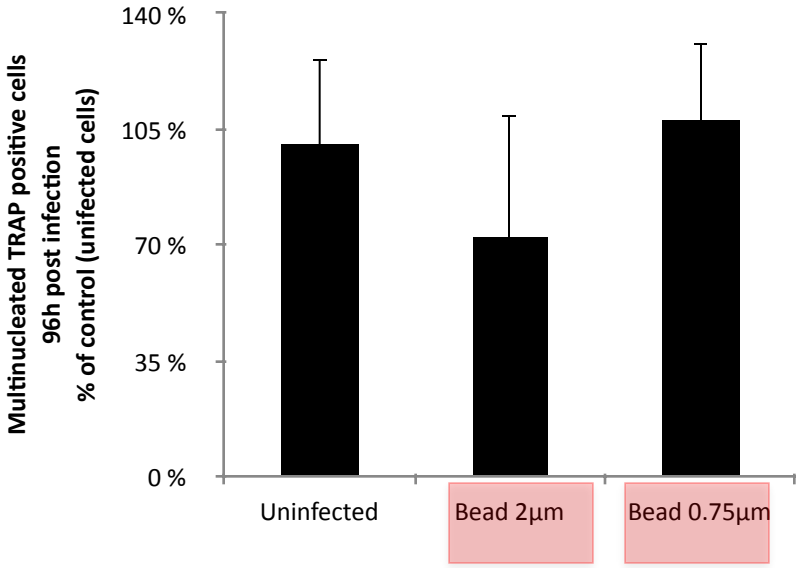
Results

Effect of particulate, membranes, secretome ?

→ Heat-killed Staphylococci



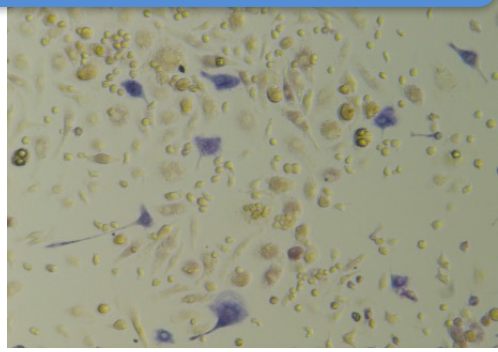
→ Latex bead 2 and 0,75µm (size of *Staphylococci*)



No effect of particulate
Low effect of killed bacteria / bacterial membranes
Major effect of secretome

Results

Characterization of mononuclear TRAP negative cells induced after *S. aureus* internalization



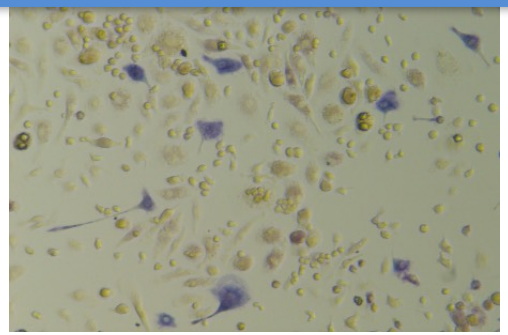
CD11b

Macrophagic differentiation

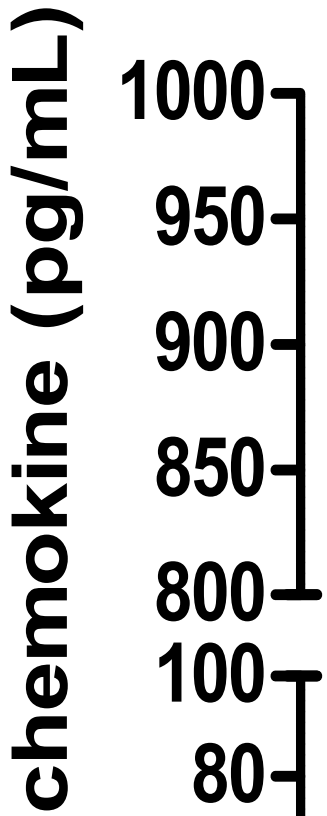
With J. Marvel and P. Parroche

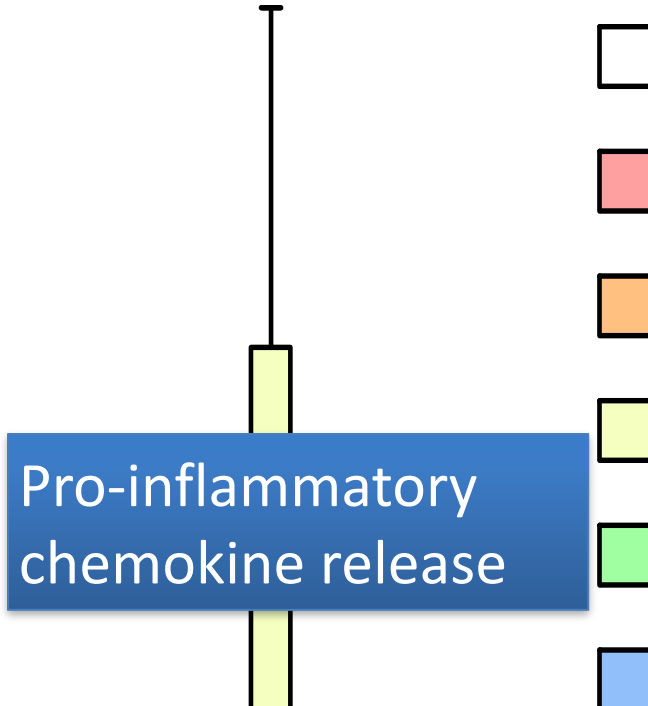
Results

Characterization of mononuclear TRAP negative cells induced after *S. aureus* internalization



