Supplementary Information

Supplementary Material and Methods

UVB.

Erythemal sensitivity is highly wavelength dependent and significantly greater doses of longer wavelengths are required to induce erythema. Therefore, to control for equivalent biological effects as determined by erythema, subjects were treated with different wavelengths of UVB using equal multiples of their MED. The irradiance of all lamps was checked prior to each exposure using a radiometer calibrated traceable to the National Physics Laboratory in the UK, and the MED for each wavelength in each patient was taken as the lowest dose to cause just perceptible erythema 24h after administration of a standard series of 10 geometrically increasing doses.

Each patient then had a dose of 0.75-3 MEDs of 311nm administered to at least one plaque of psoriasis on their lower back, and 37 of these patients also had a 3 MED dose of 290nm administered to at least one plaque. Biopsies were taken between 2 and 48h after irradiation. Patients receiving routine 311nm UVB (TL01) were given an initial dose of 70% of their MED, increasing after alternate treatments by a variable percentage; initially 40% as previously described (Kirke *et al.*, 2007). Doses were adjusted accordingly if erythema developed.

Biopsies.

Previous reports have suggested that there may be cellular difference within individual psoriatic plaques, with the active edge of a plaque having an increased vascular component (Goodfield *et al.*, 1994), and biopsies were therefore taken from small plaques (where the majority of the plaque was removed) or the edge of larger plaques. All biopsies were taken from the lower back, at least 3cm from the spine. Cell numbers were automatically counted, and colocalisation coefficients were measured using Volocity 4.01 image analysis software. For the purposes of this

study we have defined a biopsy as showing increased apoptosis if the apoptotic rate was above 0.65 cells per 1000 nucleated epidermal cells (upper inter-quartile range for un-irradiated lesional psoriasis).

Cryosections of 6μm thickness were fixed in methanol and acetone (1:1), permeabilised with 0.2% triton X-100, blocked with normal goat serum (1:60) and incubated sequentially with monoclonal rabbit anti-active caspase-3 antibody (1:500; R and D Systems), then goat anti-rabbit Oregon green 488 (1:300; molecular probes). Toto-3 (1:7000; Invitrogen) was used as a nuclear marker. Where triple labelling was performed keratinocytes were identified with AE1-AE3 (1:50; Abcam) and anti cytokeratin 14 (1:200; Novocastra) together, T cells with anti CD3 (1:100; Dako), anti CD4 (1:100; Dako) and anti CD8 (1:100; Dako), Langerhans' cells with anti Cd1A (1:50 BD Pharmingen) and langerin (1:500; Beckman), melanocytes with Melan A (1:100; Abcam) and monocytes/ macrophages with anti CD68 (1:2000; Dako). All antibodies and flurochromes were diluted in 2% bovine serum albumin.

Images of immunohistochemistry sections were taken on a BD Pathway bioimaging system for cell counts and a Leica TCP SP2 scanning confocal microscope for colocalisation experiments. Colocalisation of apoptotic cells and markers of cell origin were measured by double labelling with anti-active caspase-3 and each of the markers for cell origin. At least 130 apoptotic cells examined for each marker (n=5) (Figure 2a) and an overall colocalisation coefficient was calculated.

***In-vitro* Time-Course of Apoptosis following UVB**.

Nucview^{TM} 488 is a cell permeable caspase-3 DEVD substrate coupled to a fluorescent DNA dye. The substrate is not fluorescent but caspase-3 cleavage yields a product that binds to DNA and fluoresces at 488ex/520em, thus generating an archive of caspase-3 activity within each cell over the observation period.

A 3 x 3 montage image of each well containing cells was captured at 30 min intervals for 24h on a Pathway HT automated fluorescent microscope in wide field mode at 37^{o}C and 5% CO_2. Filter sets were: Nucview 488, Excitation 488/10 and emission 515LP; and PI, excitation 548/20 and emission 570LP. Each montage allowed approximately 100 cells to be visualised simultaneously. Results were analysed using Volocity 4 software (Improvision).

