Imiquimod for the Treatment of Superficial Basal Cell Carcinoma

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In treating many cancers, surgery remains to be the “gold standard”. For effective cancer clearance and prevention of recurrence, patients tend to side with the surgical route. Most often they are informed that surgery is the best and only choice made available to them. There are other treatments however, that can be utilized as an alternative to surgery. These include treatments that use your immune system as a way to fight off cancer, all without picking up a knife. There are topical and oral medicines that have been on the market specifically for treatment of skin cancers that induce an immune response enough to cause effective cancer clearance. A topical medication of interest, Imiquimod, is an immune response modifier that has been shown to induce a local immune response and stimulate the production of interferon and cytokines (Testerman et al., 1995)

Basal cell carcinoma, BCC, is the most common type of skin cancer and typically afflicts fair skinned people with cumulative sun exposure. They are slow growing, locally invasive malignant tumors that can be grouped into low risk and high risk growths (Telfer, Colver, & Morton, 2008). These lesions arise from uncontrolled, abnormal growths within the basal layer of the epidermis. Radiation damage to keratinocytes as well as mutations in the PTCH receptor of the hedgehog pathway contribute to the pathogenesis of this cancer (Stockfleth et al., 2003). Normally, this pathway is turned off due to the PTCH receptor inhibiting the smoothened protein, SMO, from turning on GLI transcription factors responsible for cancer cell survival and proliferation. Mutations in the hedgehog pathway are responsible for over 90% of basal cell cancers. A picture of

this pathway is shown in Figure 1.

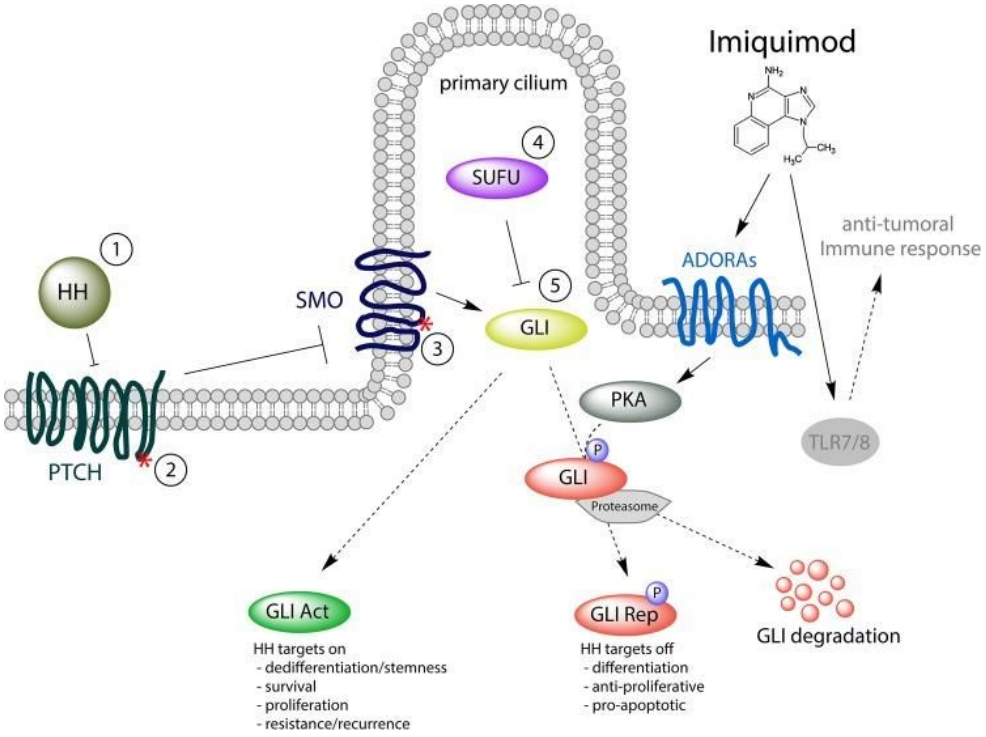

Figure 1. Hedgehog signaling pathway (Gruber, Frischauf, & Aberger, 2014).

Basal cell carcinomas can be classified histologically by their growth patterns into categories ranging from nodular to superficial to infiltrative. Superficial basal cell carcinomas, sBCC, are characterized by erythema, a scaly texture, and well-defined patches (Raasch, 2009). The incidence of basal cell carcinomas is ever increasing, nearly 10% each year worldwide and an estimated 40-50% of primary carcinomas will develop into one or more basal cells within five years (Madan, Lear, & Szeimies, 2010). Basal cells in general have low mortality but can have a significant impact on healthcare costs. The United States Medicare program spent an average of 562 million dollars in 3 years treating non-melanoma skin cancers such as BCC (Mckay et al., 2013). With the

increasing rates of skin cancer, it is important to study non-invasive methods that are both cost and cancer effective.

When deciding on treatment regimens for sBCC, a few things must be taken into account: the age of the patient, their immune status, the site of lesion, outcomes of cosmesis, and overall quality of life. These items must be assessed to determine if Imiquimod would be an appropriate treatment (Raasch, 2009). Current treatments for basal cell carcinoma include complete excision, MOHS surgery, curettage and desiccation, and cryosurgery. MOHS is known as micrographic surgery, where thin layers of cancer containing tissue are removed until there is only cancer-free tissue that remains. MOHS should be reserved for infiltrative tumors and is not recommended for superficial lesions. Surgery remains the most common treatment as it allows for adequate excision and examination of cancer margins. Surgery for treatment of sBCC results in cancer recurrence rates of less than 2%, with decent cosmetic results (Raasch, 2009). Even though surgery remains the "gold standard" of treatment, it can leave patients unhappy with facial and bodily disfigurements due to the extensive margins of the tumor (Dummer et al., 2003).