Cytokine/chemokine profile

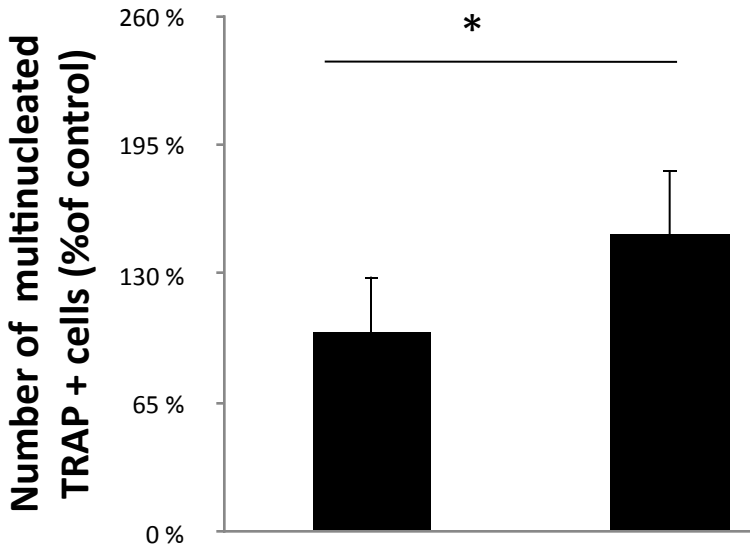




Results

Impact of pro-inflammatory cytokines on osteoclastogenesis

Uninfected OC precursors + culture supernatant from infected OC

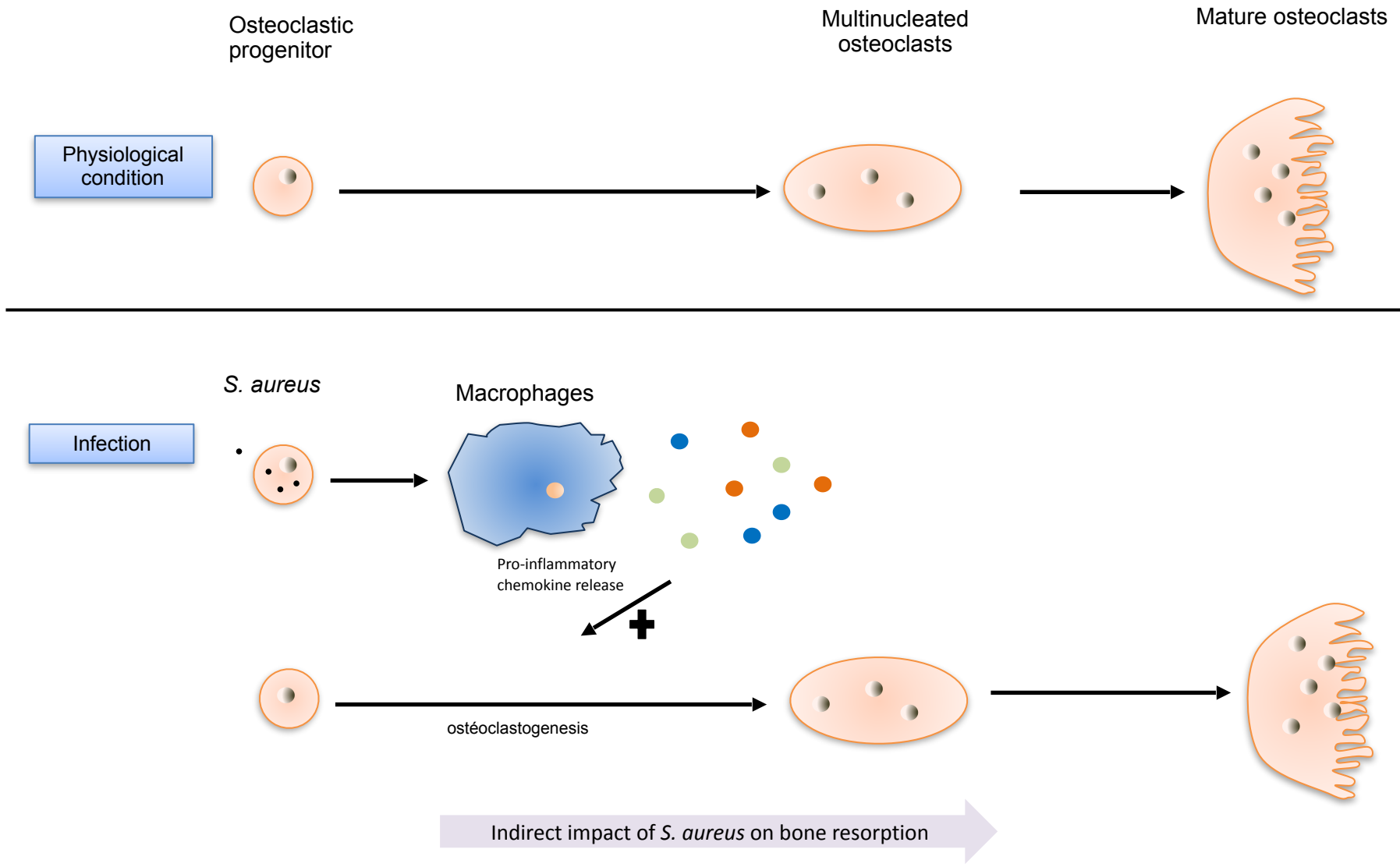


Infected OC precursors boost osteoclastogenesis

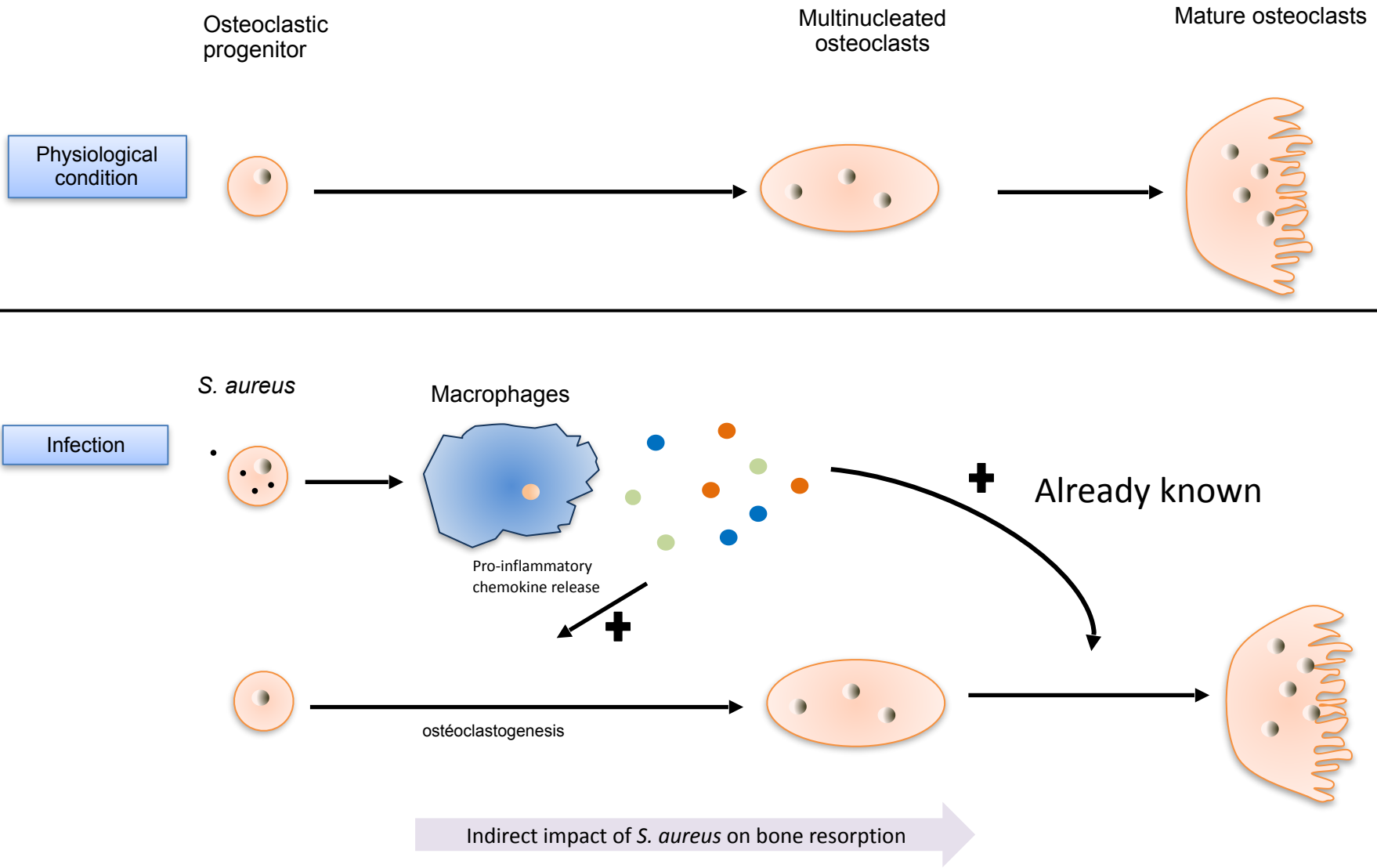
Composition of culture medium		
Culture medium 30ng/mL RANKL, 0,2% M-CSF	**50%**	**50%**
Supernatant of infected cells	0%	50%
Supernatant of uninfected cells	50%	0%

