CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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High Anti-Staphylococcal Antibody Titers in Patients with Epidermolysis Bullosa Relate to Long-Term Colonization with Alternating Types of *Staphylococcus aureus*

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TO THE EDITOR

Patients with the blistering disease epidermolysis bullosa (EB) develop wounds that are highly susceptible to bacterial colonization. Recently, we reported that over 75% of the EB patients sampled at one particular point of time were colonized with *Staphylococcus aureus* (van der Kooi-Pol *et al.*, 2012). To determine possible changes in *S. aureus* colonization over time, swabs were collected from the nares, throats, and wounds of 61 EB patients at three time points during a period of ~2 years. All *S. aureus* isolates were typed by multiple-locus variable number of tandem repeats analysis (MLVA) and *spa* typing. This revealed major fluctuations in the *S. aureus* types sampled from individual EB patients. In addition, blood samples were obtained from 13 EB patients to determine their IgG levels against 43 virulence factors or whole cells of *S. aureus*. Overall, the sera of EB patients contained higher anti-staphylococcal IgG levels than those of healthy individuals. Specifically, this applied to IgGs against nine important virulence factors, including the superantigens (SAgs) staphylococcal enterotoxin M (SEM), SEN, and SEO. Notably, EB patients carrying different *S. aureus* types contained higher levels of anti-staphylococcal antibodies than EB patients colonized by only one type.

Abbreviations: EB, epidermolysis bullosa; ET, exfoliative toxin; HlgB, gamma-hemolysin B; IsaA, immunodominant antigen A; Isd, iron-responsive surface determinant; Luk, leukocidin; LytM, peptidoglycan hydrolase; MFI, median fluorescence intensity; MLVA, multiple-locus variable number of tandem repeats analysis; Nuc, endonuclease; SAgs, superantigens; SasG, S. aureus surface protein G; SCIN, staphylococcal complement inhibitor; SE, staphylococcal enterotoxin

Our findings suggest that the immune system of EB patients is heavily challenged with *S. aureus* antigens.

EB is a genetic blistering disease that renders patients susceptible to colonization by the opportunistic pathogen *Staphylococcus aureus* (Brandling-Bennett and Morel, 2010; Graber *et al.*, 2011; Pope *et al.*, 2012). Recently, we observed that all EB patients with chronic wounds, and 75% of patients without chronic wounds, were colonized with *S. aureus* (van der Kooi-Pol *et al.*, 2012). In contrast, only ~30% of the healthy human population carries this pathogen (Wertheim *et al.*, 2005). Persistent *S. aureus* carriers have an increased risk for staphylococcal infections but, compared with noncarriers, their risk of death due to bacteremia is lower (Wertheim *et al.*, 2004). This may relate to increased levels of protective anti-staphylococcal antibodies upon long-term exposure to colonizing strains (Kolata *et al.*, 2011). Furthermore, anti-staphylococcal antibody levels were shown to increase strongly during bacteremia (Verkaik *et al.*, 2010; Kolata *et al.*, 2011). As high exposure to *S. aureus* is a potential health risk for EB patients, our present studies were first aimed at defining their *S. aureus* population over time and, second, at determining their anti-staphylococcal IgG levels.

On the basis of informed consent, 61 EB patients from the Dutch Epidermolysis Bullosa Registry were included in our studies (Supplementary Methods online). *S. aureus* colonization was determined in three rounds of sampling at half-yearly intervals. In each round, swabs were collected from three wounds, the left and right anterior nares, and the throat. A total of 43 EB patients participated in the second sampling round, 40 in the third, and 35 patients participated in all three sampling rounds. Overall, we identified 101 different *S. aureus* types by molecular typing with MLVA (Supplementary Methods online and Supplementary Table S1 online). Only 18 of these MLVA types were encountered in all rounds (Figure 1a). One hundred and eighteen strains were also *spa* typed, revealing 48 different *spa* types (Supplementary Table S1 online). Next, we compared the variations in