Imiquimod was FDA approved for sBCC in 2004 based on two double-blind clinical studies, indicated for tumors with a maximum diameter of 2.0 cm and only when surgical methods are less appropriate (Raasch, 2009). Imiquimod is a topical immune response modifier that binds to toll-like receptors 7 and 8, and induces cytokine production such as interferon (Dummer et al., 2003). It is a nucleoside analogue of the imidazoquinoline family and has been found to stimulate an immune response, secrete

cytokines, induce apoptosis, and recruit cellular infiltrates to the site of the tumor (Schon, & Schon 2007). The structure of imidazoquinoline along with the derivative producing imiquimod is shown below. These several mechanisms of action will be discussed in further detail through out the paper.

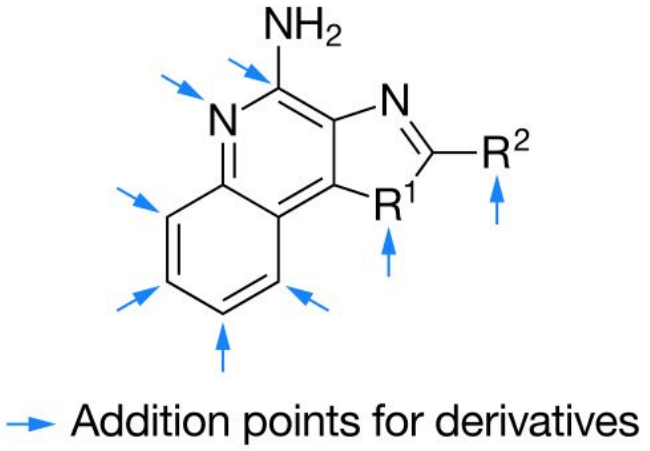

Figure 2. Imidazoquinoline molecular structure with potential sites for derivatives ("Imidazoquinoline," 2017).

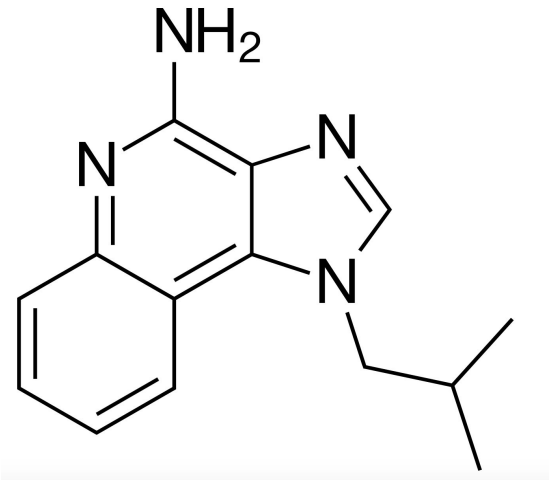

Figure 3. Molecular structure of imiquimod ("Imiquimod," 2019).

Imiquimod use is reserved for tumors that meet certain requirements as it's currently only FDA approved for superficial BCCs. Physicians and patients need to take into account tumor thickness as a predictor of imiquimod efficacy. The definition of superficial basal cell carcinoma is vague, as its defined as a tumor in which "proliferating basal cells grow down from the epidermis into the superficial dermis, while maintaining attachment to the base of the epidermis". Tumor thickness can help determine an appropriate treatment regimen. Mckay et al. (2013) sought out to better define the 'superficial' aspect of basal cells by observing recurrence rates in individuals with different tumor thicknesses following imiquimod treatment. They examined 127 sBCC biopsy samples after being treated primarily with imiquimod five times a week for six weeks. The average follow-up was 34 months and categorized into lesion recurrence or no recurrence. The average tumor thickness for non-recurrent lesions was 0.26 mm and for recurrent lesions was 0.57 mm. Using univariate analysis, there was a significant difference found between recurrence and non-recurrence in relation to tumor thickness, giving a p-value of <0.0001. For lesions measuring less than 0.40 mm, there was a significant difference between lesion recurrence and non-recurrence with the entire sample size having zero recurrence when lesions measured < 0.40 mm, producing a p-value of <0.0001. This also produced a significant p-value of <0.0001 for lesions measuring over 0.40 mm, with a recurrence rate of 58% (Table 1). This data suggest that lesions measuring under 0.40 mm are appropriate to use for imiquimod treatment, as they have reported zero instances of recurrence. For lesions measuring over 0.40 mm, there was a significant recurrence rate of 58%, illustrating that imiquimod should be reserved

for superficial lesions that do not extend deep into the dermis. These data should be used to determine treatment regimens for BCC and predict recurrence rates following imiquimod treatment to provide the patient with the necessary information before deciding to proceed with a topical route (Mckay et al., 2013).

Table 1. Analysis of tumor reoccurrence after imiquimod treatment (Mckay et al., 2013).

		Lesion recurrence			
Variable		No	Yes	N	P-value
Biopsy margin	Negative	25 (100)	0 (0)	25	0·1199
	Positive	91 (89)	11 (11)	102	
Tumour thickness (mm)		0·26 (0·09–0·61)	0·57 (0·41–1·41)	127	< 0·0001
Thickness	≤ 0·4 mm	108 (100)	0 (0)	108	< 0·0001
	> 0·4 mm	8 (42)	11 (58)	19	

The numbers provided are n (row%) or median (range).

The exact mechanism of action for imiquimod is still conjecture. It works through several different mechanisms to induce an immune response and the interplay between them is still being researched. It is known imiquimod induces autophagy and apoptosis. It is also known that imiquimod stimulates cytokine production through the activation of Toll-like receptors 7 and 8. Cytokines serve as signaling molecules that play an important role in immune system response as they can initiate a cascade that inhibit or enhance the effects of other cytokines. Toll-like receptors serve as immune system activators that mediate cytokine production. Specific to basal cell carcinoma, imiquimod also directly inhibits Hedgehog signaling through protein kinase A-mediated GLI phosphorylation. Phosphorylation of GLI is carried out by protein kinase A, a cAMP-dependent protein kinase. GLI transcription factors are responsible for promoting cancer cell survival,

proliferation, and anti-apoptotic properties. Phosphorylation of GLI transcription factors is responsible for turning off this pathway. Lastly, imiquimod induces tumor regression through cellular infiltration of CD4, CD8 cells, and dendritic cells.