Conclusion part I

- Intracellular staphylococci inhibits osteoclastogenesis independently of the presence of FnBP
- No effect of particulates but specific effect of intracellular staphylococci
- Infected osteoclastic precursors differentiate in macrophages secreting pro-inflammatory factors such as MCP-1 (pro-osteoclastogenic properties)


Osteoclastic progenitor
Multinucleated osteoclasts
Mature osteoclasts
Physiological condition
S. aureus
Macrophages
Infection
Pro-inflammatory chemokine release
ostéoclastogenesis
Indirect impact of S. aureus on bone resorption

Conclusion


Osteoclastic progenitor
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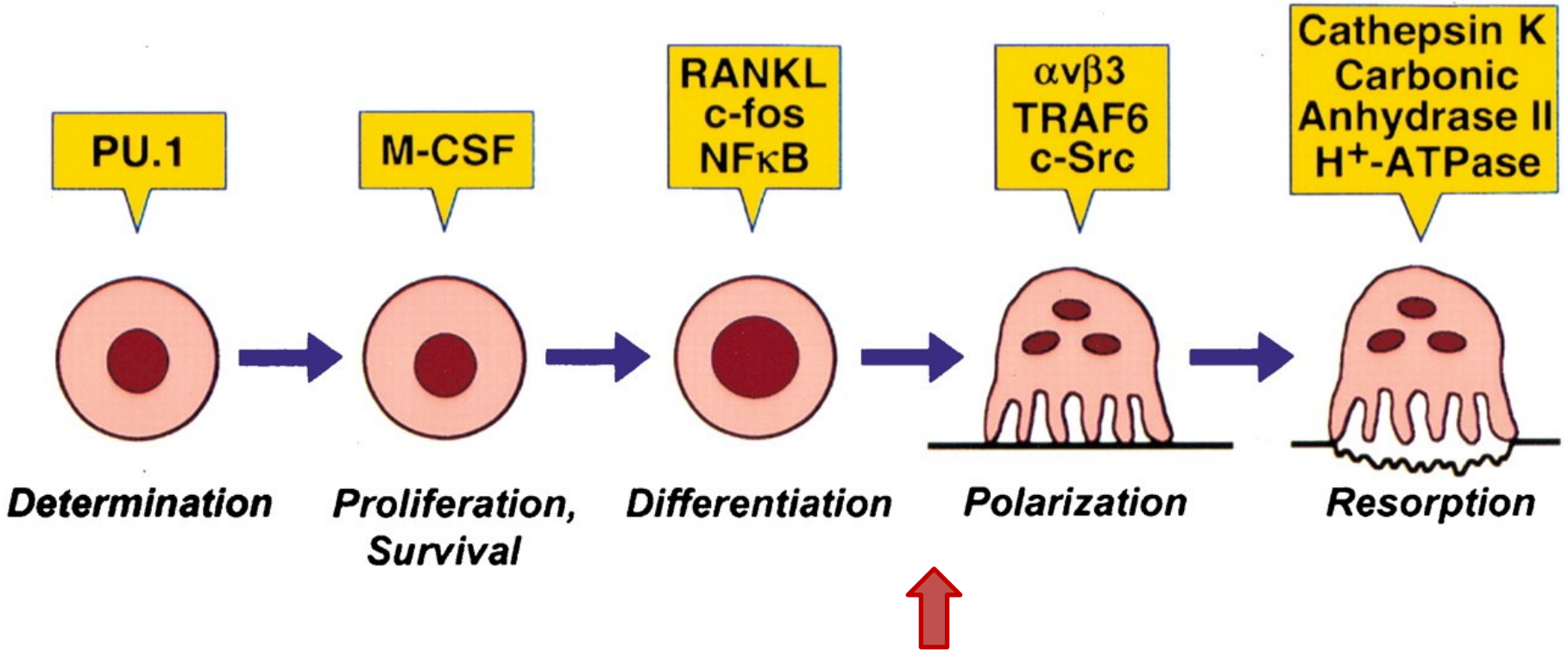
Macrophages
Infection
Pro-inflammatory chemokine release
Already known
ostéoclastogenesis
Indirect impact of S. aureus on bone resorption

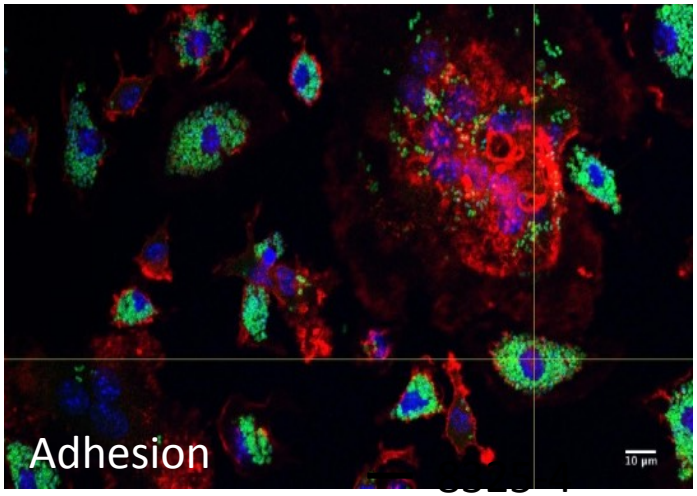
Results

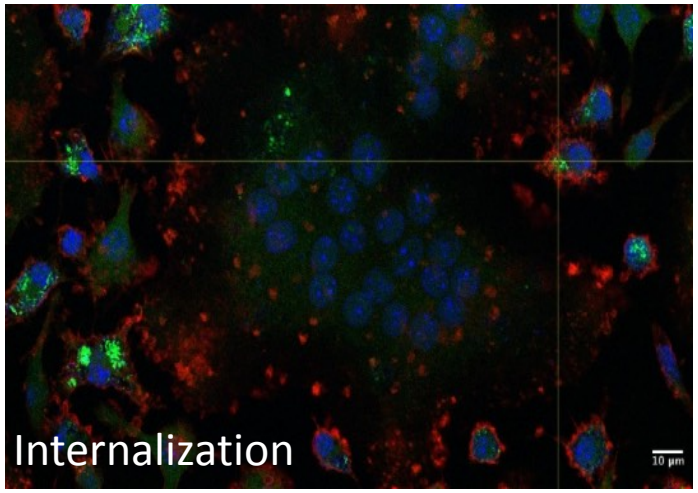
Part II : Impact of *S. aureus* on mature osteoclasts

