SERUM AND BLISTER-FLUID ELEVATION AND DECREASED EPIDERMAL CONTENT OF HMGB1 PROTEIN IN DRUG-INDUCED STEVENS JOHNSON SYNDROME/TOXIC EPIDERMAL NECROLYSIS

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SHORT TITLE: HMGB1 expression in SJS/TEN sera and skin

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CONFLICTS OF INTEREST

The authors report no conflicts of interest relating to the work.

WHAT'S ALREADY KNOWN?

High-mobility group box 1 (HMGB1) is a marker of both tissue injury and innate immune response. It's elevation in serum of patients with serious cutaneous adverse drug reactions has been reported previously but to date no analysis of its distribution in Stevens-Johnson syndrome / toxic epidermal necrolysis (SJS/TEN) patient blister-fluid and skin biopsy tissue has been described.

WHAT DOES THIS STUDY ADD?

This study confirms serum HMGB1 elevation in SJS/TEN in 3 independent cohorts. Additionally, it reports, for the first time: a) super-elevation of HMGB1 in blister-fluid and b) significantly decreased epidermal expression in pre-blistered SJS/TEN skin with suprabasal retention. This is highly distinct from healthy or maculopapular exanthem skin and may represent a viable diagnostic tool.

ABSTRACT (250 WORDS)

High-mobility group box 1 (HMGB1) is a damage-associated molecular-pattern protein indicative of cell/tissue injury and innate immune response. Stevens-Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) are serious, immune-mediated skin-blistering conditions characterised by widespread keratinocyte death and epidermal detachment.

The purpose of the study was to determine: a) serum and/or blister-fluid total HMGB1 levels in independent SJS/TEN cohorts: and b) HMGB1 expression in formalin-fixed, paraffin-embedded SJS/TEN skin vs. healthy and maculopapular exanthema (MPE).

Total serum HMGB1 was quantified by enzyme-linked immunosorbent assay (ELISA) in 3 cohorts: i) Malawian, nevirapine-induced hypersensitivity (51 cases, 102 tolerant); ii) Taiwanese SJS/TEN (n=73) (acute, maximal and recovery stage); iii) Spanish SJS/TEN (n=23) (acute reaction (blister-fluid (n=13)). FFPE skin (5 healthy, 7 maculopapular exanthema (MPE), 7 SJS/TEN) was immunohistochemically (IHC) stained and semi-quantitatively assessed for HMGB1 expression.

Serum total HMGB1 was not significantly elevated in nevirapine-induced SJS/TEN (3.98ng/ml±2.17), MPE (3.92ng/ml±2.75) or DRESS (4.73ng/ml±3.00)) patients vs. tolerant controls (2.97ng/ml±3.00). HMGB1 was significantly elevated in Taiwanese SJS/TEN patients, highest during the acute phase 32.6ng/ml±26.6 vs. maximal (19.7ng/ml±23.2; $p=0.007$) and recovery (24.6ng/ml±25.3; $p=0.027$) phases. In blister fluid from Spanish SJS/TEN patients, HMGB1 (486.8ng/ml±687.9) was significantly higher than in serum (8.8ng/ml±7.6; $p<0.0005$). Pre-blistered SJS/TEN skin demonstrated decreased epidermal nuclear HMGB1 expression in upper epidermis vs. healthy or MPE skin but retained basal/suprabasal expression.

Epidermal HMGB1 expression was decreased in SJS/TEN skin which may account for elevated serum and blister-fluid levels. Retained basal/suprabasal epidermal HMGB1 expression, from resident keratinocytes/ infiltrating inflammatory cells, may exacerbate localised injury in SJS/TEN, though further evaluation is required.

INTRODUCTION

Stevens-Johnson syndrome (SJS)/ toxic epidermal necrolysis (TEN) is characterised by blistering of the skin and mucosal membranes with significant areas of skin detachment (up to 10% for SJS and >30% for TEN). Though rare (6.52 cases per million patient years [1]), SJS/TEN can have a severe impact on patients with a significant mortality rate and many suffering from long-term debilitating sequelae, including blindness. In addition, SJS/TEN can be a resource burden on healthcare systems with the average hospital stay estimated at 7-12.6 days of which 1.7-4.9 days are spent in intensive treatment units [2].