To analyze if imiquimod induces both autophagy and apoptosis, and if these two processes work together cooperatively, Huang et al. (2010) set up a series of experiments to observe these processes and if they are induced simultaneously. Apoptosis can be observed by membrane blebbing, DNA fragmentation, and formation of apoptotic bodies essential as a self-destruction process. To observe the anti-tumor function of Imiquimod, cell viability was analyzed and quantified using an XTT assay. This assay allows for observation of live cells as they can reduce the XTT dye, producing an observable purple color. Basal cell carcinoma cells were treated with varying concentrations of Imiquimod over 24, 48, or 72 hours. The percent of viable cells decreased in a time and dose dependent manner with the lowest cell viability at 100 ug/mL of Imiquimod at 72 hours. To determine if the decrease in cell viability was attributable to apoptosis, a DNA content analysis was performed on BCC cells. After treatment with different concentrations of Imiquimod over 48 hours, there was a substantial increase in the amount of sub-G1 cells (Figure 4b). Apoptosis in cells can be characterized by DNA fragmentation, resulting in cells with reduced DNA content, indicated by the increase in sub-G1 cells. This trend continued when BCC cells were observed over 48 hours at a constant concentration of 50 ug/mL (Figure 4c). These results are consistent with apoptosis being the cause of diminished viability due to DNA fragmentation resulting in reduced DNA content (Huang et al., 2010).

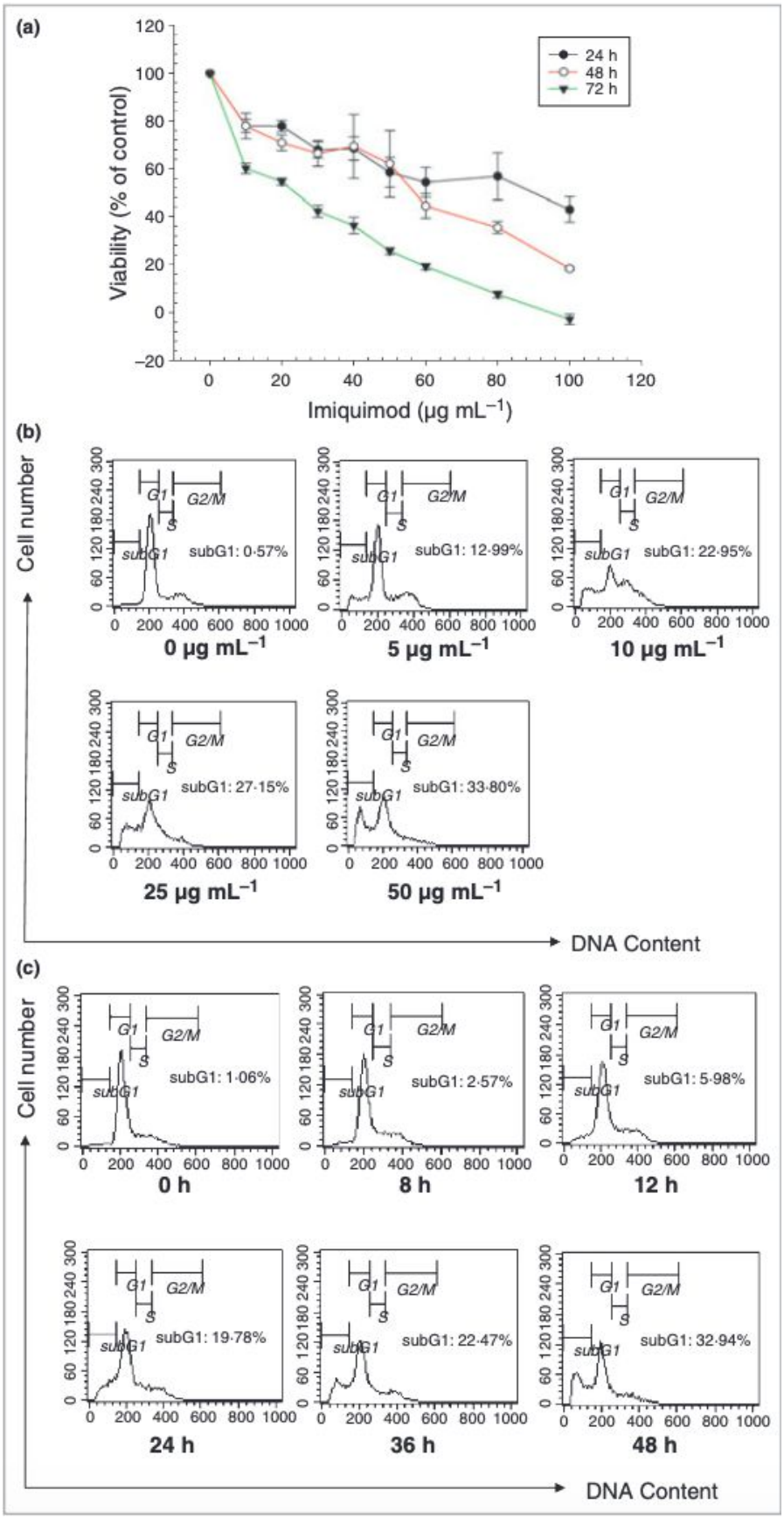
(a)
Viability (% of control)
Imiquimod (μg mL−1)
24 h
48 h
72 h
(b)
Cell number
DNA Content
G1
G2/M
S
subG1
subG1: 0·57%
0 μg mL−1
subG1: 12·99%
5 μg mL−1
subG1: 22·95%
10 μg mL−1
subG1: 27·15%
25 μg mL−1
subG1: 33·80%
50 μg mL−1
(c)
Cell number
DNA Content
subG1: 1·06%
0 h
subG1: 2·57%
8 h
subG1: 5·98%
12 h
subG1: 19·78%
24 h
subG1: 22·47%
36 h
subG1: 32·94%
48 h

Figure 4. Imiquimod induces apoptosis in basal cell carcinoma cells. (a) Assessment of cell viability through an XTT assay after treatment with varying imiquimod concentrations at different incubation periods. (b, c) DNA content analysis of BCC cells via flow cytometry after Imiquimod treatment at different concentrations and incubation periods (Huang et al., 2010).

