nature portfolio

Peer Review File

Multi-omic single-cell sequencing defines tissue-specific responses in Stevens-Johnson Syndrome and Toxic epidermal necrolysis

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In order to analyze the immunopathogenesis of SJS/TEN, the authors have performed single-cell transcriptomics (scRNA41 seq), surface protein (scCITE-seq), and TCR-sequencing of cells across unaffected skin, affected skin, and blister fluid from 17 patients with SJS/TEN. This identifies 16 different cell subtypes in lesions and blister fluid with a predominance of conventional CD8+ T cells characterized by TCR αβ clonotypes and a cytotoxic phenotype. Identification of clonal CD8+ T cells expressing two different CDR3α rearrangements suggested a pathogenic role of dual-specific T cells in these drug reactions. Myeloid cell subsets had a proinflammatory phenotype. Analysis of paired samples from unaffected and affected skin and blister fluid in one patient revealed a similar expansion of dominantly expanded TCRs among the top 50 clonotypes.

The study deals with a relevant issue. To this end, it analyzed the infiltrating cell populations by single cell analysis. Overall, it confirms the clonality of CD8+ T cells as dominant effector cells in SJS/TEN, which was already demonstrated in former studies on the same issue, and it extends the analysis to the functional phenotype of the infiltrating CD8+ T cells.

In principle, the methodological approach is correct. Nevertheless, I have some comments.

Because of the rarity of TEN, the collection of the samples is a major the challenge, that was solved here, while the single cell analysis itself is based on now established techniques. The study involves 17 patients with SJS/TEN. These are clinical phenotypes with varying degrees of severity. SJS/TEN are associated with certain HLA-clade I alleles. The patients were heterogeneous regarding culprit drug and genetic race. Therefore, the significance of the data would benefit from a differentiation of the results according to subtype/diagnosis (SJS/TEN - although the authors state that information on diseases severity is missing), HLA class I haplotype and culprit drug. The need for this is underlined by the fact that Figure 1D shows inhomogeneous distributions of cell subtypes between patients. It would be important to know whether the different composition of the cellular infiltrates might

depend on different culprit drugs, HLA-I haplotype or the disease type.

Figure 2 refers to the top 50 TCR clonotypes in the paired samples. Compared to various T-cell mediated autoimmune diseases with a few pathogenic T cell clones this is quite a huge number. It would be interesting to know how many different clonotypes were found in each patient, potentially in relation the HLA-class I haplotype and culprit drug. How can this huge number of T cell clones be interpreted?

It would be correct to discuss the data with the extensive clonality analyses performed in the study by Villani et al., Massive clonal expansion of polycytotoxic skin and blood CD8+ T cells in patients with toxic epidermal necrolysis. Sci Adv . 2021 Mar 19;7(12):eabe0013. doi: 10.1126/sciadv.abe0013.

Reviewer #2 (Remarks to the Author):

In this manuscript, Gibson A and colleagues elegantly characterise the immunological responses of skin lesions during SJS/TEN, which remains poorly understood. The study is well designed, and the data provided are compelling and exciting. I would like to congratulate the authors for such an interesting study. I hope that my comments help the authors to improve their manuscript and that they find them useful.

- I find it surprising that the UMAP in figure 1 is not dominated by stromal cells (e.g., keratinocytes, fibroblasts, hair follicle-associated cells), as these are highly abundant in the skin (Cheng JB, et al. Cell Reports, 2018; Zou Z, et al. Dev cell, 2020; Deng CC, et al. Nat comms, 2021; Stabell AR, et al, Cell reports, 2023; just to name a few). The stroma/immune cell population ratio does not seem correct, especially when healthy donors were included. I could not find any reference in the methods section to determine whether the authors performed cell enrichment (e.g., CD45+ cells) prior to single cell sequencing. Can the authors comment on this?
- I think Figure 1Ai and 1Aii would benefit from having figure 1C immediately after. It makes the comparisons easier to visualise. Once again, is it not surprising to see so many immune cells and not stromal cells in healthy skin? Figure 1D shows that ~25-30% of the cells in

healthy skin are stromal cells (fibroblasts, mesenchymal cells, and keratinocytes) but that is not immediately obvious in the UMAPs in figure 1C. Can the authors clarify or comment on these discrepancies, if any?

- The percentages in figure 1Aii are a bit confusing. How were these percentages calculated? What do they refer to (e.g., % cells within the subcluster)? It'd be also important to refer to this in 48-50. How do the proportions of these various immune subsets change in the single cell dataset across conditions and biopsy type?
- Can the authors comment on the nature of the γδ T cell populations in their dataset? Did the authors detect any CD163l, IL17, or CD27 within these cells? Are these Vγ1, Vγ6, DETCs?
- Do the T cell subsets in skin and blister fluids express residency markers? Or are these likely to be recruited?
- Lines 56-58: The authors should provide reference to support their selection of M1 and M2 markers. As far as I can tell, CD163+ macrophages are typically assigned as perivascular macrophages and can be either M1 or M2 depending on the microenvironment. It would be useful to discuss these points further in the results and discussions section.
- In figure 2Aii, which gene pathways are downregulated in keratinocytes? Would it be possible that these are associated with immunomodulation that is then switched off in SJS/TEN? In other words, can keratinocytes be setting a basal level of tolerance in the skin controlling CD8+ T cell activation? These can be speculated based on the nature of the gene pathways that are downregulated.
- Along the same lines, can the authors perform imaging (e.g., IFA, RNAscope) on tissue biopsies to determine if there is an accumulation of CD8+ T cells in the different layers of the skin and in proximity to keratinocytes. Perhaps adding a keratinocyte marker and HLA-I alongside markers of CD8 T cell activation (e.g., those detected by scCITE-seq) would be really informative to support the notion that keratinocytes are likely activating resident CD8 T cells.
- Although the results presented in figure 2 are compelling and exciting, I wonder how generalisable this concept is as it was only explored in one patient. I appreciate you see the same TCR in three different locations, but, how consistent are these clones in other patients? Addressing this would be important and really informative to determine if this response is likely to be driven by the same type of antigen(s). Can the authors comment?
- What do the authors think is the biological relevance of finding polyclonal CD8+ T cell

subsets in the blister fluid but not on the affected skin, discussed in lines 164-169? Are the authors arguing in favour of “clonal bias” in the skin due to chronic/persistent antigen stimulation? This could be clarified in the text. Also, please add a reference to the relevant figure/table.

- I struggled to understand the data provided in Figure 2Civ and v. What are we supposed to take from the circus plots provided? This needs to be clarified in the manuscript.

- The dataset provided in this study is quite impressive, but I think the authors have not extended their analysis enough to do it justice. One key question I wonder is whether the authors could apply cell-cell communication tools to identify (and potentially validate) pathways supporting key biological features of the disease, especially given that so little is known about it. For instance, what pathways define the communication between keratinocytes (and other stromal cells) and CD8+ T cells? One could argue that these sorts of predictions can provide novel biological insights into the pathways driving this response, beyond HLA-I signalling. Along the same lines, are there other cell types predicted to establish significant interactions with CD8+ T cells in the context of disease?

- I think the authors should use the discussion section to expand on some of the hypotheses derived from the transcriptomics analyses. For instance, would it be appropriate to propose keratinocytes as key modulators of T cell responses in skin? How important the stroma could in the context of SJS/TEN? Are they ways in which these data could informed future therapies or even diagnostics to predict these unwanted reactions? How about the role of macrophages? Is this likely to be modulated by CD163+ perivascular macrophages? Do these subsets express HLA-I too? There are a lot of exciting observations in this study but somehow these are not discussed in detail, or what this all means in the context of these diseases? I think this manuscript would benefit from a visual schematic depicting the key findings, e.g., skin diagram showing CD8+ T cells and keratinocytes interactions, and which pathways are overrepresented in disease? The cell-cell communication analyses could inform this.

Minor comments:

- The data provided throughout this manuscript are stunning, but the figures are perhaps a bit too cluttered. Having subsections within subsections in the same figure makes it difficult to appreciate the key message. I’d advise the authors to revisit their figures to split them

into 3 or 4 separate figures (instead of just 2 figures). I think it'll help declutter the images a bit and will most certainly help with the overall flow of the manuscript. Also, some of the text in the images is too small and it is hard to read. For instance, extended figure 3ii, extended figure 4, etc.

- Please add reference in line 96 to support this statement
- Please add reference to GLIPH2 in line 127
- Line 172-173, please check if the journal allows this sort of stamen and if not, please add this information as supplementary information.
- Line 186, please add relevant literature to support this statement.
- Please add the q values to the GO terms in figure 1Eiii. The cut-off for selection is not mentioned in the figure or the legend.
- I'm unsure as to what the different bars refer to in Figure 2Dii. For example, what is "Top TCR+", "1 Ct TCR+, "C2 TCR+", etc. This needs to be clearer either in the manuscript or the figure legend, or both.

Reviewer #3 (Remarks to the Author):

Gibson et al investigate immune responses of inflammatory skin lesions from Stevens-Johnson syndrome patients. They focus on CD8+ T cells, which are the major population of immune cells present in affected skin and blister fluid. Comparison to normal skin and burn blisters make for robust controls. Single cell RNA seq analysis revealed significant expansion of CD8+ TCR clones with enhanced effector profiles in affected skin and blisters. Overall the experiments are well performed, controlled and conclusions are accurate. However, improvements could be made to the writing and overall structure of the manuscript.

Specific comments

1. The introduction is very short for an article style publication and lacks important information required for the broad readership of Nat comms. Which drugs induce Stevens-Johnson syndrome and when are they used? How is Stevens-Johnson syndrome treated and what improvements are needed?
2. The multipart figures (especially 2C i, ii, iii, iv) are difficult to follow and detract from the significance of the results. I would suggest splitting figures 1 and 2 into 2 different figures

each. This would also give some more room for down regulated genes to be labelled on each of the volcano plots (2a and 2c).

Reviewer #4 (Remarks to the Author):

The authors are presenting a resource, single cell transcriptomes of cells + TCR profiles from blister fluid derived from 17 patients with SJS/TEN, and one skin biopsy from SJS/TEN patient, plus 2 individual controls: one healthy skin sample (from discarded edges post-surgery), one blister fluid from a burn patient. The analysis demonstrates that:

• Cytotoxic T cells are enriched at the site of SJS/TEN damage.

• A cytotoxic CD8+ subpopulation expresses the same expanded TCRαβ clonotypes in affected skin (n=1) and blister fluids (n=17) but not in unaffected skin (n=1).

• Expanded clonotypes were enriched for markers indicative of terminally-differentiated effectors driving antigen-specific response.

• Expanded oligoclonal and unexpanded polyclonal CD8+ T-cells are present in the blister fluid of SJS/TEN.

The study documents a unique resource of 119,784 transcriptomes + TCR sequences, which could be of significant benefit/interest to the field. However, the actual findings are mainly confirmatory of other studies, e.g: https://www.science.org/doi/10.1126/sciadv.abe0013, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7496676/, demonstrating the importance of CD8 T cells for SjS/TEN and the CD8 T cell clonotype expansion. It would be important to distinguish which of the reported findings are truly novel and highlight it throughout the text.

Detailed comments:

While the study presents a unique resource, there are several methodological aspects which need consideration/ correction.

1. Authors report, that their cut-off threshold for mitochondrial gene content was 50%. That value is extremely high for immune cells and suggests high level of apoptosis. That might be

specific to the disease but might be caused by the banking procedure and the use of cells from cryobank. Without a comparison with appropriate fresh controls, it is impossible to infer which is the cause. Additionally, Authors should provide QC plots, demonstrating distribution of the mitochondrial/ribosomal/doublets pre and post filtering in the supplementary material, as for such a resource, the quality of the data is of paramount importance.