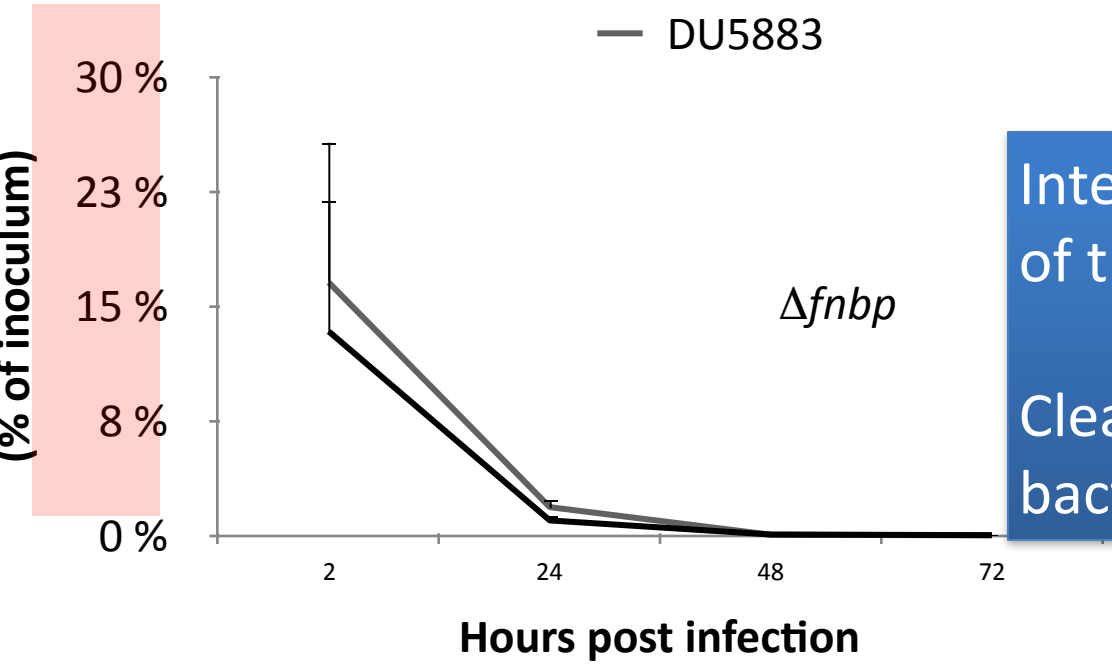


Results

Internalization and *S. aureus* intracellular persistance





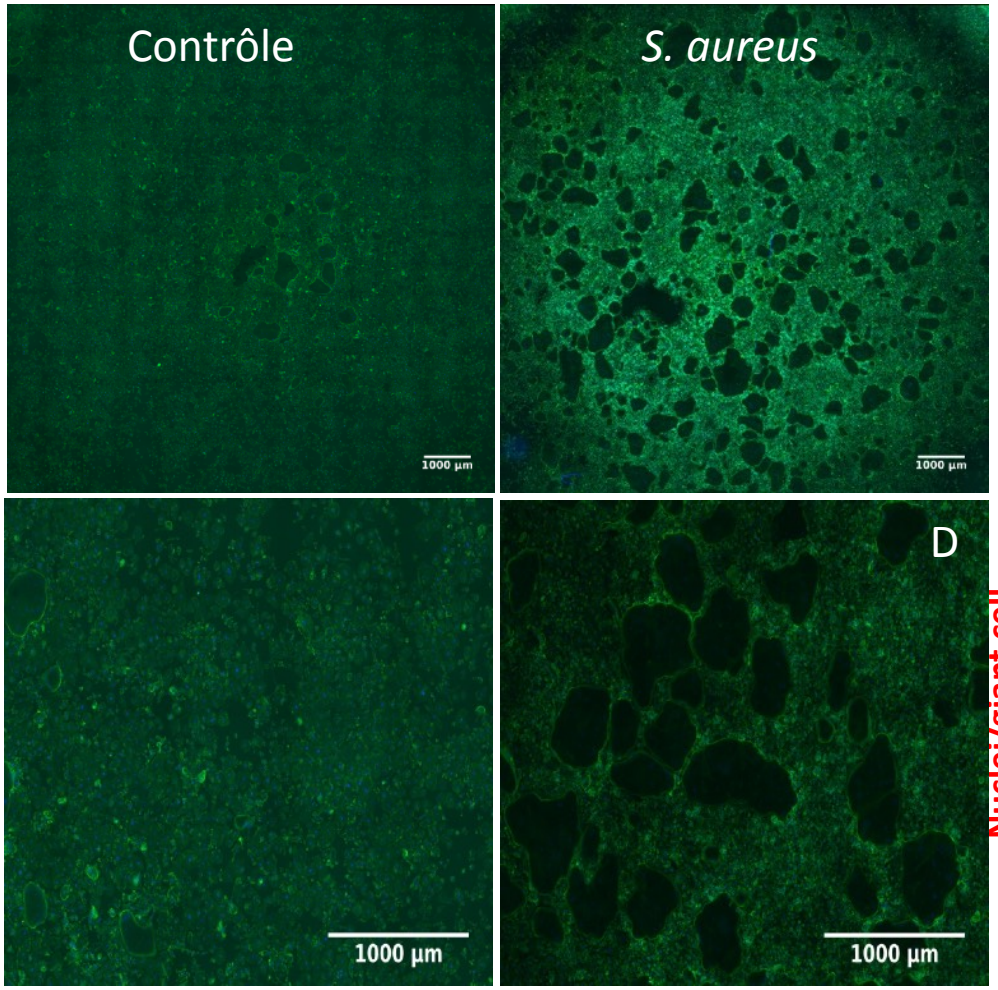


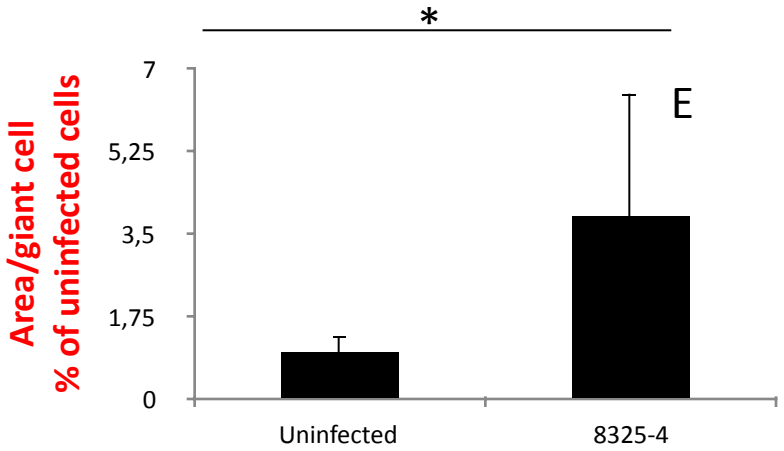
Internalization independantly of the presence of FnBP

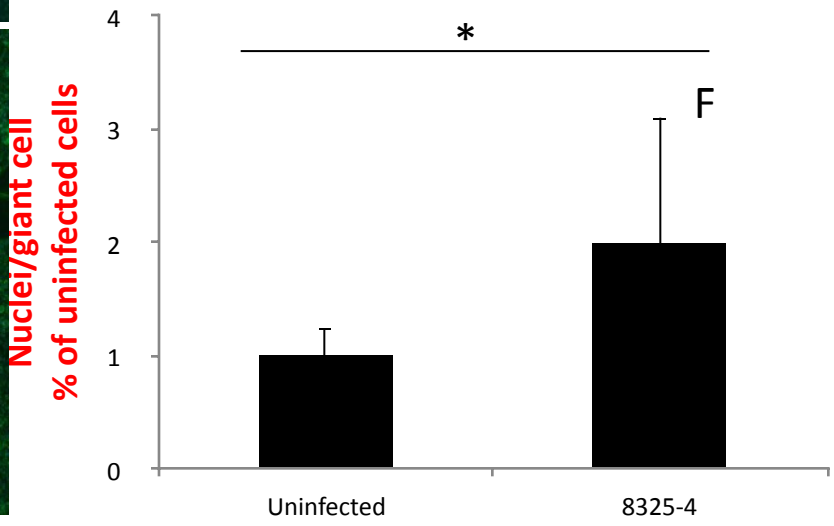
Clearance of intracellular bacteria in 72h

