Linezolid is Superior to Vancomycin in Experimental Pneumonia Caused by Superantigen-Producing *Staphylococcus aureus* in HLA class II Transgenic Mice

**Running Title:** Linezolid in experimental staphylococcal pneumonia

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Superantigens (SAg), the potent activators of the immune system, are important determinants of *Staphylococcus aureus* virulence and pathogenicity. Superior response to SAg in human leukocyte antigen (HLA)-DR3 transgenic mice rendered them more susceptible than C57BL/6 mice to pneumonia caused by SAg-producing strains of *S. aureus*. Linezolid, a bacterial protein synthesis inhibitor, was superior to vancomycin in inhibiting SAg production by *S. aureus in vitro* and conferred greater protection from pneumonia caused by SAg-producing staphylococci.
The pathogenicity and virulence of *Staphylococcus aureus* are determined by several exotoxins and the superantigens (SAg) are one such family of exotoxins. SAg are the most powerful biological activators of T lymphocytes and other cells of the immune system (9). Through this property, SAg divert the immune response against the bacterium, thereby helping in bacterial immune evasion (5, 15). At the same time, massive immune activation caused by SAg is by itself pathogenic. Higher prevalence of many exotoxins, including the SAg, in community-associated methicillin-resistant *S. aureus* (CA-MRSA) strains may facilitate infection of healthy individuals (4, 8, 10-12, 19).

Considering these factors, antibacterials such as linezolid, that inhibit staphylococcal exotoxin (including the SAg) synthesis, may be advantageous over bactericidal agents in treating infections caused by toxigenic *S. aureus*. While some murine studies have supported this hypothesis, others do not (1, 2, 14). Lack of considerable benefit with linezolid over vancomycin in mouse models of *S. aureus* infection has raised uncertainties about the potential benefits of linezolid in humans (6). Considering the enormous differences in the sensitivities of humans and conventional laboratory mice to SAg [conventional mice are believed to be $10^{11}$ times more resistant to SAg than humans, (10)], we hypothesized that the benefits of linezolid or similar antibacterial agents do not become apparent in conventional mice. On the contrary, these agents might in fact be useful in humans. Since transgenic mice expressing HLA class II molecules respond robustly to SAg similar to humans (barring certain species-level differences such as absence of emetic response in mice), they are more susceptible to *S. aureus* and *Streptococcus pyogenes* (which also produces SAg) infections than...
conventional mice (13, 16, 17). Therefore, we evaluated the activities of linezolid and vancomycin, particularly their abilities to inhibit SAg production, and compared their effectiveness in pneumonia induced by toxigenic *S. aureus* strains, using HLA class II transgenic mice.

In support of the divergent response between conventional and HLA class II transgenic mice to SAg, splenocytes from HLA-DR3 transgenic mice responded more robustly to a purified staphylococcal SAg, staphylococcal enterotoxin B (SEB) than splenocytes from B6 mice (Fig 1A). In addition, culture supernatants from a clinical *S. aureus* isolate capable of producing SAg, IDRL-7971, induced a more robust proliferation in splenocytes from HLA-DR3 transgenic mice over B6 mice (Fig 1B). Moreover, at the same inoculum size, IDRL-7971 was more lethal to HLA-DR3 transgenic mice than to B6 mice (Fig 1C, p<0.02). Sera from HLA-DR3 transgenic mice infected with IDRL-7971 had about 4-times higher levels of IL-2 than B6 mice (Fig 1D). Overall, this set of data strongly supports the involvement of SAg in the immunopathogenesis of *S. aureus*-induced pneumonia and confirms the higher susceptibility of HLA-DR3 transgenic mice to toxigenic *S. aureus* than B6 mice.