For autophagy, key proteins were observed after incubation with Imiquimod. Autophagy is the regulated degradation of cell parts through the formation of autophagosomes and lysosomes. The cellular waste is contained in a membrane called an autophagosome, which eventually fuses with a lysosome where lysosomal enzymes degrade the contained cell parts. Autophagy can be observed through LC3-I to LC3-II conjugation, indicative of autophagosome formation, necessary for cell degradation. Researchers subjected harvested BCC cells to 50 ug/mL of imiquimod over 16 hours (Figure 5a) and varying concentrations of imiquimod incubated at 4 hours (Figure 5b). The LC3 conjugation process was observed using immunoblotting with primary antibodies and incubated with the corresponding antibodies. As seen in figure 2a as time progressed, LC3-I lessened in band thickness as LC3-II increased. At time zero, LC3-II presence was hardly visible but as incubation time increased, the band darkened, indicating the conversion of LC3-1 to LC3-II and thus the formation of autophagosomes. This is also confirmed in figure 2b, with the darkening of LC3-II increasing at higher dosages of imiquimod over 4 hours, demonstrating LC3 conversion and therefore autophagosome formation. Autophagy can also be quantified by observing EGFP-LC3

puncta formation, which also indicates autophagosome formation. During autophagy, LC3 is recruited to autophagosomal membranes to fuse with lysosomes forming autophagolysosomes. Cells that are undergoing autophagy can be observed using fluorescently labeled LC3 puncta. BCC cells were cultured and transfected with pEGFP-LC3 plasmid DNA and observed with a confocal microscope. After treatment with imiquimod as seen in figure 2c, puncta formation was observed at 4, 8, and 12 hours indicated by the tight blue and green membrane formation. By inhibiting EGFP-LC3 puncta formation using 3-methyladenine, 3MA, researchers were able to observe the inhibition of puncta formation, indicating that imiquimod induced puncta formation contributes to cell death through autophagy (Figure 5). This inhibition, imiquimod with 3MA, can be seen in the microscopic image as it resembles the cells at time zero, prior to autophagosome formation (Huang et al., 2010).

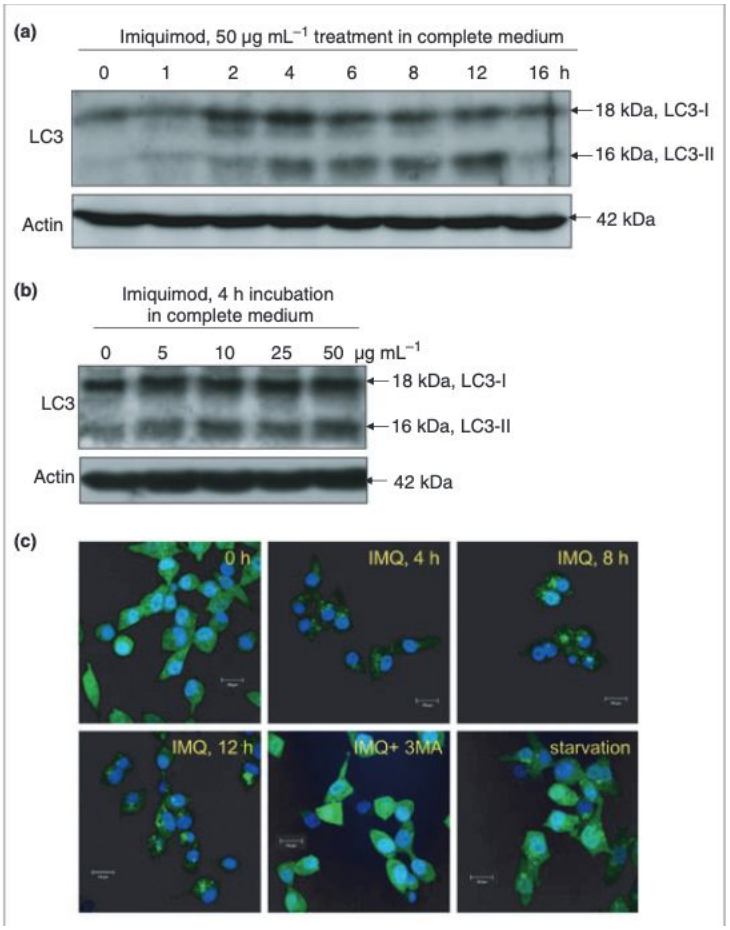

Figure 5. Imiquimod induces autophagy in basal cell carcinoma cells. (a) Conversion of autophagic protein LC3-I to LC3-II after treatment with 50 ug/mL of imiquimod. (b) Expression of LC3-I to LC3-II conversion at varying concentrations of imiquimod incubated at 4 hours. (c) Fluorescence staining of EGFP-LC3 puncta formation after imiquimod treatment (Huang et al., 2010).