Results

Impact of *S. aureus* on cellular spreading and osteoclasts fusion







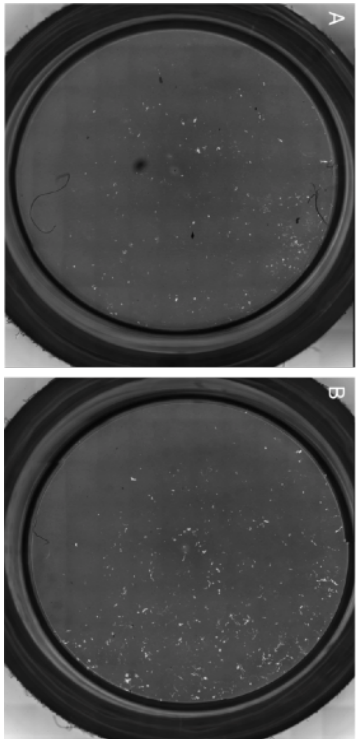
72h post infection, increase of size and fusion of osteoclasts

Results

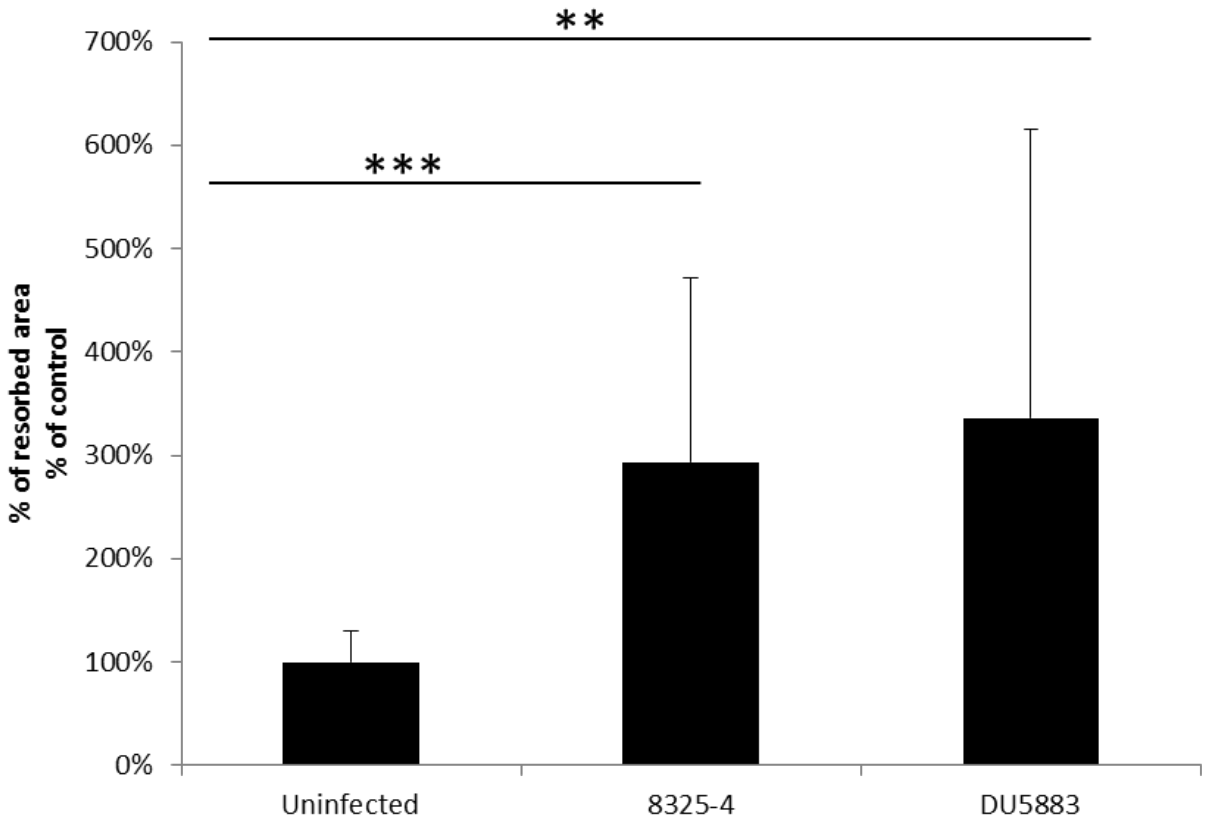
Impact on *S. aureus* bone resorption

Method : bone matrix + OC for 24h , then microscopic imaging

Control



S. aureus



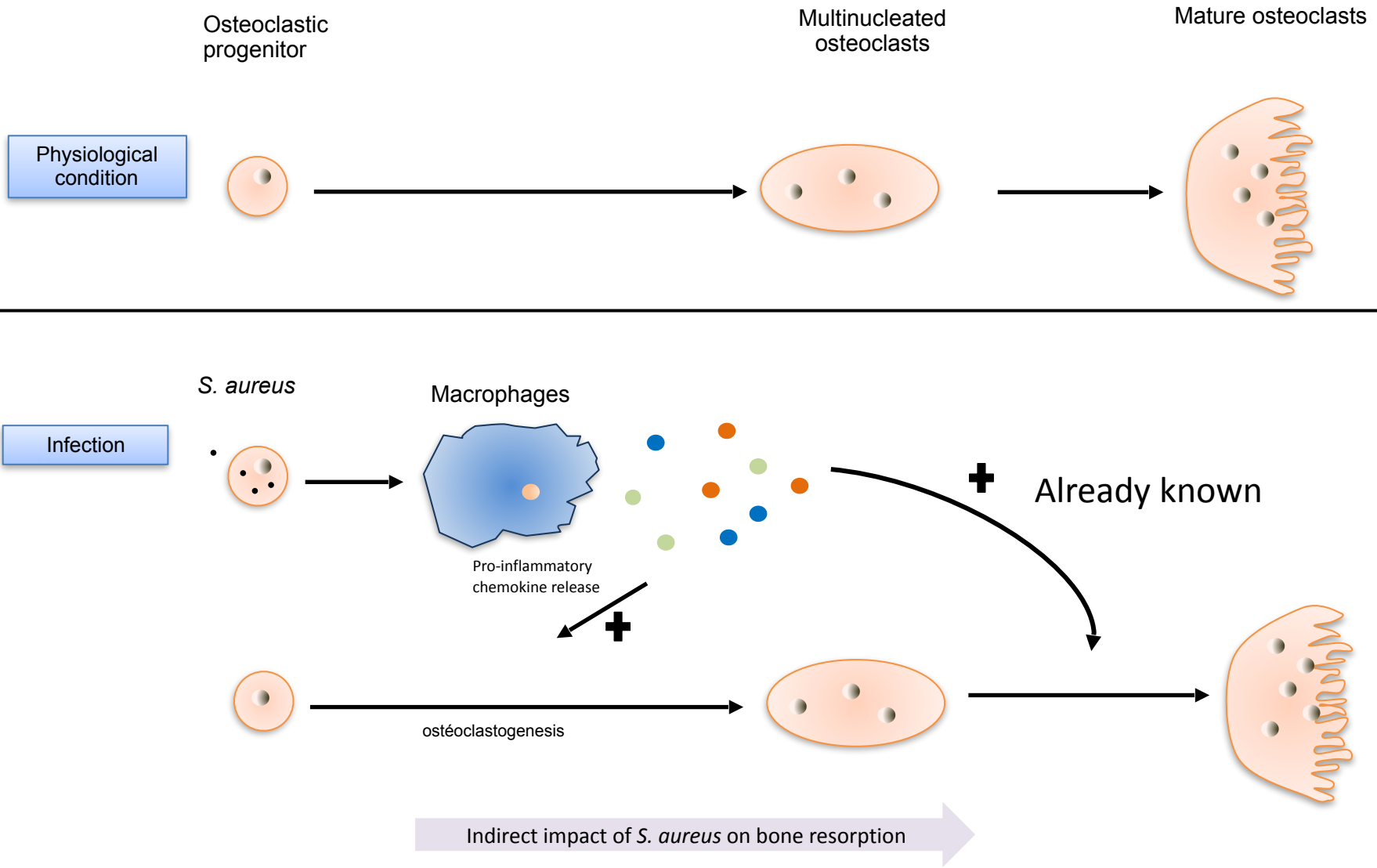
S. aureus infection enhances bone resorption of mature osteoclasts