To further demonstrate that SAg play a role in the pathogenesis of pneumonia, we used isogenic strains of *S. aureus* that either do not express SAg or express only SEB, both a generous gift from Prof. Richard Novick, New York University Medical Center, New York, NY (18). Briefly, the *S. aureus* strain RN6734, containing the intact cloned SEB, pRN5543 :: seb (pRN7114), referred to as SEB+ strain, or RN6734, containing a derivative with a large 3' deletion in SEB, pRN5543 :: seb(b.2) (pRN7116), referred to as SEB- strain, were grown in media supplemented with chloramphenicol. When tested by
ELISA, culture supernatants from the SEB+ strain were strongly positive for SEB alone, whereas cultures supernatants from the SEB- strain did not contain SEA through E (Fig 2A). We next challenged HLA-DR3 transgenic mice with these bacterial strains. As expected, the SEB+ strain was more pathogenic and induced higher mortality when compared to the SEB- strain (Fig 2B), further strengthening the notion that SAg play a role in the pathogenesis of \textit{S. aureus} pneumonia and that HLA-DR3 transgenic mice are ideal models.

We next compared the activities of linezolid and vancomycin in inhibiting the production of SAg. For this the SEB+ and SEB- \textit{S. aureus} strains described above were grown overnight in TSB in the presence or absence of linezolid or vancomycin (both at 1 μg/ml, sub-MIC). The culture supernatants were tested in an \textit{in vitro} proliferation assay using splenocytes from HLA-DR3 transgenic mice. Only the culture supernatants from SEB+ strain were able to induce strong proliferation, whereas the culture supernatants from SEB- strain induced little or no proliferation (Fig 3A). These results not only confirm the absence of SEB or other SAg in SEB- strain, but also highlight that other bacterial products that might be present in the spent medium are not mitogenic to splenocytes, further underscoring the specificity of this bioassay. Culture supernatants from SEB+ strain grown in the presence of linezolid induced less proliferation compared to supernatants from the same strain grown in the presence of vancomycin indicating better inhibition of SAg by linezolid. Greater inhibition of SAg production with linezolid was also obtained with the toxigenic clinical \textit{S. aureus} isolate, IDRL-7971 (Fig 3B, C and D).
To rule out the possibility that a reduction in SAg-induced splenocyte proliferation in the presence of linezolid (as in Figs 3A and 3B) was due to a direct inhibitory effect of linezolid on splenocytes or toxicity of linezolid to lymphocytes, splenocytes from HLA-DR3 transgenic mice were cultured with a fixed concentration of purified SEB in the presence or absence of serial 2-fold dilutions of bacterial culture medium (TSB) or linezolid or vancomycin. Addition of linezolid did not directly suppress SEB-induced splenocyte proliferation suggesting that linezolid does not have a direct suppressive/toxic effect on lymphocytes (Fig 4).

Finally, we compared the activities of linezolid and vancomycin in pneumonia induced by IRDL-7971 in HLA-DR3 transgenic mice using a previously used dosing regimen (20). As shown in Fig 5, linezolid conferred significant protection over vancomycin (p=0.0002, n=10-14 mice/group) and untreated mice (p=0.0004, n=8-10 mice/group). Surprisingly, vancomycin failed to confer significant protection from lethal pneumonia (p = 0.50, n = 8-14 mice/group). Serum cytokine analyses revealed that compared to untreated and vancomycin-treated mice infected with IDRL-7971, linezolid-treated HLA-DR3 transgenic mice infected with IDRL-7971, had significantly lower levels of IL-2, IL-6 and the chemokine, KC (Fig 6). These results suggest that linezolid played a protective role by attenuating the production of SAg in vivo.

Overall, these results support that SAg contribute to bacterial pathogenesis in vivo and that linezolid is superior to vancomycin in treating S. aureus-induced pneumonia probably because of its ability to suppress SAg production. Taking into consideration the published reports that vancomycin has poor bioavailability in the lungs and exhibits
delayed action (3, 7), linezolid might be more effective than vancomycin in humans in
treating *S. aureus* pneumonia.
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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests. Pfizer, Inc had no role in the design, interpretation and publication of the data.
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Figure 1. HLA-DR3 transgenic mice respond more robustly to staphylococcal SAg and are highly susceptible to S. aureus-induced pneumonia than C57Bl/6 mice. (A) Splenocytes from HLA-DR3 and B6 mice were cultured with the indicated concentrations of SEB and the extent of T cell proliferation was determined by standard thymidine incorporation. (B) S. aureus isolate IDRL-7971, known to produce SEB, was cultured in bacterial medium. After overnight culture, the supernatants (sup) were spun, filter sterilized and added to splenocytes from HLA-DR3 or B6 mice in serial dilutions. T cell proliferation was determined as in panel A. (C) Experimental pneumonia was induced in age-matched HLA-DR3 and C57BL/6 mice by intratracheal inoculation of IDRL-7971 (1x10⁸ cfu/mouse) in a final volume of 50 µl. Mice were closely monitored, moribund animals were removed and euthanized as per IACUC recommendations. HLA-DR3 transgenic mice were more susceptible than B6 mice (p<0.02) (n=8-10 mice/group) (D) Mice infected as in panel C were bled at 6 h post-infection and the serum levels of IL-2 was determined (n=4-6 mice/group).