Mathematical model

The keratinocyte compartment can be subdivided into stem cell, TA cell and differentiating cell compartments (Dover and Wright, 1991; Webb *et al.*, 2004). which show distinct patterns of distribution in normal and psoriatic skin. A mathematical model was created using NetLogo version 4.0.4, a 2D agent-based modelling environment, by combining known kinetic parameters derived from the literature and observed histological features characteristic of psoriasis. Previously elucidated kinetic parameters of both normal and psoriatic epidermis have been published in the literature as follows:

- Stem cells are thought to pass through the cell cycle at a slower rate than TA cells (Dover and Wright, 1991), and have been estimated to have a cell cycle time of 100-200 hours in normal epidermis (Morris and Hopewell, 1990; Potten, 1986; Potten and Bullock, 1983).
- TA cells retain a limited ability to proliferate but are committed to differentiate at some stage. They are located in the basal layer, dividing approximately 3 to 5 times (Savill, 2003; Watt, 1998) before migrating upwards, and eventually differentiating.
- TA cells may be resting or cycling, but when cycling are thought to progress through the cell cycle faster than stem cells (Potten and Bullock, 1983). The cell cycle time has been estimated using an FLM curve extended over 45h to minimize the effects of diurnal variation or cell synchronization. This showed a mean cell cycle time of 56h with a

75% coefficient of variation (Duffill *et al.*, 1976), with other estimates ranging from 50-65h (Bauer and Grood, 1975; Chopra and Flaxman, 1974; Goodwin *et al.*, 1974; Wright, 1976).

- It is thought that the overall proportion of nucleated cells in normal epidermis within the differentiating compartment is 40-66% (Bata-Csorgo *et al.*, 1993; Weinstein *et al.*, 1984). Differentiated cells continue to migrate up the epidermis until they die and become part of the stratum corneum. The transit time is the time taken for a cell to migrate from the bottom of a compartment to the top, and in normal epidermis this is estimated to be 240-336 hours for cells within the differentiating compartment (Allegra and De Panfilis, 1974; Epstein and Maibach, 1965; Weinstein and McCullough, 1973).

- The epidermal turnover time in normal human epidermis (defined as the number of cells within the epidermis / birth rate in the steady state) is estimated at 28-35 days for normal epidermis excluding stratum corneum (Bergstresser and Taylor, 1977; Epstein and Maibach, 1965; Lindwall *et al.*, 2006; Weinstein and Frost, 1971), and up to 4 times faster in psoriasis (Weinstein and Van Scott, 1965).

- The overall number of epidermal cells in psoriasis is increased by 2-5 times normal (Vanscott and Ekel, 1963; Weinstein *et al.*, 1985).

- There is an increase in cycling cells of approximately 6 times normal (Doger *et al.*, 2007), and in total epidermal mitosis of 10-23% (Duffill *et al.*, 1976; Goodwin *et al.*, 1974; Weinstein and Frost, 1968).

- The cell cycle time does not change in psoriasis compared to normal skin (van Ruissen *et al.*, 1996).

- The estimated transit time for the differentiating compartment is reduced from 240-330h in normal epidermis to as quick as 48h in psoriasis (Weinstein and McCullough, 1973).

- Several lines of evidence indicate gradients of differentiation markers within epidermis (Ishida-Yamamoto and Iizuka, 1995; Korver *et al.*,

2006; Martinsson *et al.*, 2005). Although the molecular signals that drive this process remain to be fully elucidated, the histological pattern of induction of keratinocyte differentiation is not consistent with a gradient emanating from a fixed distance beneath the stratum corneum, but rather indicates a gradient arising from the basal layer (Figure 4a).

- In psoriatic epidermis, there is a six to sevenfold increase in the number of cells in the S/G2M phase of cell cycle among CD29+ K1/K10- cells- (putative stem cell population). Furthermore, all lesional K1/K10- cells showed high PCNA positivity, indicating that all these cells had been recently induced into the cell cycle. By contrast, the proportion of cycling cells among lesional psoriatic CD29+ K1/K10+ keratinocytes was similar to normal (Bata-Csorgo *et al.*, 1993).
- Cells with the highest proliferation potential will have greater adhesion to the basement membrane and /or other cells of higher proliferative potential (Fuchs, 2008).

The above evidence and the following assumptions were used in the creation of the model, and this was then rigorously tested (Figure 4c):

- At a specified distance above the dermal papillae, differentiated cells are incorporated into the stratum corneum (not represented in the model) resulting in a 'flat' surface to the epidermis.
- Under normal conditions each stem cell divides asymmetrically to produce 1 stem cell and 1 daughter TA cell.
- The daughter cell will divide symmetrically for the user-defined number of time at the beginning of its life. It will then migrate up the epidermis.
- A gradient arising from the basal layer determines when cells differentiate (Figure 4). This was created to be consistent with the pattern of differentiation observed with immunohistochemical analysis (Korver *et al.*, 2006), while keeping the proportion of differentiating:

proliferating cells consistent with published evidence (Bata-Csorgo *et al.*, 1993; Weinstein *et al.*, 1984).