Results

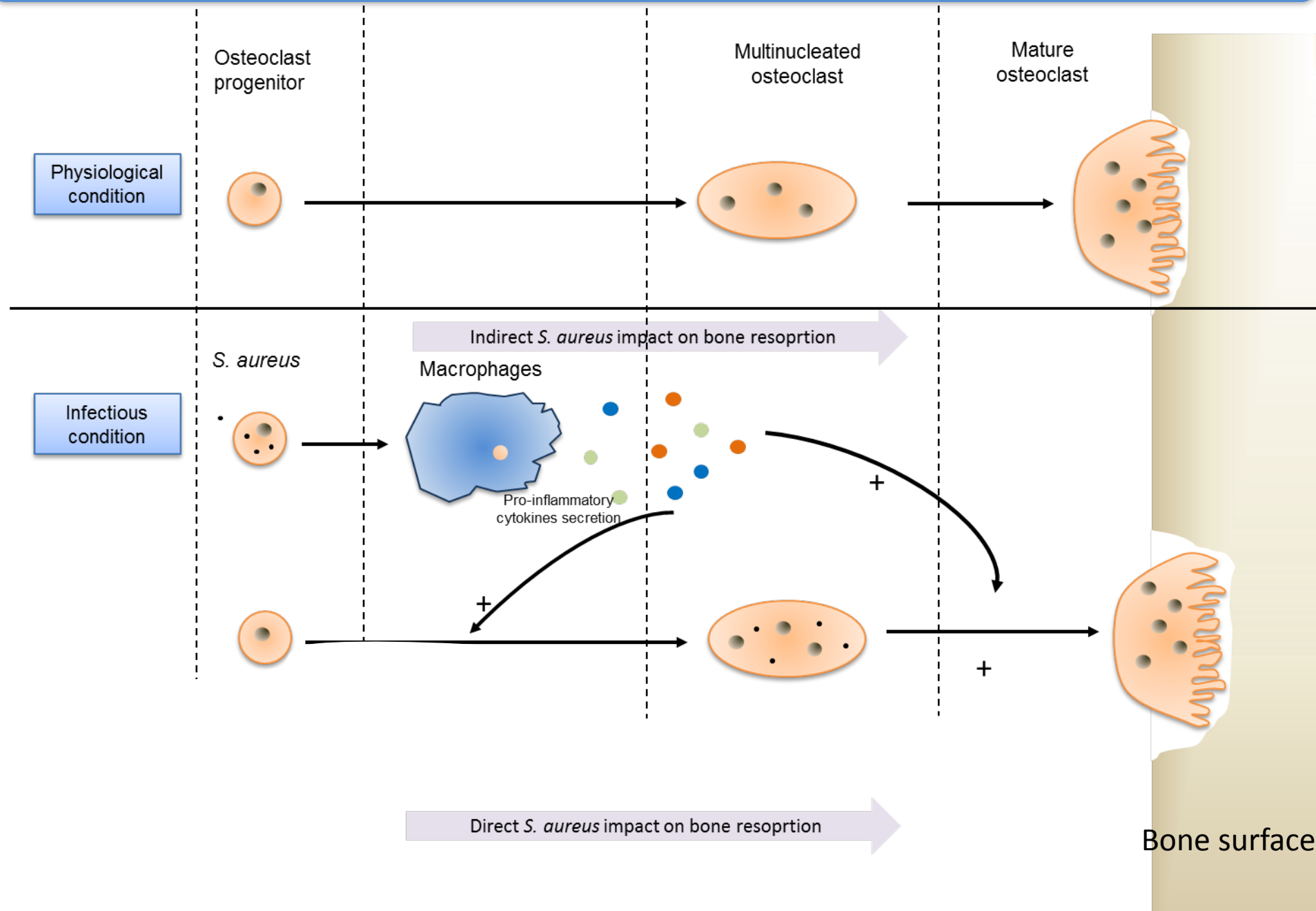
- Staphylococcal impact on mature osteoclasts is independant of the presence of FnBP

- Intracellular staphylococci modify cellular spreading and fusion and induce enhancement of bone resorption capacity