Figure 2. Pathogenic role of superantigens in S. aureus pneumonia. S. aureus strain RN6734, containing the intact cloned SEB, pRN5543::seb, pRN7114 (SEB+), or a derivative with a large 3’ deletion of SEB, pRN5543::seb(b.2), pRN7116 (SEB-) were grown in TSB supplemented with chloramphenicol (20 µg/ml). (A) Supernatants from these strains were subjected to ELISA to detect SAg (3M™ Tecra™ Staph Enterotoxin Visual Immunoassay, 3M, Minneapolis, MN). (B) Experimental pneumonia was induced in age-matched HLA-DR3 with these two S. aureus strains (1x10⁸ cfu/mouse) in a final
volume of 50 µl. Animals were monitored closely, moribund animals were removed and euthanized as per IACUC recommendations.

Figure 3. More efficient inhibition of SAg production by linezolid. SEB+, SEB- S. aureus strains described in Fig 2 or IDRL-7971 were cultured in bacterial medium (TSB) with or without the indicated amounts of linezolid or vancomycin. After overnight culture, the supernatants (sup) were spun, filter sterilized and added to splenocytes from HLA-DR3 transgenic mice. Splenocyte proliferation was determined by thymidine incorporation. Inclusion of linezolid reduced the splenocyte proliferation to a greater extent than vancomycin. This suggested that linezolid more efficiently inhibited SAg production by the SEB+ S. aureus strain (A) as well as by IDRL-7971 (B). (C) Mean percent inhibition obtained from 3-5 independent experiments as in panel B. (D) Presence of SAg in the IDRL-7971 culture supernatants were determined using 3M™ Tecra™ Staph Enterotoxin Visual Immunoassay.

Figure 4. Linezolid does not possess direct immunomodulatory activity. Splenocytes from HLA-DR3 transgenic mice were cultured with purified SEB (1 µg/ml) alone in RPMI or SEB (1 µg/ml) along with indicated serial dilutions of linezolid (starting concentration, 1 µg/ml), vancomycin (starting concentration, 1 µg/ml) or bacterial medium (TSB). Splenocyte proliferation was determined by thymidine incorporation assay.
Figure 5. Superior protection by linezolid over vancomycin from *S. aureus*-induced pneumonia in HLA-DR3 transgenic mice. Experimental pneumonia was induced in age-matched HLA-DR3 transgenic mice with IDRL-7971. Mice were left untreated or treated with vancomycin or linezolid 30 min before infection and every 12 hrs thereafter till 72 hrs. Antibiotics were administered at 200 and 150 mg/kg for linezolid and vancomycin, respectively. Mice were monitored for mortality. Linezolid versus vancomycin - p=0.0002, n=10-14 mice/group; Linezolid versus untreated mice - p=0.0004, n=8-10 mice/group; vancomycin versus untreated – p=0.50, n=8-14 mice/group). Plotting of survival curves and the statistical significance of the results were determined using the software GraphPad Prism (version 5.0d; San Diego, CA).

Figure 6. Linezolid treatment more efficiently attenuates systemic cytokine/chemokine response in HLA-DR3 with *S. aureus* pneumonia than vancomycin. Experimental pneumonia was induced in age-matched HLA-DR3 transgenic mice with IDRL-7971. Mice were left untreated or treated with vancomycin or linezolid as in Fig 5. Six hours after infection, mice were bled and serum levels of indicated cytokines/chemokine were determined by multiplex assay (Bio-Rad). Each bar represents data from 4-6 mice/group. * p<0.05 linezolid treated group compared to vancomycin treated group. No Abx – no antibiotics, Linezo – linezolid, Vanco – vancomycin.