Drugs are amongst the commonest causes of SJS/TEN. A number of predictive genetic markers for drug specific SJS/TEN have been identified [3]. Several serum protein markers are elevated in SJS/TEN patients at the time of the reaction and have the potential to act as prognostic or diagnostic markers. These include granulysin [4], Fas ligand [5], and more recently interleukin 15 [6]. However, Nakajima *et al* [7] suggested that while granulysin and Fas ligand are possible candidate biomarkers, the duration of elevation is limited and therefore false negative results for SJS/TEN are a possibility.

High Mobility Group Box-1 (HMGB1) is an example of a Damage Associated Molecular Pattern molecule (DAMP) which is critical in linking cell death to inflammation and in the progression of disease. HMGB1 sits at the intersection between infectious and sterile inflammation. It is actively released in an acetylated form from activated immune cells and passively released in the non-acetylated form during necrotic cell death [8]. Previous studies have shown that total HMGB1 is significantly elevated in serum from patients with drug reaction with eosinophilia and systemic symptoms (DRESS) [9] and drug-induced SJS [7], at the time of the reaction and may discriminate between serious

cutaneous drug eruptions and milder phenotypes (MPE).

The aim of this study was to assess total HMGB1 levels in serum samples from 3 independent cohorts of SJS/TEN patients, as well as from blister fluid in a sub-set of patients. We have also evaluated HMGB1 expression levels and distribution in formalin-fixed, paraffin embedded skin biopsy samples from SJS/TEN patients.

PATIENTS AND METHODS

Samples from 3 independent SJS/TEN patient cohorts were brought together for the purpose of this study:

Nevirapine Patient Cohort

Patients were prospectively recruited as previously described [10] after informed consent. The study was approved by the research ethics committees of the College of Medicine, Malawi, and Liverpool School of Tropical Medicine. Briefly, the study recruited 1117 antiretroviral-naive HIV-positive patients from the Queen Elizabeth Central Hospital, Blantyre, Malawi, between March 2007 and December 2008. All were self-reported black-African, over the age of 16, had no baseline jaundice, and gave informed consent. All patients were diagnosed as HIV clinical stage 3/4 or had a CD4+ count <250 cells/μL and were treated with fixed dose NVP, lamivudine and stavudine with follow up over 26 weeks. CD4+ counts and liver function test were monitored at 0, 6, 14 and 18 weeks. A total of 51 patients presented with NVP-induced cutaneous hypersensitivity fulfilling the criteria of one or more of the following phenotypes:

- Maculopapular exanthema (MPE) with no systemic manifestations and improvement only on stopping treatment.

- Drug reaction eosinophilia with systemic symptoms (DRESS): widespread rash with systemic manifestations (i.e. fever, cough or abnormal liver function tests (LFTs)).
- SJS: extensive rash, affecting ≥2 mucous membranes or blistering eruptions affecting <10% of body surface area.
- TEN: blistering rash affecting >30% of body surface area and ≥2 mucous membranes. Patients with 10-30% affected body surface area had the overlap syndrome.

Each of the 51 hypersensitivity cases were age and gender matched to 2 NVP-tolerant HIV-positive controls (n=102). Blood samples were taken from patients prior to commencement of NVP treatment (0 weeks) and then at 2, 6, 10, 14, 18 and 20 weeks. In patients where a hypersensitivity reaction occurred, a sample was taken at the time of the first presentation (acute reaction) and no further sample taken thereafter as the patient was discontinued from the study. For comparison to adverse reaction samples, tolerant control samples at the 2 week time-point were used. Serum was isolated and frozen at -80°C. The purpose of analysing this cohort was to determine variability in serum HMGB1 levels between different types of cutaneous adverse drug reactions during the acute phase compared to tolerant controls.