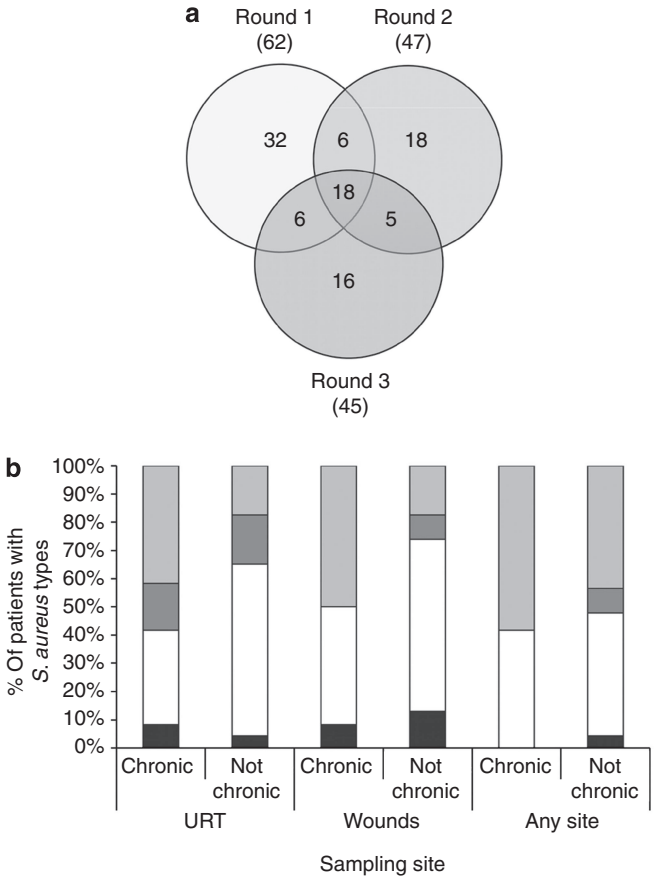

Figure 1. *S. aureus* multiple-locus variable number of tandem repeats analysis (MLVA) types identified in epidermolysis bullosa (EB) patients over a period of ~2 years. (**a**) Summary of the number of different MLVA types identified in three rounds of sampling. (**b**) Changes in the *S. aureus* MLVA types isolated from 35 EB patients in three rounds of sampling. The MLVA typing results were analyzed for individual EB patients with chronic wounds ($n=12$) or without chronic wounds ($n=23$). Black bars, percentage of patients not carrying *S. aureus*; white bars, percentage of patients colonized by the same MLVA type in all three sampling rounds; dark gray bars, percentage of patients colonized by different types in all three sampling rounds; light gray bars, percentage of patients with alternating MLVA types. URT, upper respiratory tract.

S. aureus types isolated from individual EB patients over time. This revealed that the same MLVA type was identified on ~42.5% of all sampled patients with minor variations for different sites of sampling (Figure 1b). Furthermore, 58.3% of the patients with chronic wounds and 43.5% of the patients without chronic wounds carried alternating *S. aureus* MLVA types over time. In 8.7% of the patients without chronic wounds, a different MLVA type was encountered in each sampling round. These findings show that the included EB patients are continuously challenged by different *S. aureus* types and that the carried *S. aureus* population can change rapidly. This seems to challenge the classical dogma that persistent carriers are mainly colonized by one *S. aureus* type (Wertheim *et al.*, 2005). However, our studies specifically address a patient group that is highly susceptible to *S. aureus* owing to continuous skin defects, which is different from the situation in healthy individuals.