2. The study reports results of 17 patients with SJS/TEN. It is unlikely, that all have been induced by the same medication, and it is critical to take it into account for analysis, given the different mechanisms by which drugs induce immune activation (b-lactams vs carbamazepine, requiring metabolism, vs large molecule drugs). Figure 1D demonstrates a wide spread of cellular composition, is it in any way related to patient characteristics (sex, age) or causative medication?

3. The resource includes two individual n=1 as controls (one blister fluid from burn patient, one healthy skin). Use of n= 1 is inappropriate as a comparison, and no conclusions should be drawn from it. Skin is not an appropriate control for blister fluid. Additionally, from the methods section, these were not cryobanked, but processed fresh, in contrast to the study samples, which might introduce artefactual differences. While it is understandable, that controls for this study would be difficult to obtain, studies in public domain, e.g. https://pubmed.ncbi.nlm.nih.gov/32344053/, could be used for comparison, to strengthen the argument.

4. The authors acknowledge “We could also not control all patient-specific factors such as severity and time since onset of symptoms.”, but they assert that the patients were in the acute phase of SJS/TEN. A table summarising clinical details, for individual patients, describing the differences in patient symptoms and the time from onset should be provided. The notion of “acute” disease is repeated throughout the manuscript, which requires a) adding a definition of acute used, and b) confirmation that patients conformed to that definition.

5. Line 197: “antigen-reactive polyclonal CD8+ T-cells also contribute to the effector

pathogenesis, and the total number and affinity of cytotoxic TCR may impact disease severity". It is an interesting notion, but can it be substantiated with data from the study? Does the number/affinity of TCR correlates with severity of the patient disease?

6. Skin dissociation method is unclear: Method section, lines 217:220 says tissue was processed into small cubes, and 230 says 4mm punch biopsies were thawed – which was it? How big were the cryopreserved fragments? It is also important to state the exact dissociation protocol, as it is not trivial for skin, and can have significant impact on the cell viability, cell composition, and scRNA-seq outcome. The section says biopsies from SJS/TEN patients, while presented results show n=1 for skin biopsy, is that a typo? Or were the results analysed for more that 1 patient?

Minor:

1. What do asterixes mean in Fig 1 D?

2. Fig 1 E: the significant 444 differentially expressed gene signature of CD8 Tconv cluster – From the authors description these are cluster marker genes, computed using Wilcoxon rank test vs all the other clusters not DEGs, computed for patient groups/study variables, and the nomenclature "cluster marker genes" should be used. For 1Eiii GO pathways: actual ranking missing, p/q values missing/not provided

3. Line 149: "To investigate whether T-cells with unexpanded clonotypes had a distinct 149 phenotype, we used blister fluid to increase the power for DEG analyses." – that sentence is difficult to understand, how can blister fluid be used to increase power of DEG analysis? Additionally, as in 2 above, no DEG analysis was performed, accordingly to method description.

4. Fig 2 – the title and the content does not provide data for polyclonal T cells, while a dedicated result section (line 143/173) expands on the importance of the polyclonal T cells.

5. Supplementary Fig 1 labels are upside down.

RESPONSE TO REFEREES

We would like to take the opportunity to thank the reviewers for their comments and suggestions, which have helped us to make extensive revisions that significantly improve the quality and clarity of the manuscript methods, results, outcomes, and impact of this study. In direct response to reviewer comments, our most significant changes include:

(i) A completely revised introduction to outline the current knowledge of disease immunopathogenesis and treatment strategies to demonstrate the timely and critical need for this study for unbiased signature analyses of cell populations at the site of tissue damage,

(ii) The presentation of available clinical data for all patients including culprit drug, age, sex, disease severity, and predisposing genetic (HLA) risk where known,

(iii) A larger control single-cell sequencing dataset of burn blister fluids to enable comparative signature analyses and the appropriately powered calculation of statistically enriched populations in SJS/TEN blister fluid,

(iv) Expanded analyses of keratinocyte signatures between unaffected and affected skin which propose a new hypothesis for the role of regulation and cell death mechanisms to enable the selective targeting of keratinocytes for cytotoxic death by CD8+ LAG3+ pathogenic subsets,

(v) Paired single-cell with spatial sequencing to show that the cytotoxic effector population has a signature of tissue-resident memory T-cells and line the epidermal-dermal boundary in the skin of affected patients supporting a direct interaction with keratinocytes,

(vi) New results section on the use of advanced bioinformatic cell-cell signaling and pathway analysis to identify interacting pathogenic populations and potential therapeutic targets for SJS/TEN, and

(vii) A new summary schematic to outline the model of disease proposed by this study and the shared common pathogenic cellular response.

We are extremely grateful and now include the revised manuscript and a specific reply to each of the reviewer's comments below to outline the changes made.

Reviewer #1 (Remarks to the Author):

In order to analyze the immunopathogenesis of SJS/TEN, the authors have performed single-cell transcriptomics (scRNA41 seq), surface protein (scCITE-seq), and TCR-sequencing of cells across unaffected skin, affected skin, and blister fluid from 17 patients with SJS/TEN. This identifies 16 different cell subtypes in lesions and blister fluid with a predominance of conventional CD8+ T cells characterized by TCR αβ clonotypes and a cytotoxic phenotype. Identification of clonal CD8+ T cells expressing two different CDR3α rearrangements suggested a pathogenic role of dual-specific T cells in these drug reactions. Myeloid cell subsets had a proinflammatory phenotype. Analysis of paired samples from unaffected and affected skin and blister fluid in one patient revealed a similar expansion of dominantly expanded TCRs among the top 50 clonotypes.

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In principle, the methodological approach is correct. Nevertheless, I have some comments.

<u>Comment 1.</u> Because of the rarity of TEN, the collection of the samples is a major challenge, that was solved here, while the single cell analysis itself is based on now established techniques. The study involves 17 patients with SJS/TEN. These are clinical phenotypes with varying degrees of severity. SJS/TEN are associated with certain HLA-clade I alleles. The patients were heterogeneous regarding culprit drug and genetic race. Therefore, the significance of the data would benefit from a differentiation of the results according to subtype/diagnosis (SJS/TEN - although the authors state that information on diseases severity is missing), HLA class I haplotype and culprit drug. The need for this is underlined by the fact that Figure 1D shows inhomogeneous distributions of cell subtypes between patients. It would be important to know whether the different composition of the cellular infiltrates might depend on different culprit drugs, HLA-I haplotype or the disease type.

Response to comment 1. *We thank the reviewer for their comment, which has allowed us to improve this paper and, more specifically, assess the culprit of drug-induced disease. As requested, we have now provided more information on the clinical reaction, including the suspected culprit drug, reaction sub-type/severity based on the body surface area detached (SJS, SJS/TEN overlap, or TEN), age, and sex of each patient, which we now provide in a new Extended Data Tabe 1. This table also includes the proportion of immune subsets and the putative HLA risk allele for that patient if previously described in the literature to be associated with the same causal drug. While this list is not complete, as for many drugs and populations, no HLA risk alleles have been yet associated; we also list the culprit drug for each patient in Fig 1D but also Extended Data Figure 11, which highlights that despite different drug and/or HLA associations and the private oligoclonal TCRαβ for each patient, these all occur with a common pathogenic signature of cytotoxic CD8+ T-cells. Further, we now also include a new Extended Data Figure 4 to demonstrate a lack of any detectable correlations between cell subset heterogeneity in each blister fluid sample in our dataset with the culprit drug or disease phenotype/severity (we have specifically highlighted 7 patients with cotrimoxazole-associated SJS/TEN). We also acknowledge that longitudinal studies with sequential sampling will be needed to shed light on cellular trajectories and proportions over time. Text changes are highlighted in this section and include:*

<u>Introduction, line 64 in clean doc:</u> 'Importantly, while patients varied in age, sex, culprit drug, and HLA genotype, single-cell analyses of the cellular response at the site of SJS/TEN tissue damage revealed common features across patients that were unrelated to these different predisposing factors'

<u>Results, line 98 in clean doc:</u> 'Patient samples varied in culprit drug and time since onset of reaction (Extended Data Table 1) and we theorized this might impact subset representation but were unable to detect any associations (Extended Data Fig. 4). However, we acknowledge we may have been underpowered to do so.'

<u>Results, line 248 in clean doc:</u> 'The identification of oligoclonal clonotypes in this patient was representative of CD8+ Tconv in blister fluid from all patients with SJS/TEN driven by different drugs (Extended Data Fig. 11A).'

<u>Results, line 255 in clean doc:</u> 'Notably, these three dominantly-expanded clonotypes were expressed in the same UMAP location, with the majority of those in affected skin (>97%) and blister fluid (>90%) aligned to the cytotoxic CD8+ Tconv cluster (Fig. 3Bii). This was also the predominant location of dominant TCR+ cells identified in all other patients (Extended Data Fig. 11B), indicating that the cytotoxic CD8+ Tconv cluster represents a common effector phenotype across patients with drug-induced SJS/TEN, even if they use private TCR CDR3αβ clonotypes.'

Discussion, line 386 in clean doc: 'Specifically, we observed the same cytotoxic CD8+ subpopulation expressing GNLY, GZMB, PRF1, LAG3, CD27, TIGIT, and LINC01871, but not IL7R or FASLG, in blister fluid and affected skin of patients independent of the causal drug or likely HLA risk allele.'

Discussion, line 458 in clean doc: 'Indeed, as the lymphocyte/monocyte ratio has been shown to change over time in samples obtained from a single patient with SJS/TEN[19], longitudinal single-cell studies will be required to define the dynamic progression of the disease, including the proposed transition of M1 and intermediate-like macrophages identified in this study towards populations associated with re-epithelization and repair (Fig. 6).'

Comment 2. Figure 2 refers to the top 50 TCR clonotypes in the paired samples. Compared to various T-cell mediated autoimmune diseases with a few pathogenic T cell clones this is quite a huge number. It would be interesting to know how many different clonotypes were found in each patient, potentially in relation the HLA-class I haplotype and culprit drug. How can this huge number of T cell clones be interpreted?

Response to comment 2. *As requested, we have included the drug and total number of clonotypes and cells in the cytotoxic CD8+ Tconv cluster for all patients in Extended Data Figure 11B. In the context of autoimmune disease, a distinct epitope is often known or proposed. However, for drug reactions, the exact drug-derived antigen and epitope stimulating T-cells remains unknown, and in vitro studies have shown that multiple immunogenic antigens may drive an immune response while only some of these may be relevant to disease manifestations. These neo-antigens may include diverse drug-modified peptides or an altered self-peptide repertoire binding to drug-modified HLA. To clarify this for the readership, we have included a further statement in the text and used the contrast to autoimmunity as an interesting discussion point to highlight our conclusion and the further utility of this atlas to define all relevant risk epitopes and HLA alleles in a single patient. Our model is that the drug neo-antigen stimulates several TCRs but that the expanded oligoclonal TCR clonotypes are those responsible for driving the majority of keratinocyte death. In contrast, we cannot be sure of the degree to which the polyclonal CD8+ T-cells also directly contribute to the effector pathogenesis, or the antigen stimulus, which will require further investigation. Text changes include:*

Discussion, line 466 in clean doc: 'We cannot be sure of the degree to which the polyclonal CD8+ T-cells also directly contribute to the effector pathogenesis, which will require further investigation. This more detailed understanding of the complete cytotoxic repertoire will be critical to defining cytotoxic responses in patients with different causal drugs and HLA risk alleles. In contrast to autoimmune diseases with few pathogenic epitopes and TCRs, the epitope(s) driving SJS/TEN remain undefined. Still, drugs have been shown to stimulate T-cells through diverse metabolites, drug-modified peptides, and by binding to HLA to alter the self-peptide repertoire[77].'