To examine if autophagy and apoptosis were induced simultaneously, key proteins in both processes were observed through immunoblotting of Imiquimod treated BCC cells (Figure 6). Proteins essential in autophagy such as LC3 conversion, beclin 1,

and Atg5-12 conjugation were observed. LC3 conversion as well as Atg5-12 conjugation is involved in autophagosome formation, and Beclin 1 is an essential protein implicated in autophagic cell death. Apoptotic proteins included cleaved caspase 3 and 9, and cleaved PARP. Caspases regularly demolish cell structures and organelles, with 9 being involved in the initiation phase and 3 in the execution phase of apoptosis. PARP is an enzyme that responds to DNA strand breaks and when cleaved is inactivated, thus being a hallmark of apoptosis. Both autophagic and apoptotic proteins were observed using immunoblotting after incubation at 50 ug/mL of imiquimod over 24 hours. All of the proteins were expressed simultaneously within the imiquimod treated BCC cells, with actin serving as the control. Conversion of LC3-I to LC3-II increased in a time dependent manner as well as beclin 1, and Atg5-12 conjugation (Figure 6). Cleaved caspases as well as PARP followed the same trend, increasing with time but peaking around 8 to 12 hours (Huang et al., 2010).

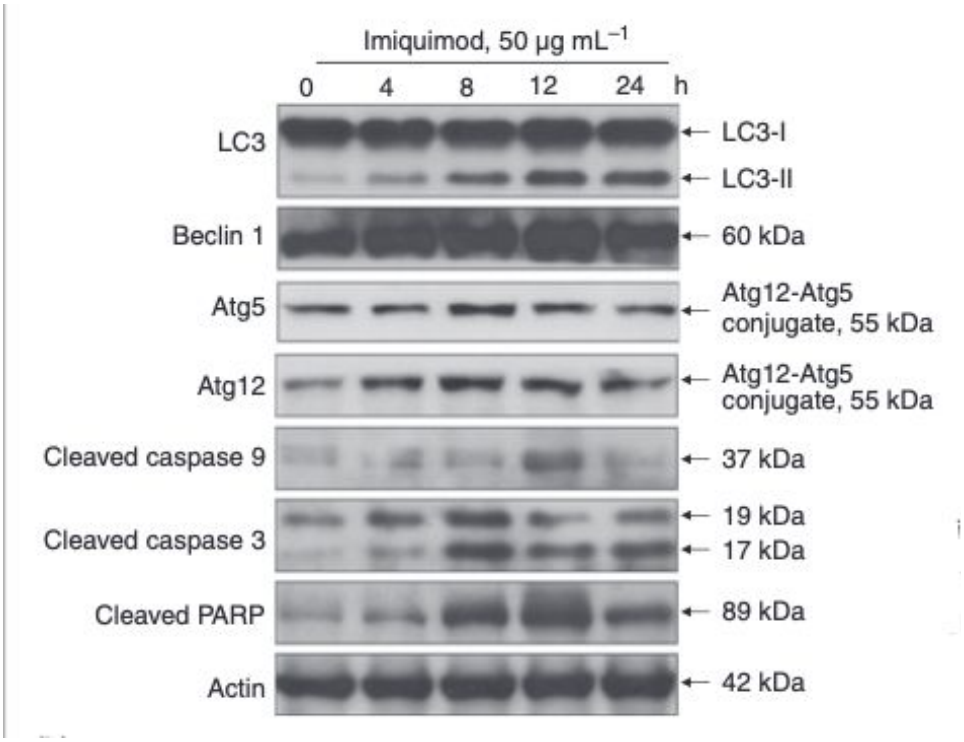

Figure 6. Simultaneous induction of apoptosis and autophagy in basal cell carcinoma cells after treatment with 50 ug/mL of imiquimod. Protein expression was determined by immunoblotting (Huang et al., 2010).

To observe the relationship between autophagy and apoptosis, imiquimod treated BCC cells were treated with caspase and PARP cleavage inhibitors. For inhibition of caspase 3, caspase 9, and PARP cleavage, imiquimod treated BCC cells were subjected with the zVAD-fmk inhibitor. Immunoblotting was used to observe the expression of these proteins and acridine orange staining to quantify the effects of the apoptotic inhibitor on the autophagy process. Acridine orange staining allows for detection and quantification of vesicular organelles used to visualize lysosomes and phagolysosomes involved in autophagy. Quantification of this staining was performed using an

enzyme-linked immunosorbent assay. BCC cells were treated over 12 hours at 0, 10, or 25 ug/mL of imiquimod and with or without treatment with 20 umol/L of zVAD-fmk (Figure 7a). When the apoptotic proteins treated with imiquimod were inhibited with zVAD-fmk, the autophagic protein LC3-I to LC3-II conversion was expressed, thus compensating for the loss of apoptotic proteins. Inhibition led to decreased expression of caspase 3, 9, and PARP indicating that the inhibition prevented cleavage of these proteins. When the apoptotic proteins weren't inhibited, they expressed appropriate cleavage and LC3 conversion wasn't expressed as intensely. There was a significant difference in acridine orange intensity between the 10 ug/mL of imiquimod treated cells and no treatment. There was a significant difference between the 10 ug imiquimod treatment and 10 ug imiquimod treatment with 20 umol of zVAD-fmk, with more acridine intensity when treated with the inhibitor, indicating phagolysosome formation. There was a significant difference in autophagolysosome formation between the 50 ug/mL and 10 ug/mL imiquimod treatment with an increase due to the higher concentration of imiquimod. There was also a significant difference between the 50 ug/mL imiquimod group and the 50 ug/mL imiquimod with 20 umol zVAD-fmk group (Figure 7b). When apoptosis was inhibited through zVAD-fmk, autophagy compensated and increased presence and formation of autophagolysosomes as compared to the non-inhibited group. These results suggest that when apoptosis is inhibited, it may accelerate imiquimod-induced autophagy (Huang et al., 2010).