- A second inverted gradient maps to the area containing actively dividing TA cells and stem cells (Figure 4). The biological nature of the gradient remains to be fully defined but soluble paracrine signals such as keratinocyte growth factor (KGF) are known to arise from mesenchymal cells and regulate keratinocyte proliferation (Rubin *et al.*, 1995; Wearing and Sherratt, 2000) and KGF is upregulated in psoriasis (Kovacs *et al.*, 2005). Where actively proliferating cells are too numerous/ few to be contained within this gradient, the basement membrane expands/ contracts in response.
- Stem cells always remain in the basal layer and do not move.
- To create 'psoriasis' the number of actively dividing stem cells were increased by a factor of 4 and the number of times a TA cell can divide was increased from an average of 4 times to 4.5 times.
- The model assumes that lesional psoriasis contains twice the number of nucleated epidermal cells compared to non-lesional skin, representing psoriasis of moderate severity (typical of the patients who are treated with UVB), however the model could be used to examine thicker psoriasis; which would take longer to clear.

Unlike previous models there is no maximum age specified for individual cells, and no cell is destined to spend a set period of time in either the proliferating or differentiating compartment (Grabe and Neuber, 2007; Grabe *et al.*, 2006). This is a major advantage, as an arbitrary value such as maximal age of a cell will have a direct effect on the thickness of the epidermis, and therefore affects the whole model.

The following data was used in the mathematical model to simulate UVB irradiation:

- An area under the curve calculation using data from Figure 1c and supplementary Figure 2 was used to calculate the rate of apoptosis.
- For 3 MEDs 311nm, each cell is randomly given a probability of undergoing apoptosis following each irradiation; the probabilities used were 13% for stem cells and 20% for TA cells (see Figure 5). Differentiating cells do not undergo apoptosis in this model as this does not fit with the distribution of apoptosis seen *in vivo* (Figure 1b and supplementary Figure 3), and does not affect the overall outcome of the model (Figure 7d). Apoptosis occurs at least 12h following irradiation, with a mean time of 24h post-irradiation.
- As the number of proliferating cells decreases in response to treatment, it is assumed that change from 5 TA divisions back to 4 will occur gradually (as immunocytes and keratinocytes produce cytokines which drive the proliferative process) and this is reflected in the model.
- If a stem cell undergoes apoptosis it will be replaced over time by symmetrical division of a neighbouring stem cell. This new stem cell will divide 20% of the time (as for normal epidermis). Therefore the same number of keratinocyte stem cells would be expected in the epidermis before and after a course of phototherapy.

To view a movie of the model or for an interactive demonstration please visit: http://research.ncl.ac.uk/psoriasis. The movie starts showing normal epidermis (position 1) which is stable over time. At 1400h (hours are shown as 'ticks' at the top of the movie) a 'cytokine stimulus' is triggered (i.e. there is an increase in proliferation inducing cytokines due to an external trigger; see results section of the main paper), and the basement membrane expands in a serial manner from position 1-7. The stimulus is withdrawn at 1900h but the psoriasis remains stable, and the

epidermis maintains homeostasis in this state. At 2300h 7 doses of 3 MEDs 311nm UVB are administered (as described above), and these are given at 56h intervals (equivalent to 3 times per week). This returns the epidermis to the normal state, where homeostasis is retained until a further stimulus is received. Note that:

1. The thickness of the epidermis is not affected after the first UV doses are administered; most affects occur towards the middle and end of the course.

2. The stratum corneum is not modelled here as it is assumed to be a consequence of the underlying epidermal changes, but in clinical practice this would result in a hyperkeratotic surface.

3. The program interface allows the user to adjust the rate of stem and TA cell division, as well as the number of irradiations and their frequency. The model can either be started in the ‘normal’ position, or as ‘psoriasis’.