Comment 3. **It would be correct to discuss the data with the extensive clonality analyses performed in the study by Villani et al., Massive clonal expansion of polycytotoxic skin and blood CD8+ T cells in patients with toxic epidermal necrolysis. Sci Adv . 2021 Mar 19;7(12):eabe0013. doi: 10.1126/sciadv.abe0013.**

Response to comment 3. *We thank the reviewer for drawing our attention to this important publication. Our findings of a common cytotoxic effector phenotype with oligoclonal expansion of*

private TCR clonotypes, irrespective of risk drug or HLA allele restriction, are consistent with their findings. We reference the Villani paper in the text as follows:

Discussion, line 391 in clean doc: 'These data are consistent with a previously reported shared drug-expanded TCR+ polycytotoxic cluster defined across patients with varied drug causality and HLA risk alleles by Villani using a 16-marker flow[60]. Here, we now provide an unbiased analysis of this and interacting populations at single-cell resolution to define a shared CD8+ T_{RM}-like cytotoxic cluster with both expanded and unexpanded TCRαβ clonotypes (Fig. 6).'

Reviewer #2 (Remarks to the Author):

In this manuscript, Gibson A and colleagues elegantly characterise the immunological responses of skin lesions during SJS/TEN, which remains poorly understood. The study is well designed, and the data provided are compelling and exciting. I would like to congratulate the authors for such an interesting study. I hope that my comments help the authors to improve their manuscript and that they find them useful.

Comment 4. I find it surprising that the UMAP in figure 1 is not dominated by stromal cells (e.g., keratinocytes, fibroblasts, hair follicle-associated cells), as these are highly abundant in the skin (Cheng JB, et al. Cell Reports, 2018; Zou Z, et al. Dev cell, 2020; Deng CC, et al. Nat comms, 2021; Stabell AR, et al, Cell reports, 2023; just to name a few). The stroma/immune cell population ratio does not seem correct, especially when healthy donors were included. I could not find any reference in the methods section to determine whether the authors performed cell enrichment (e.g., CD45+ cells) prior to single cell sequencing. Can the authors comment on this?

Response to comment 4. *We thank the reviewer for this important feedback and for highlighting the need to discuss the differences in cellular subset proportions in blister fluid and skin. We discuss the methods of skin tissue digestion that led to the variable capture of stromal populations. The total UMAP is predominated by samples and cells of blister fluid. In line with previous studies, blister fluid provides an accessible, natural, single-cell, immune-rich suspension devoid of stromal subsets. In contrast, the enzymatic protocols required for the digestion of skin biopsies may result in a variable loss of stromal cells in the single-cell suspension. We did not perform any cell enrichment before sequencing. Therefore, we now emphasize that the enzymatic skin digestion protocol is associated with preferential lymphoid cell recovery. To clarify these points and stress the variable capture of stromal subsets from skin biopsies, we first define individual populations in Figure 1Aii to better highlight the specific capture of both immune and stromal populations from the outset. Second, we have included new statements and references in the text to confirm the expected lack of stromal subsets in blister fluid, but also that, although we capture stromal subsets in skin biopsies, enzymatic protocols required for the digestion of skin biopsies may result in a variable loss of stromal cells from run to run, and therefore, the relative proportion of stromal cells within all cells and samples cannot be relied upon. To support this, we include a new Extended Figure 5 to focus on the relative proportion of immune cell subsets within immune cells of skin biopsies and cite the comparative capture of immune and stromal subsets by the study suggested by the reviewer by Zou, 'A Single-Cell Transcriptomic Atlas of Human Skin Aging', as this best aligns with our use of skin punch biopsies. These data also highlight the known*

transcriptional similarity of mesenchymal stromal cells and fibroblasts at a single-cell level, which cluster in our UMAP, and we include an additional reference to this point. Text changes include:

Results, line 71 in the clean doc: 'The total scRNA-defined uniform manifold approximation and projection (UMAP, Fig. 1Ai) includes 109,888 cells, spanning 15 subtypes, including both immune and stromal subsets. Specifically, these include CD8+ and CD4+ T-cells, natural killer (NK) cells, hematopoietic stem cells (HSC), B-cells, monocytes, macrophages, dendritic cells (DC), mesenchymal stromal cells (MSC), fibroblasts, endothelial cells, and keratinocytes (Fig. 1Aii); with the predominant representation of each of these subsets aligning to the same UMAP clusters across disease and sample types (Extended Data Fig. 1).'

Results, line 107 in clean doc: 'Consistent with previous studies, CD14+ monocytes were the predominant subset in burn blister fluid[18], B-cells represented <0.5% of cells in SJS/TEN-affected skin and blister fluid[19], and SJS/TEN blister fluid was an immune-rich reservoir devoid of stromal subsets[19]. Further, while full-thickness skin biopsies also contained immune cells, they also consisted of diverse stromal populations[20], including phenotypically similar MSC and fibroblasts[21]. However, skin digestion methods may result in a variable loss of stromal cells from run to run, and therefore, the relative proportion of stromal cells within all cells cannot be relied upon. In contrast, immune cells are relatively robust, and we, therefore, focused on the relative proportion of immune subsets within the immune cells (Extended Data Fig. 5). These data identified similar proportions of CD4+ and CD8+ T-cells in unaffected skin from a patient with SJS/TEN and normal skin from an unrelated donor. While CD4+ T-cells were reduced in affected compared to unaffected SJS/TEN patient skin, the relative proportion of the predominant CD8+ T-cell population remained the same across healthy, unaffected, and affected biopsies.'

Comment 5. I think Figure 1Ai and 1Aii would benefit from having figure 1C immediately after. It makes the comparisons easier to visualise. Once again, is it not surprising to see so many immune cells and not stromal cells in healthy skin? Figure 1D shows that ~25-30% of the cells in healthy skin are stromal cells (fibroblasts, mesenchymal cells, and keratinocytes) but that is not immediately obvious in the UMAPs in figure 1C. Can the authors clarify or comment on these discrepancies, if any?

Response to comment 5. *The idea for this split was that we initially present the total combined UMAP to orientate the reader to the positioning of individual cell subsets, and then in the lower half of Figure 1, focus on the individual samples and sample types, which aligns with the flow of the manuscript, and we hope this is acceptable to keep in this same orientation. However, as above, we valued the reviewer's observations regarding stromal populations and now elaborate that the enzymatic protocols required for the digestion of skin biopsies may result in a variable loss of stromal cells from run to run, and the proportions of stromal subsets cannot be relied upon. We emphasize that the enzymatic digestion protocol is associated with preferential recovery of immune cells relative to stromal and other cells. We now clarify this in the text and include a new Extended Figure 5 to focus on the relative proportion of immune cell subsets within immune cells of skin biopsies, without stromal subsets, which may be proportionally misleading. Text changes include:*

Results, line 107 in clean doc: 'Consistent with previous studies, CD14+ monocytes were the predominant subset in burn blister fluid[18], B-cells represented <0.5% of cells in SJS/TEN-affected skin and blister fluid[19], and SJS/TEN blister fluid was an immune-rich reservoir devoid of stromal subsets[19]. Further, while full-thickness skin biopsies also contained immune cells, they also consisted of diverse

stromal populations[20], including phenotypically similar MSC and fibroblasts[21]. However, skin digestion methods may result in a variable loss of stromal cells from run to run, and therefore, the relative proportion of stromal cells within all cells cannot be relied upon. In contrast, immune cells are relatively robust, and we, therefore, focused on the relative proportion of immune subsets within the immune cells (Extended Data Fig. 5). These data identified similar proportions of CD4+ and CD8+ T-cells in unaffected skin from a patient with SJS/TEN and normal skin from an unrelated donor. While CD4+ T-cells were reduced in affected compared to unaffected SJS/TEN patient skin, the relative proportion of the predominant CD8+ T-cell population remained the same across healthy, unaffected, and affected biopsies.'

Discussion, line 451 in clean doc: 'Limitations of this study are that, unlike the blister fluid, the composition of skin is likely to be affected by enzymatic digestion with variable recovery of stromal cell populations. This may explain why the proportion of immune cells appeared to be increased in the normal control skin biopsy compared to unaffected SJS/TEN patient skin.'

Comment 6. The percentages in figure 1Aii are a bit confusing. How were these percentages calculated? What do they refer to (e.g., % cells within the subcluster)? It'd be also important to refer to this in 48-50. How do the proportions of these various immune subsets change in the single cell dataset across conditions and biopsy type?

Response to comment 6. *These values are the percentage of that particular subset represented within the indicated cluster, i.e., 98% of CD8+ T-cells reside within the dotted circle. Our intention was to help show the majority location of a single subset. We have now amended the figure legend to clarify this for the readership. We have added a new extended data figure (Extended data figure 1) to show the representation of key subsets across conditions and biopsy types, as suggested. This also similarly highlights the majority cluster of each subset, and we add comments to this extent in the text:*

Results, line 71 in the clean doc: '*The total scRNA-defined uniform manifold approximation and projection (UMAP, Fig. 1Ai) includes 109,888 cells, spanning 15 subtypes, including both immune and stromal subsets. Specifically, these include CD8+ and CD4+ T-cells, natural killer (NK) cells, hematopoietic stem cells (HSC), B-cells, monocytes, macrophages, dendritic cells (DC), mesenchymal stromal cells (MSC), fibroblasts, endothelial cells, and keratinocytes (Fig. 1Aii); with the predominant representation of each of these subsets aligning to the same UMAP clusters across disease and sample types (Extended Data Fig. 1).'*

Comment 7. Can the authors comment on the nature of the gd T cell populations in their dataset? Did the authors detect any CD163l, IL17, or CD27 within these cells? Are these Vg1, Vg6, DETCs?

Response to comment 7. *The gamma delta population represented very few cells and was underpowered for comparative analyses between paired unaffected and affected skin. Further, the scTCRseq pipeline used in this study enables paired sequencing of CDR3alpha and beta, but not gamma delta TCRs. However, we have looked further at transcripts detected by scRNAseq. The gamma delta population expressed diverse TRGV and TRGD. They did not express high CD163 or IL17, but did express high CD27; similar to Tconv. While to the best of our knowledge, there is no direct human equivalent of DETC identified in mice, in recent years, several subsets of gamma delta T-cells have been proposed in the antitumor setting. These align with the traditional Th classification system, which includes IFN-*

responding (γδT1) and IL17-responding subsets (γδT17), of particular interest in tissue including skin, and outlined by a recent nature article which we include as an additional reference. While too few cells were identified in skin for specific analyses, those in blister fluid did not express IL17-related genes, but CD27, IFITM1, and IFITM2 were highly expressed in line with Tconv suggesting a predominantly IFN-producing inflammatory subset, and we include a brief description in text and a new extended data figure 6. Text changes include:

Results, line 135 in clean doc: 'Interestingly, distinct γδ T-cell subsets have recently been characterized, including IFN- (γδT1) and IL-17-responding subsets (γδT17) of particular importance in skin[24]. In SJS/TEN blister fluid, γδ T-cells did not express IL-17-related genes but CD27, IFITM1 and IFITM2 were highly expressed as was seen in the CD8+ Tconv subset (Extended Data Fig. 6), suggesting co-stimulation of a limited IFN-responding γδT1-like subset.'

Comment 8. Do the T cell subsets in skin and blister fluids express residency markers? Or are these likely to be recruited?