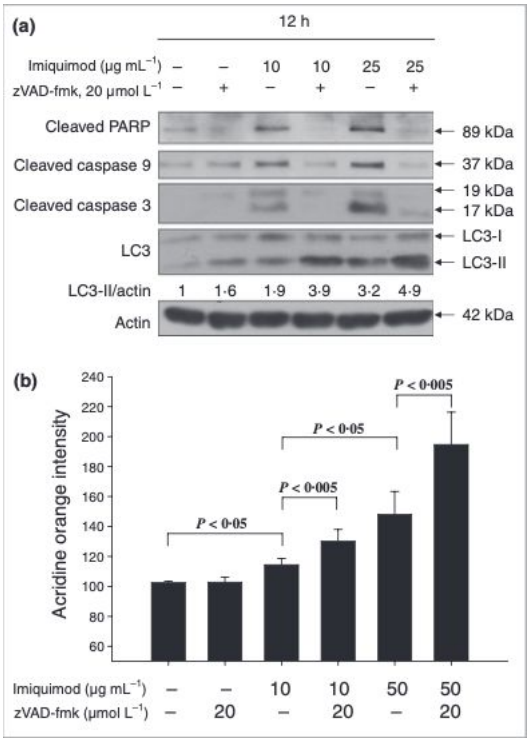

Figure 7. Inhibition of apoptosis accelerates imiquimod-induced autophagy in basal cell carcinoma cells. (a) Expression of caspase 3 and 9, PARP, and LC3 conversion at varying dosages of imiquimod over 12 hours. zVAD-fmk served as an apoptosis inhibitor. Expression was determined using immunoblotting. (b) Quantification of acridine orange staining with and without zVAD-fmk using an ELISA assay (Huang et al., 2010).

To determine if this acceleration was similar when autophagy was inhibited, researchers down-regulated proteins involved in autophagy and observed the apoptotic response. Beclin 1, Atg5, and LC3 conjugation were down regulated using respective siRNAs and expression of these proteins along with cleaved caspase 3 and cleaved PARP were observed. BCC cells were treated with no treatment or 50 ug/ml of imiquimod and

inhibition of beclin 1 or Atg5 (Figure 8a). When Atg5 and beclin 1 were down regulated, levels of cleaved caspase 3 and cleaved PARP increased. It was also observed that when subjected to down regulated Beclin 1 and Atg5 proteins, LC3 conversion decreased, showing that when these proteins are inhibited, LC3 conversion is as well. The down regulation of these autophagic proteins actually accelerated the apoptosis process by increasing the percentage of subG1 cells (Figure 8b). There was a significant difference between the 50 ug/mL Imiquimod treated group and the non-treated group, both with no siRNA down regulated proteins. There was a significant difference between the 50 ug/mL imiquimod group with the control siRNA and the 50 ug/mL imiquimod group with down regulation of Atg5 protein. There was also a significant difference between the imiquimod 50 ug/mL group with the control siRNA and down regulation of Beclin 1 (Figure 8b). Due to the inhibition and depletion of specific proteins involved in autophagy, apoptosis of BCC cells increased in the percent of subG1 cells to compensate for this loss (Huang et al., 2010).

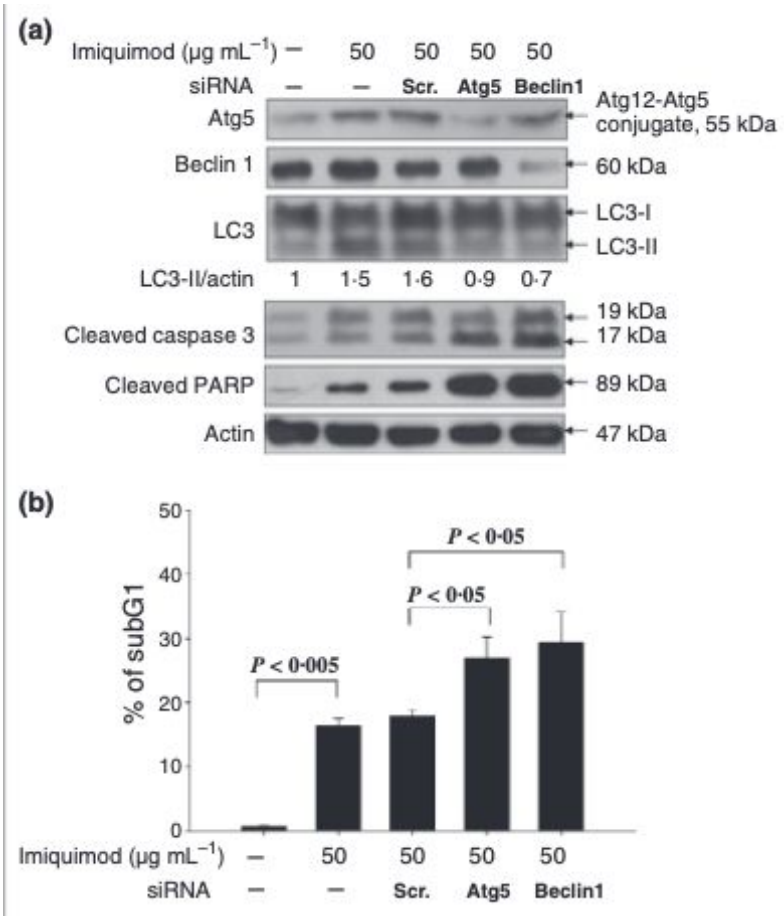

Figure 8. Inhibition of autophagy accelerates imiquimod-induced apoptosis. (a) Expression of apoptotic and autophagic proteins determined by immunoblotting. (b) Quantification of sub-G1 cells involved in apoptosis, determined by a DNA content assay (Huang et al., 2010).