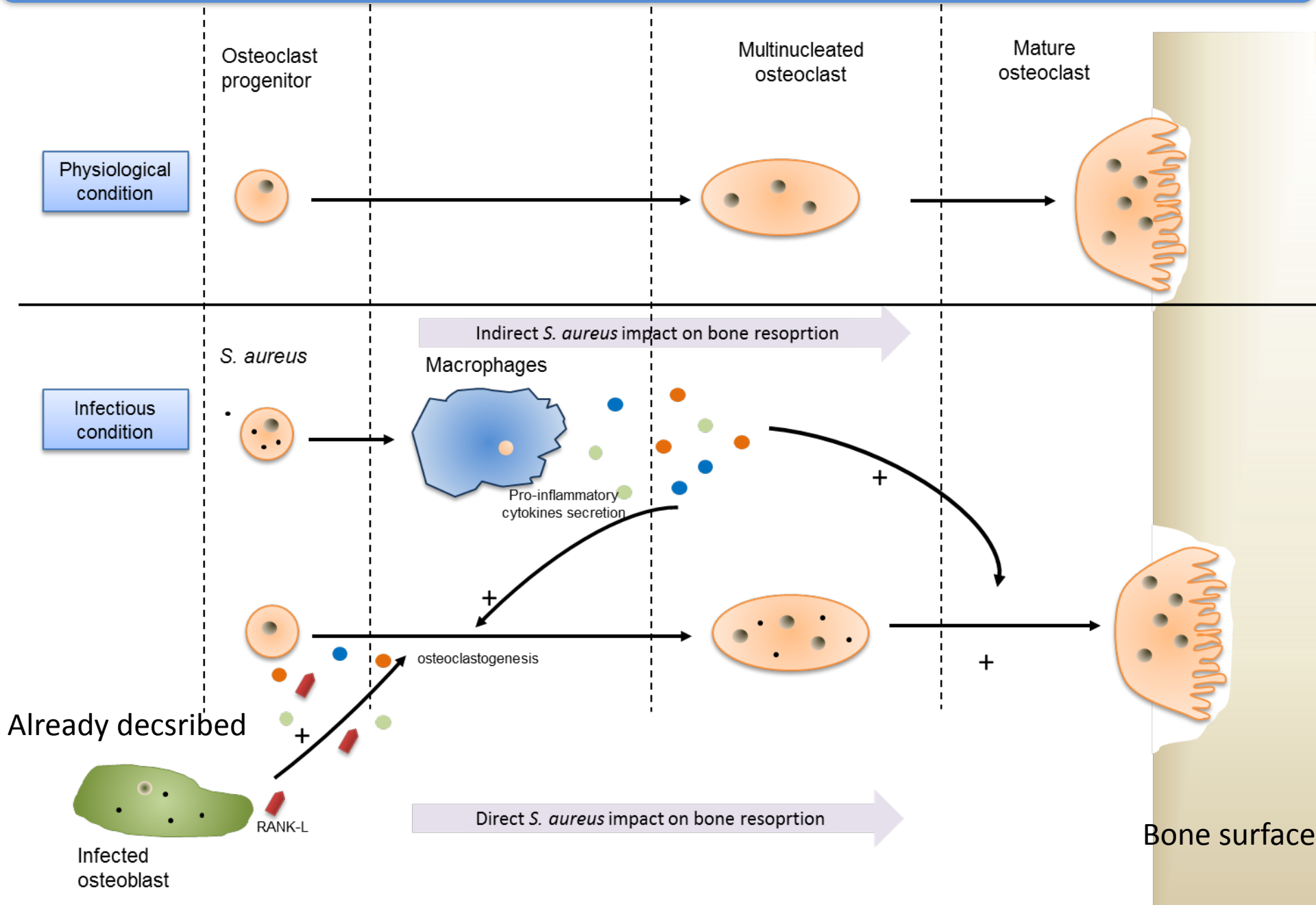
Conclusion


Osteoclastic progenitor
Multinucleated osteoclasts
Mature osteoclasts
Physiological condition
S. aureus
Macrophages
Infection
Pro-inflammatory chemokine release
Already known
ostéoclastogenesis
Indirect impact of S. aureus on bone resorption

Conclusion

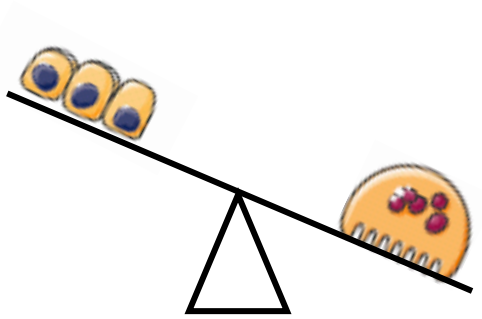

Osteoclast progenitor
Multinucleated osteoclast
Mature osteoclast
Physiological condition
Indirect S. aureus impact on bone resoprtion
S. aureus
Macrophages
Infectious condition
Pro-inflammatory cytokines secretion
+
+
+
Direct S. aureus impact on bone resoprtion
Bone surface

Conclusion


Osteoclast progenitor
Multinucleated osteoclast
Mature osteoclast
Physiological condition
Indirect S. aureus impact on bone resoprtion
S. aureus
Macrophages
Infectious condition
Pro-inflammatory cytokines secretion
+
+
+
osteoclastogenesis
Already decsribed
RANK-L
Infected osteoblast
Direct S. aureus impact on bone resoprtion
Bone surface

Take home message

Direct and indirect effects of *S. aureus* invasion of bone cells on bone homeostasy



- Invasion of osteoblasts (CA-MRSA)
  - PSM (-> autophagy ->) mort cellulaire
  - perte de fonction
- Invasion of osteoclastic precursors
  - Hijacking to macrophagic differentiation -> cytokines -> enhacement of osteoclastogenesis
- Invasion mature osteoclast
  - Increase of bone resorption

All effects are in favour of bone looseming

THANK YOU FOR YOUR ATTENTION

STAPHYLOCOCCAL PATHOGENESIS
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THANK YOU FOR YOUR ATTENTION

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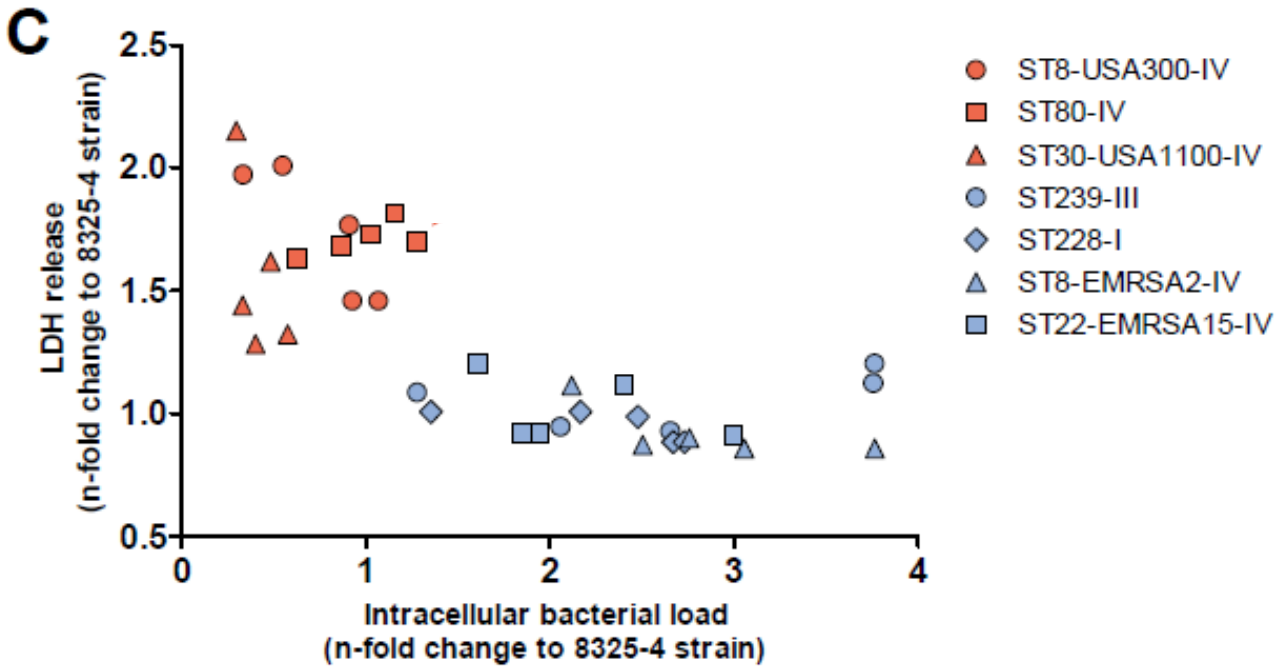
- **Pauline Nauroy**
- Margot Grignard
- Chantal Domenge, Irma Machuca Gayet, Dan Georgess

finovi
Fondation Innovations en Infectiologie

Is there a correlation between cytotoxicity and intracellular survival ?

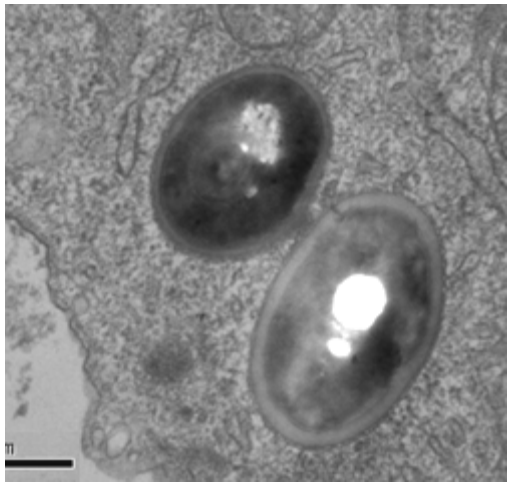


Cytotoxicity and intracellular survival were **not independently associated** in multivariate analysis controlling for the CA- or HA-MRSA status