Response to comment 8. *We thank the reviewer for raising this critical question, which we now address in a section in the text with its own heading. We also include a new part in Figure 2 to better describe the cytotoxic CD8+ Tconv cluster phenotype before more detailed signature analyses. Specifically, we now show that cells in the cytotoxic CD8+ cluster expressed genes associated with cell remodelling and/or proliferation (STMN1, TUBA1B, TUBB), but also RNA and protein for CD103 (ITGAE), CD49a (ITGA1), and CD69 (CLEC2C), indicative a population of locally-expanded cytotoxic CD8+ tissue-resident memory (T_{RM}) T-cells which are retained in the skin without the capacity to recirculate. Further, in line with a latter comment about using imaging to visualize the proximity of keratinocytes with CD8 T-cells in the affected skin, we now show immunohistochemistry and spatial transcriptomic sequencing, which confirm the close proximity of keratinocytes with cytotoxic CD8+ CD103+ (T_{RM}) populations at the site of separating epidermis in patients with SJS/TEN. These data are included as a new Extended Data Figure 7. Text changes include:*

Results, line 168 in clean doc: See entire section with sub-heading 'The cytotoxic CD8+ Tconv enriched in SJS/TEN affected skin and blister fluid are locally-proliferating CD8+ T_{RM} T-cells.'

Discussion, line 385 in clean doc: 'Using a multi-omic single-cell approach, we found clonally-expanded cytotoxic CD8+ T_{RM} T-cells at the site of SJS/TEN tissue damage.'

Discussion, line 409 in clean doc: 'Importantly, while pathway analyses propose an accessory role for stromal populations including fibroblasts in immune cross-talk and the culmination of an immunomodulatory microenvironment, our phenotypic, spatial, and cell communication analyses position the cytotoxic CD8+ TRM-like Tconv cluster at the basement membrane in direct contact with keratinocytes.'

Comment 9. Lines 56-58: The authors should provide reference to support their selection of M1 and M2 markers. As far as I can tell, CD163+ macrophages are typically assigned as perivascular macrophages and can be either M1 or M2 depending on the microenvironment. It would be useful to discuss these points further in the results and discussions section.

Response to comment 9. *We now include relevant references to justify our selection of classical M1 and M2 markers. CD163 is widely used as a classical surface marker for M2 macrophages, but as the reviewer correctly points out, it may also be present on intermediate-like macrophage populations, including perivascular macrophages. However, we propose that the upregulation of CD163 remains reflective of at least the early transition of M1 towards M2-like signatures, which aligns with the mixed surface phenotype. We agree that the specific phenotype relevant to the tissue-specific environment should be discussed. While dermal macrophage subsets are relatively newly described at a transcriptional level, we now discuss the known distinction of LYVEI+ and MHCII+ dermal subsets, whose function in literature aligns with the classical M1/M2 paradigm and pro-inflammation or wound repair, respectively. With the increased number of burn blister fluid samples in this revised manuscript, we have also identified the differential gene signature of macrophages in SJS/TEN blister fluid that aligns with inflammation and an extended intermediate-like signature presented in a new Extended Data Figure 3. Importantly, our pathway analyses in a new Figure 5 also implicate signaling in macrophages associated with M1 to M2 transition, and we now also add a further statement in the discussion to stress the need for longitudinal sampling to ascertain the dynamic progression of SJS/TEN and the development of M2-like macrophages involved in wound repair. Text changes include:*

<u>Results, line 84 in clean doc:</u> '*Macrophages expressed a mixed pro-inflammatory M1- (CD11c+) and anti-inflammatory M2 (CD11b+, CD163+)-like surface phenotype*[13,14]*. As tissue-specific macrophages are recently described, with distinct dermal lineages involved in inflammation or repair and defined by the expression of HLA class II or LYVE1, respectively*[15]*, presenting functional resemblances of the traditional M1/M2 classification, we further investigated the signature of these cells. Macrophages in SJS/TEN blister fluid were* $LYVE1^{LO}$ *HLA class* II^{HI}*, as typically seen in inflamed tissue. Indeed, compared to macrophages in burn blister fluid, those in SJS/TEN blister fluid were significantly enriched for HLA class I, HLA class II, STAT1, interferon-induced response (IFITM1-3), and C1Q involved in the phagocytosis of apoptotic cells (Extended Data Fig. 3). However, further transcriptomic analyses confirmed an extended intermediate-like macrophage signature (*$CD9^{LO}$*,* $CD163^{HI}$ $APOE^{LO}$ $APOC1^{HI}$*,* $MRC1^{HI}$*)*[16]*, supporting a mixed surface phenotype and subpopulations involved in inflammation and others with repair.'*

<u>Results, line 367 in clean doc</u>: *'…and macrophages upregulated PI3K and downregulated androgenic pathways (Figure 5Bi), previously associated with M1 to M2-like transition towards cell repair*[57]*. This is consistent with our earlier surface and transcriptomic phenotyping of an emerging intermediate-like macrophage population.'*

<u>Discussion, line 458 in clean doc</u>: *'Indeed, as the lymphocyte/monocyte ratio has been shown to change over time in samples obtained from a single patient with SJS/TEN*[19]*, longitudinal single-cell studies will be required to define the dynamic progression of the disease, including the proposed transition of M1 and intermediate-like macrophages identified in this study towards populations associated with re-epithelization and repair (Fig. 6).'*

<u>Comment 10.</u> In figure 2Aii, which gene pathways are downregulated in keratinocytes? Would it be possible that these are associated with immunomodulation that is then switched off in SJS/TEN? In other words, can keratinocytes be setting a basal level of tolerance in the skin controlling CD8+ T cell activation? These can be speculated based on the nature of the gene pathways that are downregulated.

Response to comment 10. *We agree that altered immune regulation will potentially play a role in the onset of disease, and we thank the reviewer for the suggestion to discuss this based on up- and downregulated gene signatures. First, we comment on the genes downregulated in keratinocytes, which match those similarly downregulated in keratinocytes during psoriasis, identifying initial disease similarities. However, second, as LAG3 was highly expressed on cytotoxic T-cells, we now provide additional text and data (Extended Data Figure 8) which proposes that keratinocytes (unlike other stromal subsets) in affected compared to paired unaffected skin downregulate the LAG3 ligand LGALS3, which leaves them susceptible to cell death by cytotoxic LAG3+ CD8 Tconv cells. These data suggest that keratinocytes and other stromal populations set a basal level of tolerance in the skin controlling HLA-restricted CD8+ T cell activation, as suggested by the reviewer, but the proposed downregulation of LGALS3 specific to keratinocytes enables selective targeting for cytotoxic CD8+ T-cell driven death during SJS/TEN. Importantly, LGALS3-LAG3 is previously described to confer cellular resistance to apoptosis, the LAG3-LGALS3 interaction is co-inhibitory to CD8+ T-cells, and LGALS3 is similarly downregulated in the lesional skin of patients with atopic dermatitis and psoriasis. Moreover, in psoriasis, a disease driven by auto-reactive LAG3+ CD8+ T-cells, LAG3+ cell-depleting antibody therapies have recently been shown to reduce inflammation and improve lesion severity and barrier integrity. Thus, we also include this as an additional main point in our discussion to highlight (i) why keratinocytes may be the specific target of cell death during SJS/TEN and (ii) that LAG3+ cell-depleting therapies may provide a novel therapeutic opportunity in SJS/TEN. Text changes include:*

Results, line 219 in clean doc: '*However, in keratinocytes, downregulated genes matched those similarly downregulated in keratinocytes during psoriasis (GADD45B, MT1X, DUSP1, ZFP36, MYC, BTG2)[41] including a regulatory circuit of chronic inflammation (CEBPD, ATF3)[42].*'

Results, line 226 in clean doc: 'Importantly, while HLA class I was also upregulated in macrophages, endothelial cells, fibroblasts, and MSC of the affected skin (Extended Data Fig. 8A), the checkpoint receptor LAG3 was highly upregulated in cells of the cytotoxic CD8+ Tconv cluster. Thus, as immune checkpoint receptors are known to regulate HLA-restricted drug-induced T-cell activation[43], we investigated whether decreased expression of LAG3 ligands (HLA class II, FGL1, CLEC4G, LGALS3) may identify disease-susceptible cell populations (Extended Data Fig. 8B). Notably, while CLEC4G and FGL1 were not expressed across samples, and HLA class II was high or upregulated in antigen-presenting cells of affected skin including fibroblasts and MSC, suggesting a role in immune regulation, LGALS3 was among the top 25 most downregulated genes by fold change in keratinocytes of affected skin, which was significant before false discovery rate (FDR) correction (Extended Data Fig. 8B). A protective role for LGALS3-LAG3 has been described to confer cellular resistance to apoptosis[44], and the LAG3-LGALS3 interaction is known to be co-inhibitory to CD8+ T-cells[45]. The expression of LGALS3 remained high across other stromal subsets in affected skin. These observations suggest that a decrease in the expression of LGALS3 may facilitate the selective targeting of keratinocytes for LAG3+ cytotoxic CD8+ Tconv-mediated death in SJS/TEN.'

Discussion, line 416 in clean doc: 'Specifically, while cells of the cytotoxic CD8+ Tconv cluster and dominant TCR+ cells, in particular, expressed LAG3, keratinocytes were the only stromal cell subset to downregulate the expression of its ligand LGALS3. Previous studies show that high expression of LGALS3 confers resistance to apoptosis in cancer cells[44] and the LAG3-LGALS3 interaction is co-inhibitory to CD8+ T-cells[45]. While functional studies are warranted, a similar downregulation of LGASL3 is observed in the lesional skin of patients with atopic dermatitis and psoriasis[67], where CD8+ LAG3+ cell-depleting antibody therapies have recently been shown to reduce inflammation and improve lesion severity and barrier integrity, without reported issues surrounding safety or tolerability[68]. These data are consistent with early studies in non-human primates which show that the depletion of LAG3+ cells

prevents the onset of delayed-type T-cell-mediated hypersensitivity reactions[69]. These data suggest that LAG3+ cell-depleting therapies may have benefit in SJS/TEN and that immune dysregulation represents a conserved defense mechanism enabling epidermal detachment to protect against diverse (drug-, self-) antigens (Fig. 6).'

Comment 11. Along the same lines, can the authors perform imaging (e.g., IFA, RNAscope) on tissue biopsies to determine if there is an accumulation of CD8+ T cells in the different layers of the skin and in proximity to keratinocytes. Perhaps adding a keratinocyte marker and HLA-I alongside markers of CD8 T cell activation (e.g., those detected by scCITE-seq) would be really informative to support the notion that keratinocytes are likely activating resident CD8 T cells.

Response to comment 11. *As suggested by the reviewer, we now show immunohistochemistry and spatial sequencing, which confirm the close proximity of keratinocytes with cytotoxic CD8+ CD103+ (T_{RM}) populations at the site of separating epidermis in patients with SJS/TEN. These data are included as a new Extended Data Figure 7. Further, we have also included a new Figure 5 using bioinformatic tools, including Cellchat, to predict cell interactions, which also strongly predicts the direct interaction of cytotoxic CD8+ Tconv with HLA class I on keratinocytes. Text changes include:*

Results, line 190 in clean doc: 'Indeed, immunohistochemistry confirmed that cytotoxic (GNLY+) CD8+ CD103+ T_{RM} T-cells line the basement membrane in SJS/TEN-affected skin (Extended Data Fig. 7A). To better understand whether these cells mapped to the cytotoxic cluster we performed spatial sequencing on a section of SJS/TEN affected skin, and the typical histopathological features of SJS/TEN were seen (Extended Data Fig. 7Bi). These included a dense lymphocytic population predominating in the dermis, no evidence of eosinophilia, basal keratinocyte disruption with separation of the epidermis from the dermis, and formation of a subepidermal cleft. Importantly, spatially-resolved regions of interest (ROI) nearest the detached epidermis (ROI 1, ROI 9) had the highest transcriptomic expression of CD8+ T-cells (CD3E, CD8A; Extended Data Fig. 7Bii) but also specific markers of the cytotoxic CD8+ Tconv associated with cytotoxicity (GZMB), regulation (LAG3), and activation (CD27, Extended Data Fig. 7Biii).'