To assess the anti-staphylococcal IgG levels in EB patients, we first performed whole-cell ELISAs using an *S. aureus* mutant lacking the IgG-binding proteins Spa and Sbi, and IgGs isolated from patients with chronic wounds. Sera from healthy donors were used as controls. This revealed that EB patient sera contained significantly higher anti-staphylococcal IgG levels than the controls (Supplementary Figure S1 online). To determine specific responses, the levels

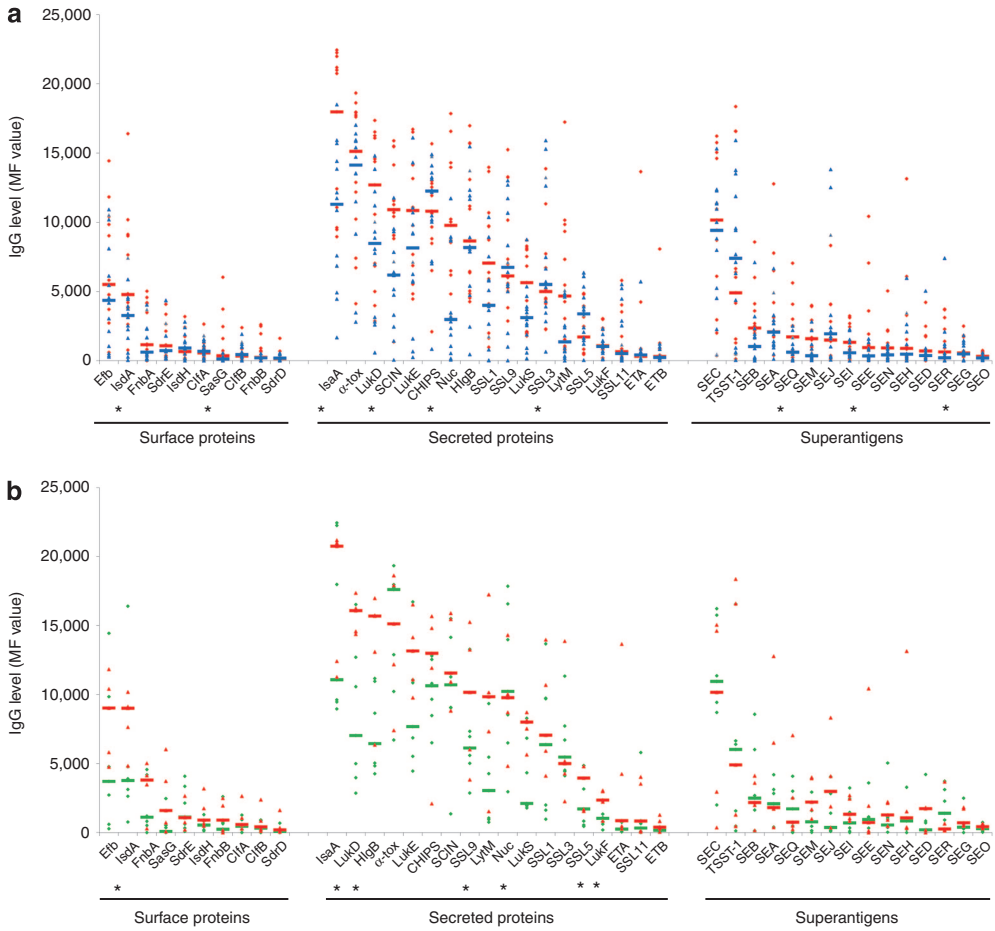

Figure 2. IgG responses of epidermolysis bullosa (EB) patients to staphylococcal antigens. (**a**) IgG levels against 43 purified *S. aureus* antigens in sera of EB patients (red diamonds; $n = 13$) or age-matched healthy controls (blue triangles; $n = 14$) were determined by Luminex assays. Median fluorescence intensity (MFI) values as indicated by color-coded bars (EB patients, red; healthy controls, blue) reflect the levels of antigen-specific IgGs. (**b**) IgG levels against 43 *S. aureus* antigens in sera of EB patients colonized by multiple multiple-locus variable number of tandem repeats analysis (MLVA) types (red triangles; $n = 5$), or EB patients colonized by only one MLVA type (green diamonds; $n = 7$) were determined by Luminex assays. The respective MFI values are indicated by red and green bars.