Taiwanese SJS/TEN Cohort

A total of 73 Han Chinese patients with SJS/TEN (Supplementary Table 1) were enrolled from 2009 to 2015 at Chang Gung Memorial Hospital in Taiwan that received referrals from other hospitals nationally (including National Taiwan University Hospital, Taichung Veterans General Hospital, National Cheng Kung University Hospital, Kaohsiung Medical University, and Chung-Ho Memorial Hospital). SJS/TEN was characterized by rapidly developing blistering exanthema with purpuric macules, accompanied by mucosal

involvement and skin detachment. Diagnosis of SJS/TEN had to be assessed with its phenotypes based on the criteria of the RegiSCAR study group [11-13]. Each enrolled subject gave written informed consent, which had been approved by the institutional review board (IRB) and ethics committee of our CGMH based on Taiwan law (IRB No.97-0509B, No.100-4657A3, No.103-2562C, and No.105-3600C). Patients' serum samples during the acute, maximum, and recovery stages of SJS/TEN were obtained and frozen at -80 °C. The time to onset of the illness in each individual patient was also evaluated. The "acute" stage was defined as <6 days from onset of illness; the "maximum" stage was defined as the time from onset to maximal skin detachment (without progression of skin detachment); the "recovery" stage was defined as the time to complete skin healing. The purpose of analysing this cohort was to determine serum HMGB1 during the acute phase of SJS/TEN but also at the maximal point of the reaction and during the recovery phase (both of which were determined retrospectively).

Spanish SJS/TEN and DRESS cohort

Cases (n=23) diagnosed with SJS, TEN, SJS/TEN or DRESS/SJS/TEN overlap were identified in different hospitals in Madrid (Spain) belonging to the PIELenRed consortium (Supplementary Table 2). SJS/TEN was characterized purpuric macules and or target-like lesions, accompanied by mucosal involvement and skin detachment. Patients were classified according to the consensus criteria [14]. DRESS was diagnosed according to the RegiSCAR scoring system [15] Patient serum and blister fluid samples were obtained at first presentation (acute) and frozen at -80°C. The study was approved by the Research Ethics Committee of Príncipe de Asturias University Hospital (the coordinating center of the PielenRed Consortium). Patients or their legal representatives gave written informed

consent. The purpose of analyzing this cohort was to compare HMGB1 levels during the acute phase of SJS/TEN in both serum and blister-fluid.

Formalin-fixed paraffin-embedded skin biopsy tissue samples.

FFPE skin samples (5 healthy controls, 7 drug-induced rash and 7 SJS/TEN) were identified from the histology archive database at Cleveland Clinic, Ohio, USA, from 2013-16 (accessed January 2017). An internal diagnosis or description search included the terms "Stevens-Johnson Syndrome" or "toxic epidermal necrolysis" and drug eruption/ drug reaction (including "dermal hypersensitivity reaction"). Specific cases were selected where a diagnosis of drug-induced SJS/TEN or maculopapular exanthem was very strongly favoured and was supported by clinical notes. For normal healthy control samples, normal skin from excision specimens were utilised. Suspected causal drugs were identified from clinical notes.

Serum HMGB1 Measurement

All serum samples were transferred to the Wolfson Centre for Personalised Medicine, University of Liverpool, UK for analysis. Total HMGB1 protein concentrations in serum and blister fluid (where available) were determined by ELISA according to the manufacturer's protocol (Oxford Biosystems, UK). Spectrophotometric analysis at 450nm was undertaken using a DTX880 Plate Reader (Beckman Coulter Inc., High Wycombe, UK). Blister fluid samples were analysed iteratively, firstly neat and then at 30-fold and 300-fold dilution in PBS until quantifiable within the detection range of the assay. Sample measurements failing to reach the manufacturer's lower limit of quantification (0.2ng/ml) or where replicates were discordant by >15% were excluded.

HMGB1 Immunohistochemistry

For immunohistochemistry (IHC), sections were dewaxed and subjected to antigen retrieval in Dako PT buffer high pH (Agilent Technologies Ltd, UK) using a computer controlled antigen retrieval workstation (PT Link; Agilent Technologies Ltd, UK) for 20 min at 98°C. Sections were then stained in an automated immunostainer (Link 48; Agilent Technologies Ltd, UK), using a primary antibody against human HMGB1 (Rabbit polyclonal, ab18256, Abcam Ltd, Cambridge, UK,; 1 in 1000) in a 1 h incubation at room temperature (RT). This was followed by a 30 min incubation at RT with the polymer peroxidase-based detection system (Anti Mouse/Rabbit Envision Flex+, Agilent Technologies Ltd, UK). The reaction was visualised with diaminobenzidine (Agilent Technologies Ltd, UK). Consecutive sections incubated with non-immune rabbit serum served as negative controls. The positive reaction was represented by a distinct brown nuclear (or rarely cytoplasmic) reaction. Positive control was represented by epidermis and follicle in normal skin.