Supplementary Figures

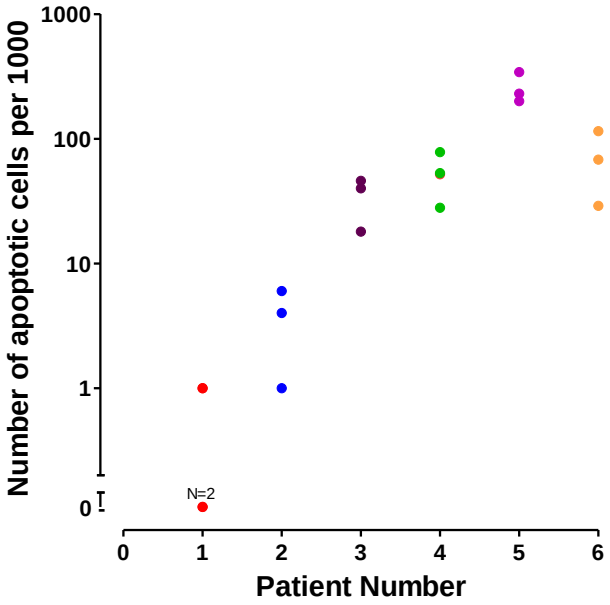

Figure S1. The number of apoptotic cells produced following a single 3 MED dose of 311nm UVB to lesional psoriatic skin *in vivo* shows intra-patient variability but is reproducible. Biopsies from different psoriatic plaques within individuals taken 24h post 311nm irradiation, a measurement error test showed significantly greater inter-patient variation than intra-patient variation ($P<0.05$). Matched un-irradiated psoriatic plaques in each patient did not show any apoptosis, confirming no pre-existing apoptosis.

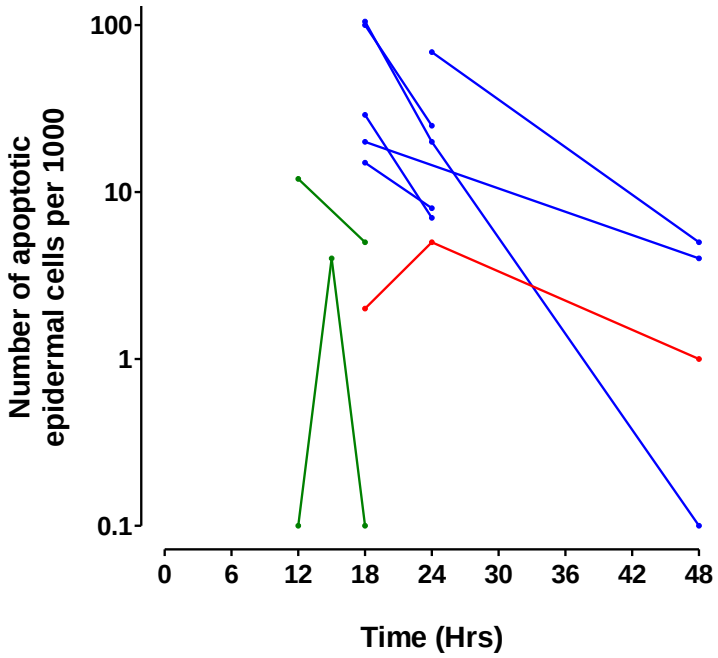

Figure S2. Individual time-course data following irradiation with 311nm in 9 representative patients. The timing of peak number of apoptotic cells varied from less than 18h (green) to 24h (red) in different patients. Note the rapid decline in the number of detected apoptotic cells with time, suggesting that these *in vivo* cells are removed from the epidermis within a matter of hours.

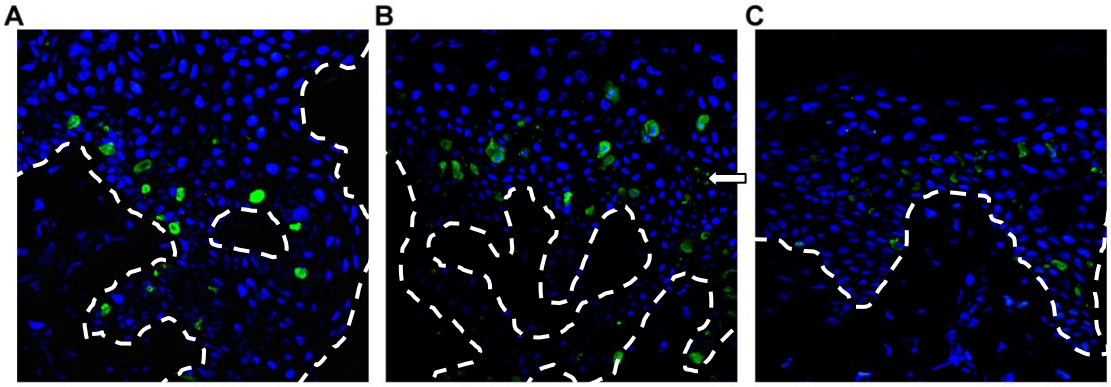

Figure S3. Apoptotic cells are located in the lower epidermis between 18 and 48h, without appearing to migrate up into the upper epidermis. Apoptotic cells (green) are located in the basal and suprabasal layers at different time points. Figure shows 3 biopsies taken from a typical individual at 3 time points: (**a**) 18h, (**b**) 24h, and (**c**) 48h post *in vivo* irradiation with 311nm. Toto-3 (blue) was used as a nuclear dye, and basement membrane shown as white line. Arrow shows fragmented apoptotic cells.

