Response to "*In vivo* attenuation and genetic evolution of a ST247-SCC*mec*I MRSA clone after 13 years of pathogenic broncho-pulmonary colonization in a patient with cystic fibrosis: implications of the innate immune response"

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To the Editor: In response to the letter from Kriegeskort *et al.* "Response to "*In vivo* attenuation and genetic evolution of a ST247-SCCmecI MRSA clone after 13 years of pathogenic bronchopulmonary colonization in a patient with cystic fibrosis: implications of the innate immune response"",[1] we confirm that at the present time the methicillin-resistant *Staphylococcus aureus* (MRSA) "slow growth" isolates from the studied cystic fibrosis patient did not correspond to small colony variants (SCVs) and were not thymidine-, hemin- or menadione-dependent. We tested the auxotrophy of our all 36 MRSA isolates following a previously defined methodology,[2] and all were able to grow in chemically defined medium agar without hemin, menadione or thymidine supplementation. Moreover, we compared the genes involved in the heme, menadione and thymidine pathways of both the initial CF-96 and the evolved CF-09 strains with the staphylococcal genomes previously deposited in the GenBank database, without significant findings. Mutations were only detected in the evolved genome of the CF-09 isolate in two genes: the iron-regulated surface determinant protein B (*isd*B) gene, which was truncated at nucleotide 588, and in the hemin transport system permease protein B, which had the amino acid change $I_{355}V$. Both genes have been implicated in human hemoglobin incorporation and their lack of functional impairment is associated with a significant decrease in virulence.[3–5]

The patient was transferred to our institution when he was 9-years old, and the initial MRSA CF-96 isolate had the same poor morphotype as the most recent MRSA isolate obtained 13 years later. It is important to note that the isolates from the sputum of the cystic fibrosis patient were obtained a long time ago and were preserved at $-80\,^{\circ}C$, thus the successive cultures on rich mediums might have reverted the SCVs morphotype. In fact, the current isolates displayed nearly normal non-pigmented and non-hemolytic growth in 24 h. Based on the genome analysis of the first and the evolved isolates, we speculated that although the colony morphology strongly suggested the existence of SCV, this poor-growth phenotype might have been produced as a consequence of the strong and sustained antibiotic pressure combined with the immunological response of the host. As we have not found genetic evidence of SCV, the poor growth might correspond to a phenotypic stage previous to the SCVs full conversion.

We are aware that a virulence attenuation of the SCVs has been demonstrated in relation to a persistence phenomenon and has also been linked to cystic fibrosis lung colonization.[2,6–8] The initial intention of our work was to analyze both the bacterial reactivity and the host response, and we found that they were attenuated during the long process of colonization/infection. In our opinion, the most relevant results related to the bacteria were the continuous changes in superficial antigen protein A, up to six times, that decreased the bacterial stimulus. The clinical relevance of this phenomenon has been previously described.[9,10] On the other hand, the monocytes of the patient exhibited a considerable decrease in their activation ability during the studied period.

In summary, we cannot rule out the existence of SCVs in the lung of the patient, but our MRSA isolates have intact heme, menadione and thymidine genetic pathways. Mutations in metabolic routes in the evolved CF-09 isolate are only related to superficial proteins, which are related to iron incorporation. The attenuation phenomenon described in our study over a 13-year period of lung

colonization was due to superficial, antigenic bacterial changes and a decrease in the inflammatory response of the innate immune system within a refractory status of the patient.

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