Semi-quantitative analysis of immunohistochemical HMGB1 expression intensity was undertaken by a pathologist with respect to distribution within the epidermal layers but also to nuclear staining as follows: Negative stain (-) was defined as absence of specific brown stain. Minimal stain (+/-) was judged as barely visible specific brown stain. Moderate stain (+) was judged as clear evident staining which was milder compared to the intensity of the keratinocytes of the normal skin. Strong stain (++) was of an intensity compared with the keratinocytes of the normal skin (positive control). Stain intensity category (- ; +/- ; + ; ++) was allocated according to the most represented pattern present in the biopsy available on the slide.

Statistical Analysis

Statistical analysis was undertaken using Prism 5 software (GraphPad Inc.) and RStudio version 1.1.414. To analyse the difference in HMGB1 levels between serum and blister fluid in the Spanish cohort and difference in HMGB1 levels with time in the Taiwanese cohort, linear mixed models were fitted using the *lmer* function in R package *lme4*. This approach allowed for more than one measurement from the same individual. For the Spanish cohort, HMGB1 levels were log-transformed to ensure model residuals were normally distributed. Comparison of data between unpaired sample groups (i.e. between hypersensitivity phenotypes) was undertaken using a Kruskal-Wallis test. A threshold cut-off, based on the recently reported 97.5% quantile reference ranges [16], was used to classify samples as elevated (>2.3ng/ml). Analysis of the resulting binary outcome between phenotype groups was by Chi-squared test. To adjust for multiple testing, the Bonferroni approach was undertaken, adjusting the p-value threshold for nominal significance, 0.05, by the number of tests. Since not all outcomes tested are independent (e.g. combined cutaneous hypersensitivity phenotype and the specific hypersensitivity reaction phenotype groups), we believe this to be a conservative approach.

RESULTS

Serum and blister fluid HMGB1 concentrations.

Nevirapine-induced cases and controls: Of the initially identified 51 nevirapine cutaneous hypersensitivity cases and 102 tolerant controls, sera from 40 cases (22 MPE, 9 DRESS, 9 SJS/TEN) and 70 controls at time of reaction were available for analysis of total HMGB1 (Figure 1). Using the Kruskal-Wallis test, we found that mean concentrations (±SD) of total HMGB1 in each of the 3 nevirapine-induced cutaneous

phenotypes (MPE: 3.92±2.75ng/ml, DRESS: 5.25±2.65ng/ml SJS/TEN: 3.98±2.17 ng/ml, and combined (4.17±2.81ng/ml) were higher (though not significantly) than those in the tolerant control group (2.97ng/ml±3.00; P>0.05) (Figure 1A).

Using the Chi-squared test, we found that the percentage of individuals with elevated levels above our defined upper limit of normal (ULN) (>2.3ng/ml) was higher in the combined cutaneous hypersensitivity (75%, p=0.002), MPE (72%, p=0.027), DRESS (89%, p=0.029) and SJS/TEN (78%, p=0.042) compared to the tolerant group (46%). Only the combined cutaneous hypersensitivity phenotype remained significant after correction for multiple testing (p<0.0125).

Taiwanese SJS/TEN patients: Serum from 73 Taiwanese SJS/TEN patients was analysed for HMGB1 (Figure 2). This included 73 acute reaction samples, 59 samples at time of maximal reaction and 66 samples from the recovery phase. The mean total serum HMGB1 (±SD) during the acute phase of the reaction was 32.6±26.6ng/ml which was higher than at both the maximal reaction time (19.7 ±23.2ng/ml; p=0.0002) and recovery (24.6ng/ml ±25.3; p=0.0003). Both these differences were statistically significant after applying Bonferroni adjustment (p-value threshold 0.017 for three pairwise comparisons). No statistically significant difference was observed between maximal reaction and recovery.