Discussion, line 411 in clean doc: '…our phenotypic, spatial, and cell communication analyses position the cytotoxic CD8+ T_{RM}-like Tconv cluster at the basement membrane in direct contact with keratinocytes.'

Methods, line 593 in clean doc: see the entire section called 'Immunohistochemistry and spatial sequencing'.

Comment 12. Although the results presented in figure 2 are compelling and exciting, I wonder how generalisable this concept is as it was only explored in one patient. I appreciate you see the same TCR in three different locations, but, how consistent are these clones in other patients? Addressing this would be important and really informative to determine if this response is likely to be driven by the same type of antigen(s). Can the authors comment?

Response to comment 12. *We expand on this important point by referring to recent literature where, with the notable exception of carbamazepine-induced SJS/TEN, it is shown that the drug-expanded TCR*

*is unique (private) to risk drug and HLA-matched patients. This has been shown for HLA-B*58:01-restricted allopurinol SJS/TEN in South Asian populations where different dominant drug-reactive TCR are identified in different patients. Thus, while we expect to see private oligoclonal expansion of dominant TCR clonotypes, we do not expect this to be the same clonotype between patients even with the same ethnicity, drug, and risk HLA, and we have added additional statements throughout the manuscript to help clarify this for the readership. We think this is a key utility of this single-cell resource in the future, which will allow us to identify relevant effector signatures and utilize different patients with the same HLA- and drug-restricted SJS/TEN to explore shared epitope reactivities of different private TCR between patients.* However, we also clarify that although patients may have disease driven by a different drug and risk HLA allele restriction in our study in a new Extended Data Table 1, a private expanded TCR is seen in all patients and is expressed on the same common pathogenic cluster of cytotoxic CD8+ Tconv (Extended Data Figure 11). *We have also included the number of clonotypes observed in the cytotoxic CD8+ Tconv cluster for each patient blister fluid in this same figure. Text changes include:*

Introduction, line 44 in clean doc: 'Importantly, drug exposure in someone carrying a specific HLA class I risk allele for that drug is necessary for the development of SJS/TEN[6]. Moreover, for most drugs, with the notable exception of carbamazepine which has a public TCR clonotype, private drug-antigen-expanded TCR clonotypes are identified in the blister fluid of patients with drug-induced SJS/TEN that are rare in the peripheral blood[7]. In other words, different oligoclonal expanded TCRs are seen in the blister fluid of different patients even if they have been exposed to the same drug and have the same HLA risk allele.'

Results, line 257 in clean doc: 'This was also the predominant location of dominant TCR+ cells identified in all other patients (Extended Data Fig. 11B), indicating that the cytotoxic CD8+ Tconv cluster represents a common effector phenotype across patients with drug-induced SJS/TEN, even if they use private TCR CDR3αβ clonotypes.'

Comment 13. What do the authors think is the biological relevance of finding polyclonal CD8+ T cell subsets in the blister fluid but not on the affected skin, discussed in lines 164-169? Are the authors arguing in favour of "clonal bias" in the skin due to chronic/persistent antigen stimulation? This could be clarified in the text. Also, please add a reference to the relevant figure/table.

Response to comment 13. *We acknowledge the unclear wording and to clarify, we do similarly find polyclonal CD8 in the skin and the blister fluid, but there was variability in the unexpanded repertoire between samples. Our model is that the drug neo-antigen stimulates several TCRs but that the expanded oligoclonal TCR clonotypes seen in both affected skin and blister fluid are those responsible for initiating and driving most of keratinocyte death. In contrast, we cannot be sure of the degree to which the polyclonal CD8+ T-cells also directly contribute to the effector pathogenesis or the antigen stimulus for these cells. This will require further investigation but could relate to diverse drug-derived antigens or a broader array of new antigens produced by the pathological process. We now add new comments to describe this in the text and the uncertainty regarding the antigenic reactivity of unexpanded but cytotoxic clonotypes. Text changes include:*

Results, line 306 in clean doc: 'We cannot be sure if this represents a clonal bias between the skin and overlying blister fluid or whether there has been an incomplete capture of the TCR repertoire in the affected skin. Either way, we can be more confident that oligoclonal TCR CDR3αβ expansion that is seen in both blister fluid and affected skin is more likely to be directly relevant to the killing of keratinocytes

and most worthy of further investigation. Indeed, between blister fluids, only up to 10% of polyclonal T-cells in the cytotoxic CD8+ Tconv cluster shared a TCR, raising the possibility that at least some had not been triggered by the initiating drug-neo-antigen but either by 'bystander' cytokine activation[50] or a broader array of new antigens produced by the pathological process.'

Discussion, line 466 in clean doc: 'We cannot be sure of the degree to which the polyclonal CD8+ T-cells also directly contribute to the effector pathogenesis, which will require further investigation. This more detailed understanding of the complete cytotoxic repertoire will be critical to defining cytotoxic responses in patients with different causal drugs and HLA risk alleles. In contrast to autoimmune diseases with few pathogenic epitopes and TCRs, the epitope(s) driving SJS/TEN remain undefined. Still, drugs have been shown to stimulate T-cells through diverse metabolites, drug-modified peptides, and by binding to HLA to alter the self-peptide repertoire[77].'

Comment 14. I struggled to understand the data provided in Figure 2Civ and v. What are we supposed to take from the circus plots provided? This needs to be clarified in the manuscript.

Response to comment 14. *We have amended the figure and legend to clarify, and this data is now part of Figure 4. Figure 4Ai shows the clonality of CD8+ Tconv cluster 3 across samples, indicating how dominant the top 3 TCRs are among the top 50 TCR, and figure 4Aii then elaborates upon this to show the total proportion of cells in CD8+ Tconv cluster 3 that express either the top TCR or an n=1 TCR. This shows that while the dominant TCR+ cells have been of focus in recent years, unexpanded polyclonal TCR may contribute toward cytotoxic response. We have added additional text in the figure legend to clarify this point. These are two ways to show similar data, but we feel each makes the other clearer to demonstrate the clonality and breadth of expanded and unexpanded clonotypes in the cytotoxic cluster across samples, which we hope is now clear.*

Comment 15. The dataset provided in this study is quite impressive, but I think the authors have not extended their analysis enough to do it justice. One key question I wonder is whether the authors could apply cell-cell communication tools to identify (and potentially validate) pathways supporting key biological features of the disease, especially given that so little is known about it. For instance, what pathways define the communication between keratinocytes (and other stromal cells) and CD8+ T cells? One could argue that these sorts of predictions can provide novel biological insights into the pathways driving this response, beyond HLA-I signalling. Along the same lines, are there other cell types predicted to establish significant interactions with CD8+ T cells in the context of disease?

Response to comment 15. *We thank the reviewer for raising this important point. We now include a new section on predicted cell-cell communications and signaling pathways to elucidate potential therapeutic targets as a new Figure 5. This focuses on cell-cell (receptor-ligand) analyses using Cellchat and LIANA and core pathway signa*ling analyses *using PROGENy and these findings suggest potential therapeutic targets for further investigation. For Cellchat, we specifically look at the key interaction between cytotoxic CD8+ Tconv and keratinocytes, which provides novel interactions previously undescribed (PKM-CD44) or without investigation in the skin (MIF), and also strengthened the hypothesis that keratinocytes present antigen on HLA class I to the cytotoxic CD8 Tconv cluster and that the dominant TCR+ cells are regulated by KLRC1/KLRD1. We then utilise PROGENy to highlight key upregulated and downregulated common core pathways in other diverse subsets, alluding to a role for other stromal populations in immune cross-talk including fibroblasts. In particular, this helps to show activation of the JAK-STAT pathway in both T-cells and fibroblasts. We then provide data showing the*

exact JAK and STAT genes expressed in these key subsets, of importance for understanding pathological mechanisms and the potential utility of clinically available selective JAK and STAT inhibitors. Text changes include:

Results, line 320 in clean doc: See new section under new sub-heading 'Cell communication analyses support the direct HLA class I presentation of epitopes by keratinocytes to cytotoxic CD8+ T-cells.'

Discussion, line 409 in clean doc: 'Importantly, while pathway analyses propose an accessory role for stromal populations including fibroblasts in immune cross-talk and the culmination of an immunomodulatory microenvironment, our phenotypic, spatial, and cell communication analyses position the cytotoxic CD8+ T_{RM}-like Tconv cluster at the basement membrane in direct contact with keratinocytes.'

Discussion, line 431 in clean doc: 'Differential and CellChat analyses also supported a direct CD8-keratinocyte interaction, including antigen presentation via HLA class I and potentially HLA-E but also MIF and its receptors. Intriguingly, MIF was first discovered in 1966 during in vitro studies of delayed hypersensitivity[70], and although elevated in the serum of SJS/TEN patients, the expression has not been similarly investigated in the skin. While MIF is pleiotropic, it can recruit and activate T-cells, with inhibition shown to suppress autoreactive CD8+ T-cells in patients with vitiligo[71]. Further, MIF signaling is known to counteract the anti-inflammatory action of glucocorticoids[72], potentially explaining the limited efficacy of corticosteroids in patients with established SJS/TEN. These data suggest the need for functional studies of MIF and its ligands to investigate their potential as therapeutic targets given the critical interaction between keratinocytes and cytotoxic CD8+ T-cells in SJS/TEN. Our data also suggest that selective targeting of the JAK-STAT pathway may be able to dampen the T-cell response during SJS/TEN. However, JAK-STAT signaling is also important in other cell types, and we identify upregulation of STAT1, STAT2, and STAT3 in fibroblasts of affected SJS/TEN skin. As the expression of STAT3 in fibroblasts is important in re-epithelialization and wound repair[73], these data highlight the use of single-cell data to understand both the efficacy and potential toxicities of proposed therapeutic interventions, and a need for selective JAK and STAT inhibition to appropriately target pro- and not anti-inflammatory processes. Importantly, specific modulators of MIF[74], JAK, STAT, and PKM[75] are established in the treatment of cancer and may provide novel tissue-specific strategies for therapeutic intervention in SJS/TEN.'

Comment 16. I think the authors should use the discussion section to expand on some of the hypotheses derived from the transcriptomics analyses. For instance, would it be appropriate to propose keratinocytes as key modulators of T cell responses in skin? How important the stroma could in the context of SJS/TEN? Are they ways in which these data could informed future therapies or even diagnostics to predict these unwanted reactions? How about the role of macrophages? Is this likely to be modulated by CD163+ perivascular macrophages? Do these subsets express HLA-I too? There are a lot of exciting observations in this study but somehow these are not discussed in detail, or what this all means in the context of these diseases? I think this manuscript would benefit from a visual schematic depicting the key findings, e.g., skin diagram showing CD8+ T cells and keratinocytes interactions, and which pathways are overrepresented in disease? The cell-cell communication analyses could inform this.