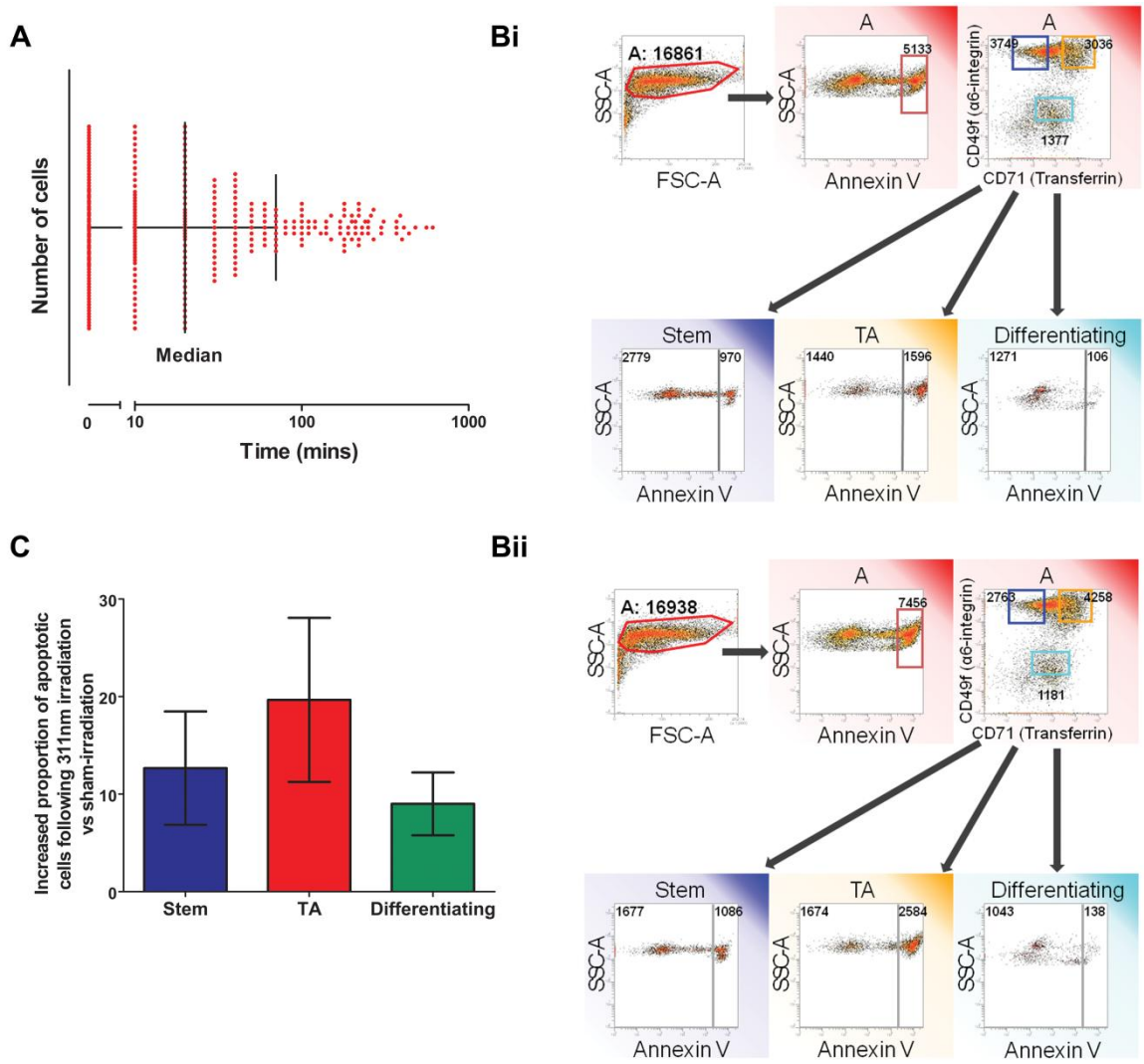

Figure S4. Apoptosis induced by 311nm UVB occurs rapidly, and in cells of all proliferative potential. (**a**) The time between apoptosis onset (membrane-permeable caspase-3 substrate activation) and PI entry (indicating breach of cell membrane integrity and therefore imminent cell death) shows wide variation. 100 cells were analysed in 3 separate experiments, each showing a median time of 20 min for apoptosis onset to cell death. All 300 cells are shown here with median and inter-quartile range. (**b**) Flow cytometric analysis of the apoptotic effect of a single 6 SED irradiation of 311nm UVB on normal human skin *ex vivo*. Keratinocytes were harvested from fresh skin 18h after irradiation and stained for apoptosis (annexin-V-FITC), and markers of proliferative potential; CD49f-PE and CD71-APC. Putative stem cells were identified as $CD49f^{+}APC^{-}$ (blue gate), TA cells as $CD49f^{+}APC^{+}$ (orange gate), and differentiating cells as CD49f– (aqua gate). The top left panels show all cells, with nucleated cells (gate A) used for analysis. The top middle and right panels show the actual number of apoptotic cells, and differential expression of proliferative markers from all nucleated cells gated in A. The lower panels show the actual numbers of apoptotic cells according to the expression of proliferation markers. (**bi**) Representative example of sham-irradiated epidermis, and (**b.ii**) skin irradiated *ex vivo* in the same patient. (**c**) The increase in actual number of apoptotic cells within each compartment was compared between irradiated and sham-irradiated controls, and expressed as a proportion in each experiment (n=3). Mean and standard error shown.