Spanish SJS/TEN patients: The mean serum total HMGB1 (n=22) in the Spanish SJS/TEN cohort (Figure 3) was 8.8 ±7.6ng/ml. Blister fluid levels (n=13) were significantly higher at 486.8ng/ml±687.9 (p<0.001) than serum levels. In the 12 individuals where samples from both sites were available, there was no correlation between matched serum and blister fluid HMGB1 levels (R^2=0.036) (data not shown).

Skin tissue expression of HMGB1.

Immunohistochemical staining of FFPE skin sections showed that, compared to healthy control and MPE skin, SJS/TEN patients exhibited significantly less nuclear expression of total HMGB1 in the epidermis of non-blistered SJS/TEN skin (Figure 4). Indeed, expression appeared to be limited to the basal/suprabasal layer (Figure 5), an observation that was consistent in all SJS/TEN individuals regardless of the site of biopsy. (Table 1). Prominent HMGB1 expression was also observed in infiltrating inflammatory cells in the skin from both MPE and SJS/TEN patients.

DISCUSSION

The data from this study show that total serum HMGB1 levels are elevated in patients with SJS/TEN, with levels being higher in blister fluid than in paired serum samples. Furthermore, our data highlight the changes occurring in lesional skin from SJS/TEN patients with HMGB1 expression being seen in the basal/suprabasal layer, but was reduced overall in the epidermis. However, the elevation in serum HMGB1 may be confounded by underlying disease as the effect in Malawian patients with advanced HIV disease was absent when compared with non-HIV patients.

In our nevirapine hypersensitivity cohort from Malawi, although HMGB1 levels at the time of reaction in SJS/TEN and DRESS patients were elevated, they were not significantly higher than those in both tolerant and MPE patients (Figure 1A). Creation of a binary outcome with a threshold cut-off (>2.3ng/ml) (Figure 1B) provided some indication that HMGB1 may be able to discriminate between phenotypes to a small degree, but this is unlikely to be useful clinically. By contrast, in HIV-negative patients with SJS/TEN from both Taiwan and Spain, serum HMGB1 levels were significantly

elevated, with levels in the Spanish cohort (8.8ng/ml) similar to that previously observed in Japanese SJS/TEN patients at the time of the reaction [7,9]. The HMGB1 concentrations were much higher in Taiwanese patients (36.2ng/ml; Figure 2) than in the Spanish patients; the reasons for this unclear, but given the small sample sizes, it is perhaps best not to over-interpret the differences as these may be due to sample collection and storage differences rather than true biological variability. One variable that can be discounted is inconsistency in the SJS/TEN phenotype definition since both the Spanish and Taiwanese cohorts utilised the same criteria (RegiSCAR), while in the nevirapine cohort from Malawi, the diagnostic criteria are closely aligned. It is important to note that the 3 independent cohorts studied were brought together retrospectively, from studies of differing design, to address different questions relating to HMGB1 elevation. This limits our ability to assess differences between the different cohorts.

The different results obtained in HIV-positive patients from Malawi compared with the HIV-negative patients from Taiwan and Spain may be related to the confounding effect of HIV infection. Our Malawian patients were at an advanced stage of HIV infection (HIV clinical stage 3/4 and/or had a CD4+ count <250 cells/μL). HIV-infection has previously been shown to cause elevated HMGB1 serum levels [17], while HMGB1 itself may enhance HIV replication [18]. By contrast, HMGB1 levels tend to be reduced by antiretroviral therapy (ART), albeit long-term therapy [19], but with little data available over the short-term particularly in those at more advanced stages of HIV disease [20]. Given the confounding effects of HIV disease and concomitant ART, it is perhaps not surprising that the total HMGB1 levels were lower than those seen in the Taiwanese and Spanish patients, and did not sufficiently distinguish between control, MPE and SJS/TEN

patients. Indeed, a significant 46% of tolerant controls exhibited HMGB1 serum levels above our nominal ULN (>2.3ng/ml) which further highlights the potential influence of co-medication and co-morbidity on serum HMGB1 levels. It is also important to acknowledge that elevation in serum HMGB1 is not specific to cutaneous hypersensitivity reactions, and has been observed in HIV[17], epilepsy [21] and a number of other inflammatory and autoimmune diseases[22].