Response to comment 16. *We deeply appreciate all of the reviewer's feedback on all comments and now expand the results and discussion to these points as requested. Specifically, we now add (1) a new extended data figure 8 and discuss the role of immune tolerance and cell death resistance pathways to*

propose that, while all stromal cells also upregulate HLA class I in the affected skin, downregulation of LGALS3 by keratinocytes permits targeted cell death by HLA-restricted LAG3+ cytotoxic T-cells, (2) a role for stromal subsets including fibroblasts in immune cross-talk and immune repair via STAT3, (3) a new figure 5 using CellChat and PROGENy to identify specific pathways with therapeutic modulators currently commercially available including MIF, PKM, TNF, and JAK-STAT, (4) and a need for longitudinal studies which will define the dynamic progression of disease and role of M2 macrophages. We have now included a schematic figure as a new Figure 6 to present the major findings and a model of disease predisposition and cellular response, including pathways leading to repair. Text changes include:

Results, line 226 in clean doc: 'Importantly, while HLA class I was also upregulated in macrophages, endothelial cells, fibroblasts, and MSC of the affected skin (Extended Data Fig. 8A), the checkpoint receptor LAG3 was highly upregulated in cells of the cytotoxic CD8+ Tconv cluster. Thus, as immune checkpoint receptors are known to regulate HLA-restricted drug-induced T-cell activation[43], we investigated whether decreased expression of LAG3 ligands (HLA class II, FGL1, CLEC4G, LGALS3) may identify disease-susceptible cell populations (Extended Data Fig. 8B). Notably, while CLEC4G and FGL1 were not expressed across samples, and HLA class II was high or upregulated in antigen-presenting cells of affected skin including fibroblasts and MSC, suggesting a role in immune regulation, LGALS3 was among the top 25 most downregulated genes by fold change in keratinocytes of affected skin, which was significant before false discovery rate (FDR) correction (Extended Data Fig. 8B). A protective role for LGALS3-LAG3 has been described to confer cellular resistance to apoptosis[44], and the LAG3-LGALS3 interaction is known to be co-inhibitory to CD8+ T-cells[45]. The expression of LGALS3 remained high across other stromal subsets in affected skin. These observations suggest that a decrease in the expression of LGALS3 may facilitate the selective targeting of keratinocytes for LAG3+ cytotoxic CD8+ Tconv-mediated death in SJS/TEN.'

Discussion, line 416 in clean doc: 'Specifically, while cells of the cytotoxic CD8+ Tconv cluster and dominant TCR+ cells, in particular, expressed LAG3, keratinocytes were the only stromal cell subset to downregulate the expression of its ligand LGALS3. Previous studies show that high expression of LGALS3 confers resistance to apoptosis in cancer cells[44] and the LAG3-LGALS3 interaction is co-inhibitory to CD8+ T-cells[45]. While functional studies are warranted, a similar downregulation of LGASL3 is observed in the lesional skin of patients with atopic dermatitis and psoriasis[67], where CD8+ LAG3+ cell-depleting antibody therapies have recently been shown to reduce inflammation and improve lesion severity and barrier integrity, without reported issues surrounding safety or tolerability[68]. These data are consistent with early studies in non-human primates which show that the depletion of LAG3+ cells prevents the onset of delayed-type T-cell-mediated hypersensitivity reactions[69]. These data suggest that LAG3+ cell-depleting therapies may have benefit in SJS/TEN and that immune dysregulation represents a conserved defense mechanism enabling epidermal detachment to protect against diverse (drug-, self-) antigens (Fig. 6).'

Discussion, line 443 in clean doc: 'However, JAK-STAT signaling is also important in other cell types, and we identify upregulation of STAT1, STAT2, and STAT3 in fibroblasts of affected SJS/TEN skin. As the expression of STAT3 in fibroblasts is important in re-epithelialization and wound repair[73], these data highlight the use of single-cell data to understand both the efficacy and potential toxicities of proposed therapeutic interventions, and a need for selective JAK and STAT inhibition to appropriately target pro- and not anti-inflammatory processes. Importantly, specific modulators of MIF[74], JAK, STAT, and PKM[75] are established in the treatment of cancer and may provide novel tissue-specific strategies for therapeutic intervention in SJS/TEN.'

Results, line 320 in clean doc: See new section under new sub-heading 'Cell communication analyses support the direct HLA class I presentation of epitopes by keratinocytes to cytotoxic CD8+ T-cells.'

Discussion, line 393 in clean doc: 'Here, we now provide an unbiased analysis of this and interacting populations at single-cell resolution to define a shared CD8+ T_{RM}-like cytotoxic cluster with both expanded and unexpanded TCRαβ clonotypes (Fig. 6).'

Minor comments:

Comment 17. The data provided throughout this manuscript are stunning, but the figures are perhaps a bit too cluttered. Having subsections within subsections in the same figure makes it difficult to appreciate the key message. I'd advise the authors to revisit their figures to split them into 3 or 4 separate figures (instead of just 2 figures). I think it'll help declutter the images a bit and will most certainly help with the overall flow of the manuscript. Also, some of the text in the images is too small and it is hard to read. For instance, extended figure 3ii, extended figure 4, etc.

Response to comment 17. *We have now split the original figures into 4 as requested and increased the text size where possible.*

Comment 18. Please add reference in line 96 to support this statement.

Response to comment 18. *Reference is now included.*

Comment 19. Please add reference to GLIPH2 in line 127

Reply to comment 19. *Reference is now included for GLIPH2.*

Comment 20. Line 172-173, please check if the journal allows this sort of statement and if not, please add this information as supplementary information.

Response to comment 20. *We have now added this data for scCITE-seq of CD127 to the main text as Fig. 4Biii and linked this to the text.*

Comment 21. Line 186, please add relevant literature to support this statement.

Response to comment 21. *Reference is now included.*

Comment 22. Please add the q values to the GO terms in figure 1Eiii. The cut-off for selection is not mentioned in the figure or the legend.

Response to comment 22. *We have now added the p and q values to the GO terms in this pathway analysis (now Figure 2D) and included the cut-off for selection in the figure legend, which included all genes above significance and 0.6log2FC.*

Comment 23. I'm unsure as to what the different bars refer to in Figure 2Dii. For example, what is "Top TCR+", "1 Ct TCR+, "C2 TCR+", etc. This needs to be clearer either in the manuscript or the figure legend, or both.

Response to comment 23. *This is now in Figure 4B and we have revised the figure descriptors, including changing 'Top TCR' to 'Cytotoxic expanded TCR+' and colored the text red to identify clonally-expanded TCR+ cells of the cytotoxic cluster in line with the naming and coloring of this subset in the rest of the manuscript. We have similarly changed 'C2 TCR+' to 'Cluster 2 TCR+', and have similarly changed the figure legend to help clarify all populations for the readership.*

Reviewer #3 (Remarks to the Author):

Gibson et al investigate immune responses of inflammatory skin lesions from Stevens-Johnson syndrome patients. They focus on CD8+ T cells, which are the major population of immune cells present in affected skin and blister fluid. Comparison to normal skin and burn blisters make for robust controls. Single cell RNA seq analysis revealed significant expansion of CD8+ TCR clones with enhanced effector profiles in affected skin and blisters. Overall the experiments are well performed, controlled and conclusions are accurate. However, improvements could be made to the writing and overall structure of the manuscript.

Comment 24. The introduction is very short for an article style publication and lacks important information required for the broad readership of Nat comms. Which drugs induce Stevens-Johnson syndrome and when are they used? How is Stevens-Johnson syndrome treated and what improvements are needed?

Response to comment 24. *We have now included further details on clinical presentation, histopathology, and prior knowledge of immunopathogenesis in the introduction regarding the common drugs associated with SJS/TEN, the typical time to onset following first drug exposure, and the current treatment strategies. Text changes include:*

Introduction, line 23 in clean doc: please see various changes throughout the introduction

Comment 25. The multipart figures (especially 2C i, ii, iii, iv) are difficult to follow and detract from the significance of the results. I would suggest splitting figures 1 and 2 into 2 different figures each. This would also give some more room for down regulated genes to be labelled on each of the volcano plots (2a and 2c).

Response to comment 25. *We have now split the original figures into 4 to help with readability and sizing and included the downregulated genes on each volcano, including a short description for each in the text. Text changes include:*

Results, line 214 in clean doc: '*…identified downregulated DEG associated with T-cell inactivation and quiescence (TSC22D3, ZFP36, ZFP36L2)*[40]'

Results, line 219 in clean doc: 'However, in keratinocytes, downregulated genes matched those similarly downregulated in keratinocytes during psoriasis (GADD45B, MT1X, DUSP1, ZFP36, MYC, BTG2)[41] including a regulatory circuit of chronic inflammation (CEBPD, ATF3)[42].'

Results, line 266 in clean doc: Furthermore, the most downregulated DEG in dominant TCR+ CD8+ Tconv included ZFP36L2 and BTG1, aligned with T-cell quiescence/inactivation[47], and IL7R, which is downregulated upon antigen-driven TCR-dependent activation[29].

Reviewer #4 (Remarks to the Author):

The authors are presenting a resource, single cell transcriptomes of cells + TCR profiles from blister fluid derived from 17 patients with SJS/TEN, and one skin biopsy from SJS/TEN patient, plus 2 individual controls: one healthy skin sample (from discarded edges post-surgery), one blister fluid from a burn patient. The analysis demonstrates that:

- ***Cytotoxic T cells are enriched at the site of SJS/TEN damage.***
- ***A cytotoxic CD8+ subpopulation expresses the same expanded TCRαβ clonotypes in affected skin (n=1) and blister fluids (n=17) but not in unaffected skin (n=1).***
- ***Expanded clonotypes were enriched for markers indicative of terminally-differentiated effectors driving antigen-specific response.***
- ***Expanded oligoclonal and unexpanded polyclonal CD8+ T-cells are present in the blister fluid of SJS/TEN.***

The study documents a unique resource of 119,784 transcriptomes + TCR sequences, which could be of significant benefit/interest to the field. However, the actual findings are mainly confirmatory of other studies, e.g: https://www.science.org/doi/10.1126/sciadv.abe0013, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7496676/, demonstrating the importance of CD8 T cells for SjS/TEN and the CD8 T cell clonotype expansion. It would be important to distinguish which of the reported findings are truly novel and highlight it throughout the text.

Reply to remarks: The reviewer's point is well-taken. We have distinguished the novel findings from what was previously known throughout the revised manuscript and elsewhere in these responses to reviewer comments.

Comment 26. While the study presents a unique resource, there are several methodological aspects which need consideration/ correction. Authors report, that their cut-off threshold for mitochondrial gene content was 50%. That value is extremely high for immune cells and suggests high level of apoptosis. That might be specific to the disease but might be caused by the banking procedure and the use of cells from cryobank. Without a comparison with appropriate fresh controls, it is impossible to infer which is the cause. Additionally, Authors should provide QC plots, demonstrating distribution of the mitochondrial/ribosomal/doublets pre and post filtering in the supplementary material, as for such a resource, the quality of the data is of paramount importance. Maybe add the % mitochondrial value of most cells?