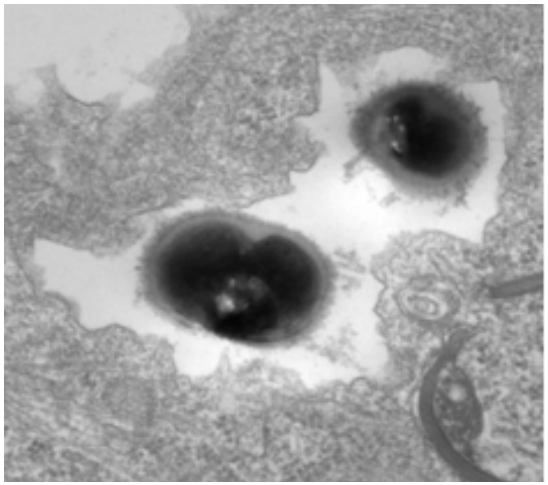
Quantification escape / autophagy en MET

- Gentamicin protection assay
- Cells harvested 6h post-infection and processed for TEM
- TEM-based scoring of bacteria based on surrounding membranes:

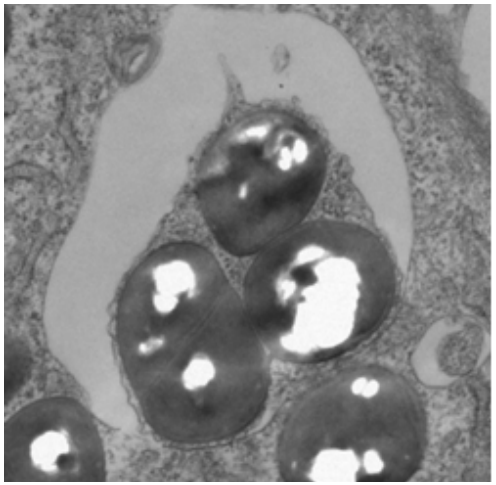
NO MEMBRANE
Free in cytoplasm



SINGLE MEMBRANE
Enclosed in phagosome

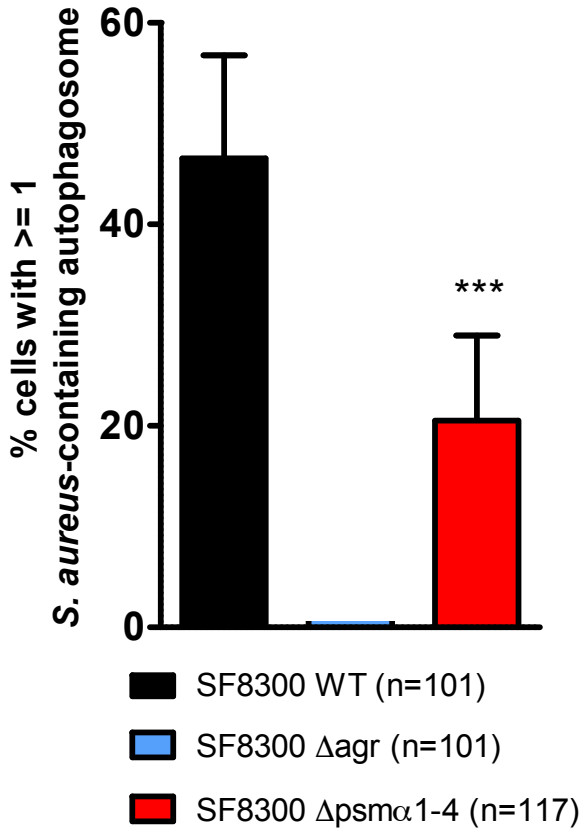


DOUBLE MEMBRANE
Enclosed in autophagosome



Dupieux *et al*. In prep.

Phagosomal escape and autophagy induction



46.5% of infected cells contained at least 1 wild type *S.aureus*-containing autophagosome

psmα deletion:
2.27-fold reduction ($p < 0.001$)

PSMs induce/hijack autophagy

Fisher's exact test, exact binomial 95%CIs, 2 indep. Exp.

Part II

Impact of staphylococcal regulators on the cytotoxic phenotype of CA-MRSA

Role of major regulatory systems ?

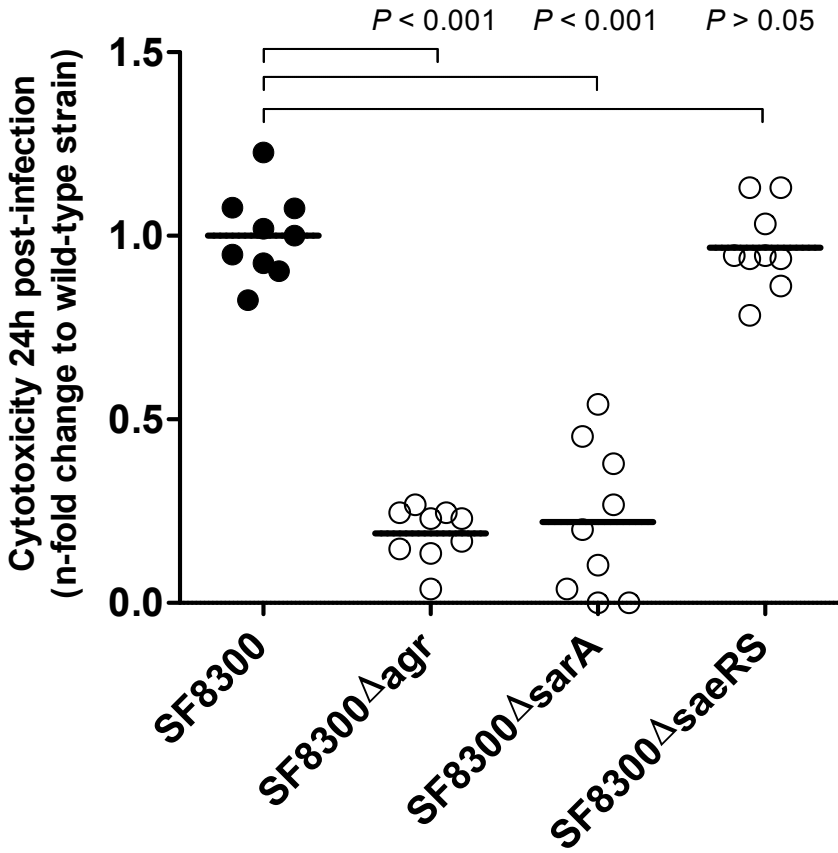
Materials

- isogenic bacteria deleted for *agr* or *sar*A or *sae*RS
- mesure of cytotocity after osteoblast invasion

Conclusion

- **Functional *agr*A and *sar*A regulators are required for cytotoxicity in strain SF8300**

- **No impact of *sae*RS deletion**



Profiles of regulation in CA-MRSA :

. *agr* : upregulates **PSMs**, alpha-toxin and PVL
. *sar*A: upregulates **PSMs** and alpha-toxin
. *sae*RS: upregulates alpha-toxin, **not PSMs**

=> regulatory requirements of cytotoxicity seem consistent with a predominant role of PSMs

Part III.

Role of CA-MRSA virulence determinants in osteoblast damage

Major virulence determinants of CA-MRSA:

- **Panton-Valentine leukocidin** (PVL): recruits and kills neutrophils
- **Alpha-toxin** (HLA) overexpression: haemolysis
- **Phenol-soluble modulins** (PSMs) overexpression: recruit and kill neutrophils BUT controversial
-

Profiles of regulation in CA-MRSA :

. *agr* : upregulates **PSMs**, alpha-toxin and PVL

. *sar*A: upregulates **PSMs** and alpha-toxin

. *sae*RS: upregulates alpha-toxin, **not PSMs**

⇒regulatory requirements of cytotoxicity seem consistent with a predominant role of PSMs