Serum HMGB1 levels in Taiwanese SJS/TEN patients were significantly higher during the acute phase of the reaction than at both the maximal and recovery phases (Figure 2), suggesting activation of this pathway at the time of presentation indicative of tissue damage, before treatment (symptomatic and specific) is given that may lead to alterations in levels. Interestingly, for the first time, we have shown that blister fluid levels of HMGB1 in SJS/TEN patients were 55-fold higher than in serum indicating that voiding of HMGB1 from the keratinocytes in the epidermis is likely to contribute to the significantly elevated blister fluid levels (Figure 3).

Our data from skin biopsies showed the previously unreported observation of decreased expression of nuclear HMGB1 in the epidermis of pre-blistered SJS/TEN skin coupled with the expression of HMGB1 in the basal and suprabasal layers (Figure 5). Although this observation has been made in a small sample (n=7) of biopsies from SJS/TEN patients, the consistency of the finding across all patients (Table1) would suggest that it is a true observation. Keratinocyte death is likely to explain the decrease in epidermal expression, and subsequent increased levels of circulating serum total HMGB1. However, given that, histologically SJS/TEN is characterised by separation of the epidermis and dermis, the role of HMGB1 retained at the suprabasal layer warrants

further investigation. The dramatic difference between epidermal distribution of HMGB1 in SJS/TEN compared to control and MPE skin makes IHC analysis of HMGB1 a potential diagnostic marker for SJS/TEN, but this would require further investigation. Interestingly, our data also suggest that keratinocyte release of HMGB1 in SJS/TEN skin may occur prior to epidermal detachment (Figure 4) and thus HMGB1 serum elevation may actually precede it, raising the possibility that it may represent a good early marker of cell death/tissue damage in SJS/TEN. Again, this will need further study.

Our study is limited by the fact that we have measured total HMGB1 but not its post-translationally modified HMGB1 acetylated isoform. In its hyper-acetylated form, HMGB1 can be actively secreted from multiple cell types (macrophages, NK cells, dendritic cells for example) as part of an innate immune response [23]. Additionally, in its un-acetylated form, it can be passively secreted from necrotic or damaged cells with both mechanisms leading to significantly elevated extracellular HMGB1 levels [24]. Given the activation of the innate immune response in SJS/TEN [25], activated macrophages are likely to be a source of HMGB1 (in its acetylated form) [26]. It is entirely plausible that elevation of serum un-acetylated HMGB1 (passive released from dying keratinocytes) may differentiate between SJS/TEN patients and individuals with milder phenotypes (MPE and DRESS) where widespread cell/tissue injury is not observed. This will require careful assessment of the HMGB1 isoforms, which we plan to undertake in the near future through the development of a new assay.

Our data suggest that, unlike granulysin and IL15 [6], total HMGB1 serum levels do not correlate with SJS/TEN severity scores (SCORTEN) (data not shown). This coupled with the observation that levels remain high even in the recovery phase (Figure 2) suggests

that its utility as a prognostic marker is likely to be limited. Furthermore, confounding by other concomitant conditions such as HIV infection may lead to changes in HMGB1 levels which may be difficult to interpret in relation to the occurrence of SJS/TEN. However, it is important to note that HMGB1 is associated with different physiological functions related to the innate immune response and dependent on its redox state [26,27]. Consequently, HMGB1 may be important in the pathogenesis of the keratinocyte damage seen in SJS/TEN, exacerbated by the infiltration of activated innate and adaptive immune cells, and the subsequent localized release of cytotoxic proteins, including granulysin to augment keratinocyte injury. This is perhaps suggested by our skin biopsy analyses, but will require further work to fully understand the role of HMGB1 in the pathogenesis of SJS/TEN.

In conclusion, this study has shown that HMGB1 is elevated in patients with SJS/TEN, with levels being higher in blister fluid than in serum, but this may not be the case in all patient groups, particularly where the underlying concomitant disease (for example advanced HIV disease as in our Malawian patients) and its treatment can affect HMGB1 levels. Our data from skin biopsies also, for the first time, demonstrate that HMGB1 expression is depleted in the epidermis of SJS/TEN patients, with some HMGB1 being retained in the basal layer, in proximity to the epidermal/dermal junction, providing suggestive evidence of a putative role for HMGB1 in the pathogenesis of epidermal separation, although further work will need to be undertaken to elucidate this.