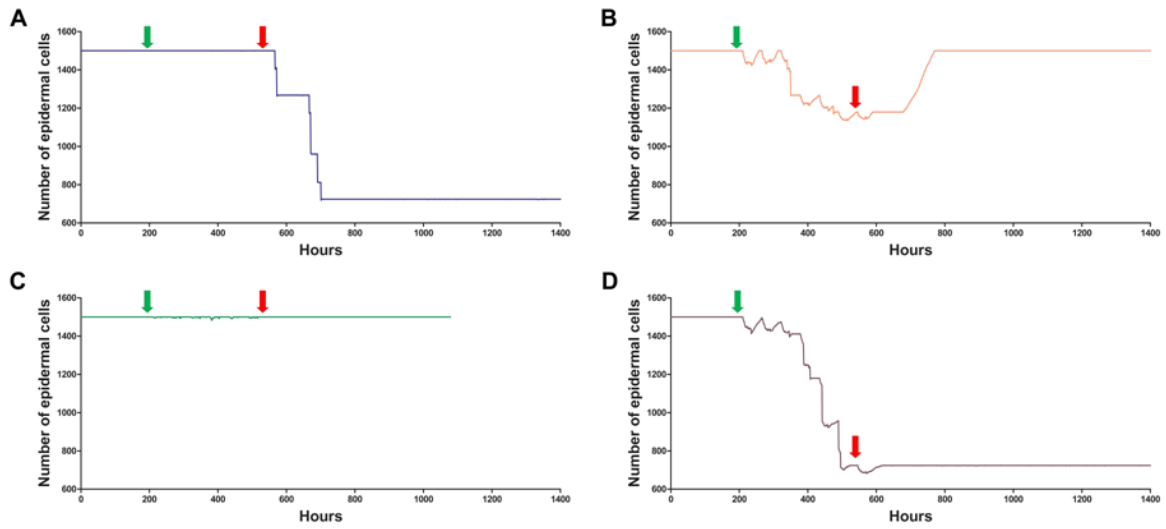

Figure S5. Both stem and TA cell apoptosis is required for clearance to occur during a course of UVB, and for lasting remission, *in silico*. Model simulations of 7 UVB treatments (3 MEDs) when apoptosis occurs in specific cell compartments only. **(a)** apoptosis occurring in only stem cells. There is a delay of over 500h between the first irradiation (green arrow) and the beginning of plaque clearance. **(b)** apoptosis occurring in only TA cells. Although there is a fairly rapid 'clinical' response seen with this model, resolution in the plaque is limited and temporary. **(c)** apoptosis occurring in differentiating cells alone. In this model, there is minimal impact on the epidermis, even when over 20 irradiations were given (not shown here). **(d)** apoptosis occurring in both stem and TA cell without an effect on differentiating cells. This model fits well with the clinical situation, as the initial treatments have little impact on plaque resolution, but complete clearance can be achieved at the end of the last (7^{th}) treatment, and can be maintained. Green arrows show time of first irradiation, and red arrows show the final treatment.

Supplementary data references

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