Response to comment 26. *While we agree that the initial cut-off of 50% mitochondrial content is high, this was subject to further refinement before analyses as a tailored strategy for initial QC which we have now clarified in the text. Importantly, this was only a first pass to remove highly apoptotic/dead populations to allow for initial QC of cells in the earlier stages of cell death, which may be important in the context of severe immune-inflammatory disease. We then applied a second-pass approach to remove low-mid apoptotic cells using a validated mitochondrial/ribosomal ratio. The remaining cells at this point had previously been included in our submitted manuscript, with only 2% of cells having >10% mitochondrial gene content. Importantly, the energy threshold for human tissue is of recent debate and can be particularly high in the context of the high metabolic stress and tissues such as the heart (30%) etc, and a standardized mitochondrial DNA threshold of 10% is recently recommended for scRNA-seq analyses across human tissues (PMID: 32840568). Thus, in the revised manuscript, we have removed the remaining 2% of cells above 10% mt gene content from downstream analyses. The median mitochondrial gene content for all 'live' cells is 3%, and this matches the immune cell subset-specific median mitochondrial DNA content for CD4+ T-cells, CD8+ T-cells, NK cells, monocytes, and macrophages. We now include additional step information for QC in text in the methods, and provide the QC plots for the mitochondrial/ribosomal/doublets pre- and post-filtering as requested (Extended Data Figure 15) which show the number of cells excluded in each step in our QC pipeline. In Extended Data Figure 15, we also include the cell proportions in each sample, which show the comparative distribution between each QC filtering step, and overall helps to show that these filtering steps did not largely change the distribution of cells and clusters identified. We also include an additional comment in the methods to clarify cell viability using the average % mt content for the readership. Importantly, the use of cryopreserved cells in single-cell studies is well-established, and multiple studies have shown that although cryopreservation can induce some limited cell death, upon dead cell exclusion, there are minimal transcriptomic differences between single-cell sequencing of fresh and cryopreserved human cells, and cryopreservation minimizes the high individual run cost of single-cell sequencing and run-to-run (batch) variation as a major limitation to running one sample at a time, which would be the case for fresh samples given the rarity of SJS/TEN at a local level. Further, it is shown that 'DMSO cryopreservation is the method of choice to preserve cells for droplet-based single-cell RNA sequencing', which is the method used in this study. We now clarify our QC methods in the text including the average mitochondrial gene content of cells analyzed in the final UMAP after filtering, and we add a further statement to the methods to highlight our selection of cryopreservation procedures. Text changes include:*

<u>Methods, line 502 in clean doc:</u> '*Cryopreservation using DMSO is the recommended cell preservation procedure for droplet-based single-cell sequencing, which, after dead cell exclusion, is widely reported to result in minimal batch variation and transcriptomic differences between fresh and cryopreserved human cells*[78,79]*.'*

<u>Methods, line 556 in clean doc</u>: *'The hashtag negatives or doublets, as well as cells with low (<500) UMIs, were removed. Cells with <100 genes and >50% mitochondrial content were removed to filter low-quality, dead, or dying populations for initial QC (QC1, Extended Data Fig. 15), with a second pass filter excluding apoptotic populations characterized by a low percentage of ribosomal genes and a high percentage of mitochondrial genes, using a defined mitochondrial-ribosomal RNA ratio of >0.47*[48] *(QC step 2, Extended Data Fig. 15). Importantly, a standardized-mitochondrial DNA threshold of 10% is recommended for scRNA-seq of human tissue*[83]*, which was met by 98% of 'live' cells identified in our dataset. The remaining 2% of cells were excluded from downstream analyses (QC step 3, Extended Data Fig. 15). The median mitochondrial gene content for all 'live' cells was 3%, and this matched the immune cell subset-specific median mitochondrial DNA content for CD4+ T-cells, CD8+ T-cells, NK cells, monocytes, and macrophages. Downstream transcriptome-based graph clustering and principal*

component analyses (PCA) with cell phenotype consensus calling were performed using R Seurat v4.1.1 package[23], without input of TCR, BCR, mitochondrial, ribosomal, or sex-linked genes to avoid bias due to known expression variability. Batch correction for 10x run, sample type (skin or blister fluid), and cell cycle phase were performed using harmony[84]. While most cell subsets formed distinct clusters, keratinocytes predominantly aligned to a shared cluster including a minority of T-cells, NK cells, and monocytes at the UMAP origin defined by lower UMI; indicative of cell stress. However, these cells met all other criteria for inclusion and were retained for pathogenic importance in the context of drug-induced stress during SJS/TEN. Experimental doublets were identified and removed using a majority consensus benchmark[85] of three independent bioinformatic algorithms (DoubletFinder[86], scDBLfinder[87], scDS[88]; QC Step 4), and subsets with <50 scRNA-defined cells were removed (QC step 5, Extended Data Fig. 15).'

<u>Comment 27.</u> The study reports results of 17 patients with SJS/TEN. It is unlikely, that all have been induced by the same medication, and it is critical to take it into account for analysis, given the different mechanisms by which drugs induce immune activation (b-lactams vs carbamazepine, requiring metabolism, vs large molecule drugs). Figure 1D demonstrates a wide spread of cellular composition, is it in any way related to patient characteristics (sex, age) or causative medication?

Response to comment 27. *We thank the reviewer for their comment, which has allowed us to improve this paper and more specifically provide data regarding the likely culprit drug. We also thank the reviewer for pointing out the important distinction between small and large-molecule drug-induced disease. Large molecules such as immune checkpoint inhibitors represent a small but emerging cause of an SJS-like illness and may involve different disease mechanisms. Thus, we have reviewed and removed any patients potentially associated with large molecules and now clarify this to be unbiased single-cell analyses of small molecule-drug-induced SJS/TEN only (n=15 patients). Further, we have now provided additional data on the casual drug, reaction sub-type/severity based on the body surface area detached (SJS, SJS/TEN overlap, or TEN), age, and sex of each patient, which we now provide in Extended Data Tabe 1. This table also includes the putative HLA risk allele for that patient if previously described in the literature as associated with the same causal drug. While this list is not complete, as for many drugs and populations, no HLA risk alleles have been described, we also list the culprit drug for each patient in Fig 1D but also Extended Data Figure 11, which highlights that despite different drug and/or HLA, the private oligoclonal TCRαβ for each patient is expressed on a common pathogenic signature of cytotoxic CD8+ T-cells as the focus of this study. Further, we also include a new Extended Data Figure 4 to demonstrate a lack of observed correlation between cell subset heterogeneity in each blister fluid sample in our dataset with the culprit or disease phenotype/severity (by highlighting the 7 patients with co-trimoxazole-induced SJS or TEN) and propose that longitudinal studies will now be required to understand cellular trajectories and proportions over time. Text changes include:*

<u>Abstract, line 5 in clean doc:</u> '…we performed (sc) single-cell transcriptome, surface proteome, and T-cell receptor (TCR) sequencing on unaffected skin, affected skin, and blister fluid from 15 patients with small molecule drug-induced SJS/TEN.'

<u>Introduction, line 64 in clean doc:</u> 'Importantly, while patients varied in age, sex, culprit drug, and HLA genotype, single-cell analyses of the cellular response at the site of SJS/TEN tissue damage revealed common features across patients that were unrelated to these different predisposing factors.'

<u>Methods, line 98 in clean doc:</u> 'Patient samples varied in culprit drug and time since onset of reaction (Extended Data Table 1) and we theorized this might impact subset representation but were unable to

detect any associations (Extended Data Fig. 4). However, we acknowledge we may have been underpowered to do so.'

Methods, line 248 in clean doc: 'The identification of oligoclonal clonotypes in this patient was representative of CD8+ Tconv in blister fluid from all patients with SJS/TEN driven by different drugs (Extended Data Fig. 11A).'

Methods, line 255 in clean doc: 'Notably, these three dominantly-expanded clonotypes were expressed in the same UMAP location, with the majority of those in affected skin (>97%) and blister fluid (>90%) aligned to the cytotoxic CD8+ Tconv cluster (Fig. 3Bii). This was also the predominant location of dominant TCR+ cells identified in all other patients (Extended Data Fig. 11B), indicating that the cytotoxic CD8+ Tconv cluster represents a common effector phenotype across patients with drug-induced SJS/TEN, even if they use private TCR CDR3αβ clonotypes.'

Discussion, line 386 in clean doc: 'Specifically, we observed the same cytotoxic CD8+ subpopulation expressing GNLY, GZMB, PRF1, LAG3, CD27, TIGIT, and LINC01871, but not IL7R or FASLG, in blister fluid and affected skin of patients independent of the causal drug or likely HLA risk allele.'

Discussion, line 458 in clean doc: 'Indeed, as the lymphocyte/monocyte ratio has been shown to change over time in samples obtained from a single patient with SJS/TEN[19], longitudinal single-cell studies will be required to define the dynamic progression of the disease, including the proposed transition of M1 and intermediate-like macrophages identified in this study towards populations associated with re-epithelization and repair (Fig. 6).'

Comment 28. The resource includes two individual n=1 as controls (one blister fluid from burn patient, one healthy skin). Use of n= 1 is inappropriate as a comparison, and no conclusions should be drawn from it. Skin is not an appropriate control for blister fluid. Additionally, from the methods section, these were not cryobanked, but processed fresh, in contrast to the study samples, which might introduce artefactual differences. While it is understandable, that controls for this study would be difficult to obtain, studies in public domain, e.g. https://pubmed.ncbi.nlm.nih.gov/32344053/, could be used for comparison, to strengthen the argument.

Response to comment 28. *We apologize for any miscommunication and clarify that all samples were cryopreserved, which we hope is now clear in the revised text. As stated by the reviewer the identification of true controls for this type of investigation and disease is problematic, and there are very few studies in the public domain which currently truly reflect the disease and methods presented herein. Indeed, while we have similarly considered the collection of induced blisters and interstitial fluid as the study cited by the reviewer, we believe burn blister fluid represents the most relevant control population we could obtain as a non-antigen-driven generalized inflammatory blister control compared to the drug antigen-driven T-cell mediated inflammation observed in SJS/TEN. Furthermore, the standard of care in most developed countries is for SJS/TEN patients to be admitted to burns centers, and thus this direct comparison is of high clinical relevance. Thus, we heed concerns from the reviewers regarding further controls and believe this has allowed us to significantly improve the manuscript. During the review process, we have been able to obtain three further burn blisters so that we have n=4 for appropriately controlled comparison, and we now include additional data and text in this regard. These include the differential analyses of macrophages in burn and SJS/TEN blister fluid and the statistical comparison of cells in burn and SJS/TEN blister fluid using scCODA to show an increased representation of CD8+ T-cells in SJS/TEN. We have also included additional text and references to*

highlight that our data mirror the minor representation of similarly cytotoxic CD8+ T_{RM} T-cells reported in public published scRNA-seq datasets of full-thickness skin biopsies from healthy individuals. Text changes include:

Introduction, line 62 in clean doc: 'Normal skin (n=1) and burn blister fluid (n=4) were included as healthy tissue and non-antigen-driven generalized inflammatory blister controls, respectively.'

Results, line 90 in clean doc: 'Indeed, compared to macrophages in burn blister fluid, those in SJS/TEN blister fluid were significantly enriched for HLA class I, HLA class II, STAT1, interferon-induced response (IFITM1-3), and C1Q involved in the phagocytosis of apoptotic cells (Extended Data Fig. 3).'

Results, line 102 in clean doc: '…were CD8+ T-cells (Fig. 1Di), which were the most significantly enriched subset in SJS/TEN blister fluid (n=15) compared to burn blister fluid (n=4) (scCODA, p<0.05)[17].'

Results, line 173 in clean doc: 'Importantly, our data mirror the minor representation of similarly cytotoxic CD8+ T_{RM} T-cells reported in published scRNA-seq datasets of full-thickness skin biopsies from healthy individuals[31,32], and this population has demonstrated the capacity to proliferate in response to local antigen encounter in the skin[33].'

Comment 29. The authors acknowledge "We could also not control all patient-specific factors such as severity and time since onset of symptoms.", but they assert that the patients were in the acute phase of SJS/TEN. A table summarising clinical details, for individual patients, describing the differences in patient symptoms and the time from onset should be provided. The notion of "acute" disease is repeated throughout the manuscript, which requires a) adding a definition of acute used, and b) confirmation that patients conformed to that definition.

Response to comment 29. *We now list the culprit drug, age, sex, risk HLA (where defined in literature), disease phenotype/severity (based on percentage of the body surface area detached), and time since onset of symptoms for each patient in a new Extended Data Table 1. We also list the culprit drug for each patient in Fig1D but also Extended Data Figure 11, which highlights that despite different drug and/or risk HLA allele, the private oligoclonal TCRαß for each patient is expressed on a common pathogenic signature of cytotoxic CD8+ T-cells as the focus of this study. We agree that 'acute' can mean different things in a clinical setting and now include a definition for this in the text, defined as the period of symptomatically progressing or non-resolving disease within 10 days of hospitalization. All patients with blisters and/or skin sampled were captured during a period of acutely progressing disease. Text changes include:*

Results, line 98 in clean doc: 'Patient samples varied in culprit drug and time since onset of reaction (Extended Data Table 1) and we theorized this might impact subset representation but were unable to detect any associations (Extended Data Fig. 4). However, we acknowledge we may have been underpowered to do so.'