Imiquimod directly induces apoptosis through a decrease in cell viability and an increase in percentage of subG1 cells after treatment. It also induces autophagy as seen in the increase in expression of LC31 to LC32 conversion. When this process is inhibited, imiquimod induced EGFP-LC3 puncta formation is not observed. Not only does imiquimod induce both these processes but also it does so simultaneously. By

immunoblotting specific proteins to these processes, simultaneous expression was observed and increased in density over time. Apoptosis and autophagy have a relationship that also operates cooperatively. When one process is inhibited, the other compensates for that loss by accelerating its own functions. The effect imiquimod exerts on BCC cells operate through these routes and cooperatively work together to cause cancer regression and cell death (Huang et al., 2010).

Specific to basal cell carcinoma, imiquimod directly inhibits hedgehog signaling through stimulation of adenosine receptors and protein kinase A-mediated GLI phosphorylation. Over 90% of BCCs are caused through an abnormal activation of the hedgehog pathway, usually due to mutations in the PTCH receptor or SMO protein (Pellegrini, Maturo, Di Nardo, Ciciarelli, Gutiérrez García-Rodrigo, & Fargnoli, 2017). Normally, the PTCH receptor inhibits SMO from activating this pathway and transcribing oncogenic GLI factors responsible for cell proliferation, differentiation, and maintenance. Imiquimod directly binds to adenosine receptors, ADORAs, which affects protein kinase A, PKA. PKA mediates downstream signaling, and through activation by ADORAs, phosphorylates GLIs, glioma-associated oncogenes. The phosphorylation of GLI is a key repressive step in the hedgehog pathway, as it turns off transcription factors involved in cancer cell survival. The role of ADORAs in this process is the main focus as this is how imiquimod exerts its function in turning this pathway off. To study if imiquimod does operate through ADORAs, researchers studied the effects of GLI phosphorylation levels after imiquimod treatment in the presence of an ADORA agonist and antagonist (Wolff et al., 2013).

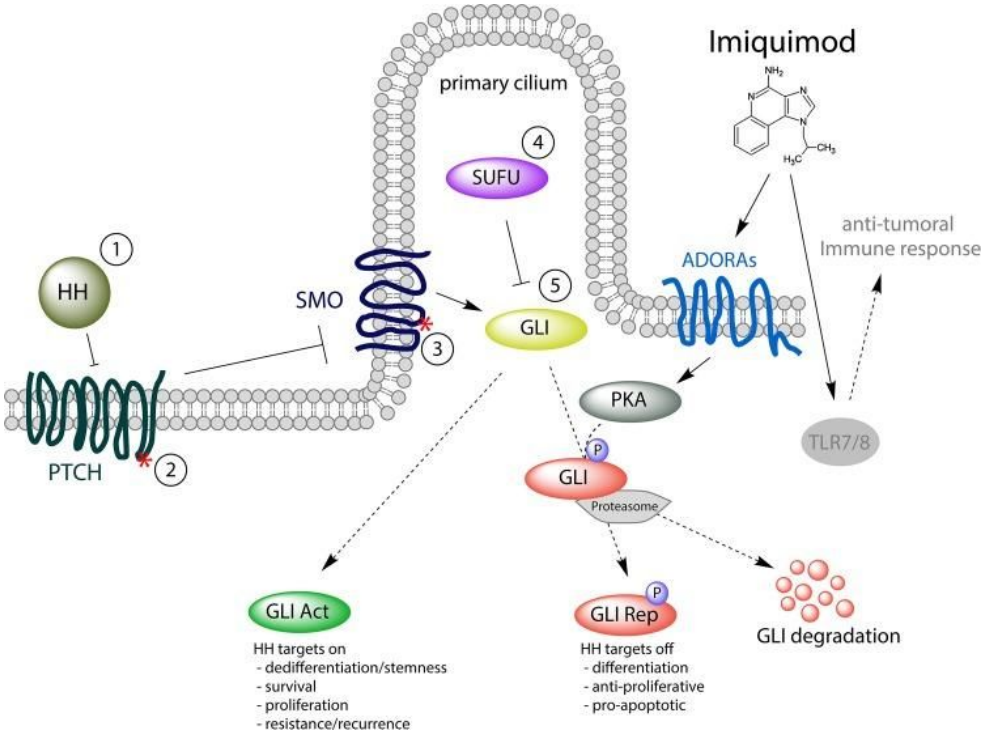

Figure 9. Hedgehog signaling pathway (Gruber, Frischauf, & Aberger, 2014).

Researchers analyzed GLI phosphorylation levels using a western blot analysis and quantified the results in a bar graph. GLI phosphorylation levels were measured on GLI2-expressing human keratinocytes after exposure to ADORA agonist and antagonist. For the western blot, GLI2 was precipitated with antibody against human GLI2. Phosphorylation levels were viewed in both short and long exposure time, allowing for observation of fainter bands if necessary (Figure 10b). GLI2 human keratinocytes were either treated with 10 ug of imiquimod for 6 hours or no treatment. Within these groups, the cells were then treated with an ADORA agonist and antagonist at either 1 um or 10 um, indicated by the black triangles. CGS was the ADORA agonist and SCH the

antagonist. With no imiquimod treatment and presence of the agonist, phosphorylation levels increased unlike with the antagonist. With imiquimod treatment alone, phosphorylation levels increased 10-fold as compared to the non-treatment group, as made evident by the differing axes. With imiquimod treatment and the agonist, phosphorylation levels increased within both short and long exposure. With imiquimod and the antagonist, these levels notably decreased with the most significant change in 10 ug of the antagonist (Figure 10c). These results suggest that imiquimod exerts its activity through ADORA receptors and when these receptors are inhibited, GLI phosphorylation levels are decreased (Wolff et al., 2013).