of serum IgGs from 13 EB patients against 43 purified *S. aureus* antigens were measured using Luminex technology (Figure 2). As controls, the sera from 14 age-matched healthy individuals were used. For most antigens, the median fluorescence intensities (MFI) were higher in EB patients than in the control group. In particular, the MFI levels for IgGs against the surface proteins IsdA (iron-responsive surface determinant A) and SasG (*S. aureus* surface protein G), the secreted proteins IsaA (immunodominant antigen A), SCIN (staphylococcal complement inhibitor), Nuc (endonuclease), and LytM (peptidoglycan hydrolase), and the SAgs SEM, SEN, and SEO were statistically significantly higher in EB patients. The increased IgG levels against IsaA, SCIN, Nuc, and LytM could be explained by the fact that these proteins are expressed by many *S. aureus* types (Ziebandt *et al.*, 2010). In addition, the *egc* gene cluster–encoded SAgs SEM, SEN, and SEO are among the most prevalent SAgs of *S. aureus* (52–66%) (Holtfreter *et al.*, 2007). Intriguingly, persistent carriers, bacteremia patients, and furunculosis patients were found to develop no, or only low levels of, antibodies against these SAgs (Grumann *et al.*, 2011; Holtfreter *et al.*, 2011; Burian *et al.*, 2012). This suggests that EB patients are more significantly challenged by *egc* SAgs than healthy carriers and bacteremia or furunculosis patients.

To determine whether carriage of multiple *S. aureus* strains has an impact on anti-staphylococcal IgG levels, we compared patients colonized by one MLVA type ($n = 7$) with patients colonized by multiple MLVA types ($n = 5$). Interestingly, the highest MFI levels were observed for IgG's from patients carrying multiple MLVA types. This was particularly evident for IgGs against IsdA, LukD (leukocidin D), HlgB (gamma-hemolysin B), LytM, LukS, LukF, and ETA (exfoliative toxin A) (Figure 2b). Notably, the incidence of LukS/F is very low, and thus, conceivably, the respective Luminex signals represent cross-reactive IgGs against the more common HlgA/B or LukE/D proteins (Gravet *et al.*, 1998). A significant correlation between anti-staphylococcal IgG levels in serum, wound fluid, and sterile blister fluid was revealed in samples from one EB patient

(Supplementary Figure S2 online). Here, the largest difference concerned 4-fold lowered anti-IsaA levels in wound fluid. This implies that future studies on anti-staphylococcal immune responses in EB patients can be based on noninvasively sampled wound fluid.

In conclusion, EB patients are highly challenged with very diverse *S. aureus* types, and carriage of multiple *S. aureus* types seems to elicit the highest humoral responses in these patients. However, we cannot exclude the alternative possibility of increased humoral and reduced cell-mediated immunity in EB patients, which might have an impact on *S. aureus* carriage. Notably, EB patients do not frequently suffer from *S. aureus* bacteremia, and none of the patients who donated blood was treated for staphylococcal bacteremia in the 5 years before blood donation. This suggests that their high anti-staphylococcal antibody titers may be protective against invasive *S. aureus* infections, which would be consistent with the protective effects of IsaA-specific antibodies in mice (Lorenz *et al.*, 2011).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

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Keratin Intracellular Concentration Revisited: Implications for Keratin Function in Surface Epithelia

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TO THE EDITOR

In 1978, Sun and Green carried out a densitometry-based analysis of PAGE samples and reported that keratin proteins account for 25–35% of total cellular proteins in human epidermal keratinocytes serially passaged in primary culture. This represents an astounding figure with very significant implications for the structural support role of keratin intermediate filaments in epidermis, and its regulation by post-translational modifications and/or interaction with associated proteins. We report here on an effort to investigate