Methods, line 490 in clean doc: 'All samples were collected from patients either without a prior history of drug allergy or during acute SJS/TEN. Acute disease was defined as the period of symptomatically progressing or non-resolving disease and within 10 days of hospitalization (Extended Data Table 1).'

Comment 30. Line 197: "antigen-reactive polyclonal CD8+ T-cells also contribute to the effector pathogenesis, and the total number and affinity of cytotoxic TCR may impact disease severity". It is

an interesting notion, but can it be substantiated with data from the study? Does the number/affinity of TCR correlates with severity of the patient disease?

Response to comment 30. *We agree that this is too speculative and do not have sufficient direct evidence to support this statement. We have reworded the text to reflect better the need for study of the total cytotoxic repertoire to define disease without specific inference on disease severity. Text changes include:*

Discussion, line 468 in clean doc: 'This more detailed understanding of the complete cytotoxic repertoire will be critical to defining cytotoxic responses in patients with different causal drugs and HLA risk alleles.'

Comment 31. Skin dissociation method is unclear: Method section, lines 217:220 says tissue was processed into small cubes, and 230 says 4mm punch biopsies were thawed – which was it? How big were the cryopreserved fragments? It is also important to state the exact dissociation protocol, as it is not trivial for skin, and can have significant impact on the cell viability, cell composition, and scRNA-seq outcome. The section says biopsies from SJS/TEN patients, while presented results show n=1 for skin biopsy, is that a typo? Or were the results analysed for more that 1 patient?

Response to comment 31. *Thank you and we apologize for the confusion and discrepancies. We have now removed the inaccuracies and clarified that all skin was collected as 4mm punch biopsies, which were cryopreserved before sequencing. We describe the dissociation protocol in the methods (beginning line 511 in the clean document) in full, including enzymes utilized, concentrations, and incubation times. We have expanded on this to highlight that we did not dissociate the dermis and epidermis, which are separated in some studies. However, in this study, full-thickness skin biopsies were processed whole, and we note the protocol is optimized for lymphoid cell recovery and skin digestion methods are associated with a variable recovery of stromal subsets from run to run, which cannot be relied upon, unlike more resilient immune populations. The mention of 'patients' is also a typo, as the skin investigation directly compares paired unaffected and affected skin biopsies from a single patient, which we have now amended. Text changes include:*

Methods, line 512 in clean doc: 'Cryopreserved 4mm punch biopsies from SJS/TEN patients and healthy skin from unrelated donors were quickly thawed in a water bath (37°C) and full thickness biopsies were subjected to enzymatic digestion without separation of the epidermis and dermis to obtain a single-cell suspension using published methods optimized for lymphoid cell recovery[80] and single-cell sequencing[81].'

Minor:

Comment 32. What do asterixes mean in Fig 1 D?

Response to comment 32. *Asterisks indicate that the data is an average of multiple time-paired blister fluids from the same patient. We have now clarified this in the figure itself and the figure legend.*

Comment 33. Fig 1 E: the significant differentially expressed gene signature of CD8 Tconv cluster – From the authors description these are cluster marker genes, computed using Wilcoxon rank test vs all the other clusters not DEGs, computed for patient groups/study variables, and the nomenclature

"cluster marker genes" should be used. For 1Eiii GO pathways: actual ranking missing, p/q values missing/not provided

Response to comment 33. *We have amended this text to describe these as 'cluster marker genes' on lines 148 and 202 of the clean document and included the rank and q values for the GO terms in the pathway analyses now shown in Fig2D.*

Comment 34. Line 149: "To investigate whether T-cells with unexpanded clonotypes had a distinct phenotype, we used blister fluid to increase the power for DEG analyses." – that sentence is difficult to understand, how can blister fluid be used to increase power of DEG analysis? Additionally, as in 2 above, no DEG analysis was performed, accordingly to method description.

Response to comment 34. *For clarity we have simplified this wording in the text to focus on blister fluid. Text changes include:*

Results, line 289 in clean doc: 'Using blister fluid, we investigated whether T-cells with unexpanded clonotypes had a distinct phenotype.'

Comment 35. Fig 2 – the title and the content does not provide data for polyclonal T cells, while a dedicated result section (line 143/173) expands on the importance of the polyclonal T cells.

Response to comment 35. *We have now split the figures to give more space and make them more dedicated to a particular section. Figure 4 is now focussed on expanded and unexpanded polyclonal clonotypes which is now reflected in the title of Figure 4 as 'Cytotoxic CD8+ Tconv consist of TCR-dependant expanded and unexpanded clonotypes.'*

Comment 36. Supplementary Fig 1 labels are upside down.

Response to comment 36. *We have corrected the label orientation for this figure (now Extended data Figure 2).*

We have made additional amendments to this revised draft in response to issues raised collectively by the reviewers and these are summarized below:

1. *After focussed analyses of small molecule drug-induced SJS/TEN patients and further filtering of cells to remove any above 10% mitochondrial gene content, we have reclassified CD8+ Tconv cluster 4 as inactive and cluster 5 as a population with cluster markers genes associated with the inflammasome. We now provide updated text to this extent, which importantly, has further clarified the stand-out importance of cluster 3 as the only cluster associated with antigen-induced cytotoxicity and proliferation. The number of cell subsets was also reduced from 16*

subtypes to 15 subtypes as a small previously defined population of neutrophils was removed by the new QC filtering. Text changes include:

<u>Results, line 71 in clean doc:</u> 'The total scRNA-defined uniform manifold approximation and projection (UMAP, Fig. 1Ai) includes 109,888 cells, spanning 15 subtypes, including both immune and stromal subsets.'

<u>Results, line 151 in clean doc:</u> '…migration and post-transcriptional regulation of the inflammasome (cluster 5; NEAT1, MALAT1, FMNL1, CBLB, INPP5D, USF2, MYH9, IKNA, IKZF3)[26]'

2. *Under an existing IRB approval new burn blister fluid samples were provided for analysis for additional controls and to address reviewer comments and Mark W Fear and Fiona M Wood who contributed these samples have been included as additional authors.*

3. *We have included a new Extended Data Figure 13 which shows that CD8+ Tconv expressing two TCR clonotypes have slightly elevated total gene and UMI counts which suggests the possibility that these cells represent physically interacting T-cell-T-cell doublets as suggested by the work of Sun et al, which is referenced. Text changes include:*

   <u>Results, line 275 in clean doc:</u> 'We considered the possibility that T-cells that appeared to express more than one TCRαβ were in fact two or more physically interacting T-cells as suggested by the work of Sun et al[48]. We found that these "dual expressing TCR+ cells" had higher gene and unique molecular identifier (UMI) counts (Extended Data Fig. 13) than single TCRαβ-expressing T-cells, indicating that at least some of these are PICs. These data were mirrored in affected skin (Extended Data Fig. 12ii-iii); suggesting the possibility that dual TCRαβ+ PIC T-cells[49 28] play a role in the auto-presentation of drug neoantigen, contributing to the pathogenesis of SJS/TEN.'

4. *To better reflect the newly revised manuscript and revised conclusions from additional analysis, we have completely revised the abstract.*

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have responded to my comments. The risk HLA allele is now reported for 6 of the 15 patients. The risk alleles for carbamzepine (patient 8: SJS, HLA-A*31:01, patient 15: TEN, HLA-B*15:21), allopurinol (HLA-B*58:01) and nevirapine (HLA-C*04:01) correspond to the known risk alleles for the respective drugs. Unfortunately, the HLA alleles for the remaining patients are missing.

The authors have also added Extended Data Figure 4. It is a scatter plot showing the relationship between immune cell subset representation and sampling day. The legend does not indicate whether each point corresponds to a different patient, or if within the same patient different sampling days are included. Furthermore, macrophages are only indicated for 6 of the 7 patients for co-trimoxazole. This should be clarified.

In Extended Data Table 11, the drug and total number of clonotypes are now indicated. This makes the information about the clonal expansion of CD8+ T cells more visible.

I have one more comment regarding the preparation of single cells from cryopreserved tissue samples by enzymatic digestion that may affect the detectability and expression levels of RNA and not only the composition of the isolated skin cells, as noted by another reviewer. Actually, according to today's technical possibilities, a transcriptome analysis can be performed directly from laser-dissected single cells of fixed tissue sections. It should therefore be mentioned in the discussion that the mode of sample preparation may have affected the results of single-cell transcriptomics.

Reviewer #2 (Remarks to the Author):

I have now gone over the revised version of the manuscript and appreciate that the authors have incorporated all my feedback, which I'm pleased was useful to improved their manuscript. I would also like to highlight how important the validations included in this version are, as I am sure this manuscript will be a reference in the field. I have no other comments and am delighted to support this manuscript for publication in its current form. I look forward to seeing this manuscript in press.

Best wishes and well done!

Juan Quintana.

Reviewer #3 (Remarks to the Author):

The manuscript has been significantly revised and improved. The authors have address all of my comments and questions.

RESPONSE TO REFEREES

We would like to take the opportunity to once again thank all reviewers for their comments and suggestions, which have helped us to make extensive revisions that significantly improve the quality and clarity of the manuscript methods, results, outcomes, and impact of this study. Please see our direct response to the most recent reviewer comments below.

Reviewer #1 (Remarks to the Author):

Comment 1: The authors have responded to my comments. The risk HLA allele is now reported for 6 of the 15 patients. The risk alleles for carbamazepine (patient 8: SJS, HLA-A*31:01, patient 15: TEN, HLA-B*15:21), allopurinol (HLA-B*58:01) and nevirapine (HLA-C*04:01) correspond to the known risk alleles for the respective drugs. Unfortunately, the HLA alleles for the remaining patients are missing.

Response to comment 1: Thanks, we have now included HLA class I typing for all patients in supplementary table 1. Where the risk allele has been previously published, we have bolded this in the table.

Comment 2: The authors have also added Extended Data Figure 4. It is a scatter plot showing the relationship between immune cell subset representation and sampling day. The legend does not indicate whether each point corresponds to a different patient, or if within the same patient different sampling days are included. Furthermore, macrophages are only indicated for 6 of the 7 patients for co-trimoxazole. This should be clarified.

Response to comment 2: *Thanks, we have now amended the figure legend as requested for supplementary figure 4 to clarify that 'Each dot represents an individual patient (n=15)' and included the statement 'Macrophages represented ≥0.5% of cells in blister fluids from 6/7 patients with co-trimoxazole-induced SJS/TEN (Supplementary Fig. 4)' in the results (line 111 of manuscript document).*

Comment 3: In Extended Data Table 11, the drug and total number of clonotypes are now indicated. This makes the information about the clonal expansion of CD8+ T cells more visible. I have one more comment regarding the preparation of single cells from cryopreserved tissue samples by enzymatic digestion that may affect the detectability and expression levels of RNA and not only the composition of the isolated skin cells, as noted by another reviewer. Actually, according to today's technical possibilities, a transcriptome analysis can be performed directly from laser-dissected single cells of fixed tissue sections. It should therefore be mentioned in the discussion that the mode of sample preparation may have affected the results of single-cell transcriptomics.

Response to comment 3: *Thanks, to address this important point we now include an additional two sentences in the limitations section of our discussion: 'Importantly, there are different methods available for cell preparation, including the laser-dissected isolation of single cells from fixed tissue sections. It is possible that these non-enzymatic methods of sample preparation will differentially affect the results of single-cell transcriptomics and warrant future comparative study.' (line 460 of manuscript document).*