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TABLES

Table 1. Semi-quantitative analysis of HMGB1 IHC staining in healthy, MPE and SJS/TEN skin biopsy sections from the Cleveland Clinic archival cohort. N.P. denotes not present in the biopsy section analysed. Intensity: (-)=negative; (-/+) =minimal; (+) = moderate; (++)=strong.

					IHC HMGB1 Expression				
Phenotype	**Biopsy Site**	**Age**	**Gender**	**Causal Drug**	**Epidermis**	**Nuclear stain within Epidermis**	**Follicle/ Adnexae**	**Dermis**	**Inflammatory Infiltrate**
Healthy	Thigh	23	F	n/a	++	all layers	++	+/-	N.P.
Healthy	Forehead	19	M	n/a	++	all layers	++	+	N.P.
Healthy	Arm	61	F	n/a	++	all layers	++	+/-	N.P.
Healthy	Back	58	F	n/a	++	all layers	++	+/-	N.P.
Healthy	Inferior Breast	40	F	n/a	++	all layers	N.P.	+	N.P.
MPE	Arm	81	F	vancomycin	+	all layers	++	+	++
MPE	Arm	61	M	meropenem	++	all layers	++	+/-	++
MPE	Arm	59	F	simvastatin	++	all layers	++	+/-	++
MPE	Arm	22	M	apiprazole	++	all layers	++	+/-	++
MPE	Scalp	78	F	undetermined	++	all layers	++	+/-	++
MPE	Abdomen	59	F	levetiracetam	++	all layers	++	+	++
MPE	Abdomen	62	M	piperacillin /tazobactam	++	all layers	++	+	++
SJS	Back	62	M	ceftaroline	+/-	Basal/Suprabasal layer	++	+/-	++
SJS	Arm	51	M	vancomycin	+/-	Basal/Suprabasal layer	N.P.	+/-	++
SJS	Lip	23	F	sulfamethoxazole/ trimethoprim	+/-	Basal/Suprabasal layer	N.P.	+	++
SJS/TEN	Thigh	54	M	vancomycin	+/-	Basal/Suprabasal layer	++	+/-	++
SJS/TEN	Abdomen	39	F	piperacillin/ tazobactam	+/-	Basal/Suprabasal layer	++	+/-	++
TEN	Arm	24	F	ketorolac	+/-	Basal/Suprabasal layer	+/-	-	++
TEN	Chest	28	F	sulfamethoxazole/ trimethoprim	+/-	Basal/Suprabasal layer	++	+/-	++

FIGURE LEGENDS

Figure 1. Serum total HMGB1 concentration in the Malawian, nevirapine-exposed HIV cohort at time of reaction. **A)** represents concentration (ng/ml) for different cutaneous hypersensitivity phenotypes (2 weeks after drug initiation for tolerant). The dashed line indicates the defined ULN (2.3ng/ml). **B)** shows the number of individuals whose serum concentration was above the ULN (black bars) or below the ULN (grey bars). Statistical significance, determined by Kruskal-Wallis test (A) and Chi-squared (B) is indicated by * ($p<0.05$), **($p<0.01$).

Figure 2. Total HMGB1 in serum from the Taiwanese SJS/TEN cohort during the acute phase of the reaction; maximal point of reaction and during the recovery phase. The dashed line indicates the notional ULN (2.3ng/ml). Statistical significance, determined by linear mixed modelling is indicated by *** $p<0.001$).

Figure 3. Total HMGB1 in serum and blister fluid from the Spanish SJS/TEN cohort at time of reaction. The dashed line indicates the notional ULN (2.3ng/ml). Statistical significance, determined by linear mixed modelling is indicated by *** ($p<0.001$). Circles represent SJS/TEN cases and triangles DRESS/TEN overlap.

Figure 4. Immunohistochemical staining of HMGB1 in healthy, maculopapular exanthema and SJS/TEN skin. (100X magnification).

Figure 5. HMGB1 staining at the epidermal basal/suprabasal layer in healthy, maculopapular exanthema and SJS/TEN skin. Black arrow indicates the stratum basale; white arrow the stratum spinosum and arrowhead the inflammatory infiltrate. The dashed line indicates the dermal/epidermal junction. (400X magnification).