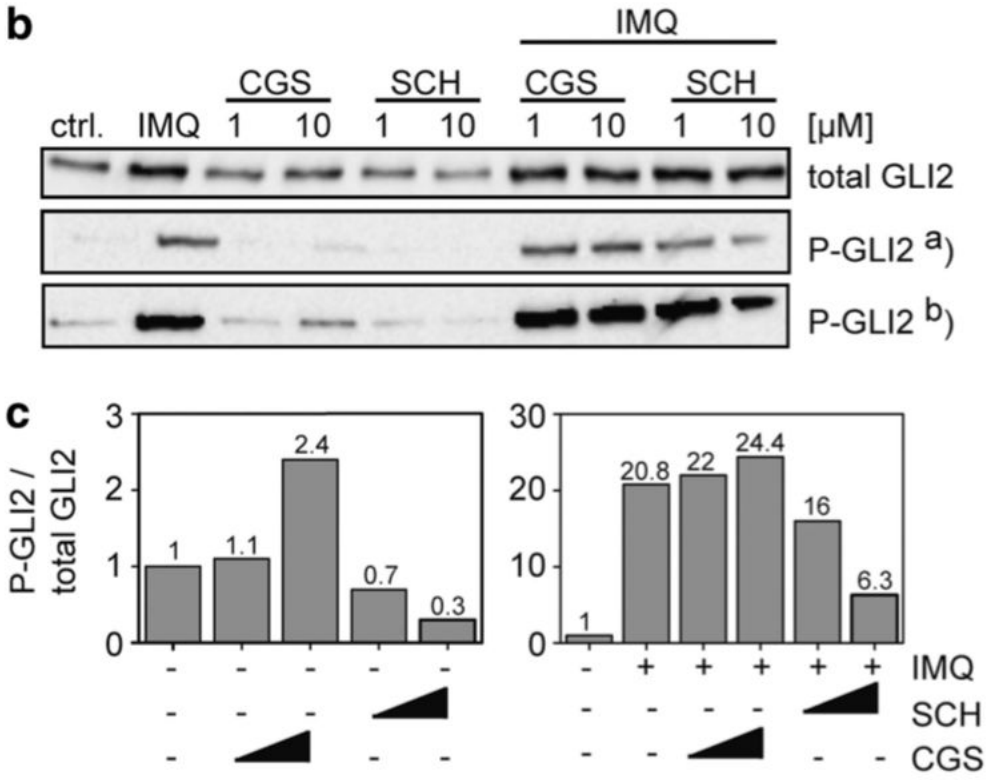

Figure 10. Modulation of ADORA receptor affects GLI phosphorylation levels and contributes to repression of the hedgehog-signaling pathway. (b) Protein expression observed using a western blot. P-GLI2a: short exposure, P-GLI2b: long exposure. SCH: ADORA antagonist, CGS: ADORA agonist. (c) Quantification of western blot; phosphorylation ratio values indicated at top of each bar (Wolff et al., 2013).

Imiquimod directly inhibits the hedgehog-signaling pathway through GLI phosphorylation mediated by PKA and ADORA receptors. This uncovers a once unknown mechanism of action for imiquimod specific to basal cell carcinoma. Imiquimod accomplishes GLI destabilization and repressor formation through binding to ADORAs. When these receptors are inhibited, GLI phosphorylation is decreased and thus GLI transcription factors are expressed. ADORAs remain necessary for imiquimod to exert its function and turn off the abnormal activation of this pathway. Since imiquimod operates through a different pathway than other medicines that target the PTCH receptor or SMO protein, if used in conjunction can work through multiple pathways for more effective cancer clearance (Wolff et al., 2013).

Imiquimod has also been shown to induce basal cell carcinoma regression through infiltration with various immune system cells that operate over different time courses. To analyze whether cellular infiltration is another mechanism by which imiquimod exerts its antitumor activity, researchers examined imiquimod-treated BCCs for macrophages, neutrophils, dendritic cells, CD4 T cells, and CD8 cells. Twelve patients with superficial BCCs were treated with 5% imiquimod five times weekly and four were treated with

placebo. Excisions were performed in four patients at week 1, 2, and 4. A 4mm punch biopsy was taken from the center of the excisions for immunohistochemical analysis and marked for the following cell types: CD4 T cells, CD3 T cells, CD8 T cells, dendritic cells, macrophages, neutrophils, and HLA-DR cells. CD4 cells are helper cells that differentiate and trigger an immune response, while CD8 cells are known as "killer cells" that secrete cytokines such as tumor necrosis factor and interferon. Dendritic cells act as messengers between the innate and adaptive immune system by activating T cells and initiating an immune response. HLA-DR is a receptor that presents antigens to the immune system to elicit or suppress a T-cell response. Cellular infiltrates were based on averages of positive cells. It was found that following imiquimod treatment, CD4 cells infiltrated early at week one, nearly twofold as compared to the placebo. This trend continued in the following two and four weeks with a significant difference between week 4 and the placebo group. CD8 cells infiltrated later on, with a significant difference between weeks two and four and the placebo group. Dendritic cells continuously increased over time with a significant difference between weeks two and four and the placebo group. Macrophages increased at week one and decreased slightly, with a significant difference between weeks one and two and the placebo group. Neutrophils were present in low numbers and increased the most at week one with no significant differences between the placebo and treatment groups. HLA-DR cells increased over two weeks and then slightly declined with a significant difference between the placebo group and week one, two, and four treatment groups (Figure 11). These results suggest that CD4 cells play a critical role in the mechanism of action for imiquimod and CD8 cells

more likely have a suppressive role than cytotoxic due to the later infiltration. The continuous increase in dendritic cells represent the rising immune response after imiquimod treatment. This study showed that imiquimod works through an immune-mediated mechanism as it recruits several, various cellular infiltrates with different time courses contributing to an imiquimod-mediated tumor-regressive effect (Barnetson, Satchell, Zhuang, Slade, & Halliday, 2004).

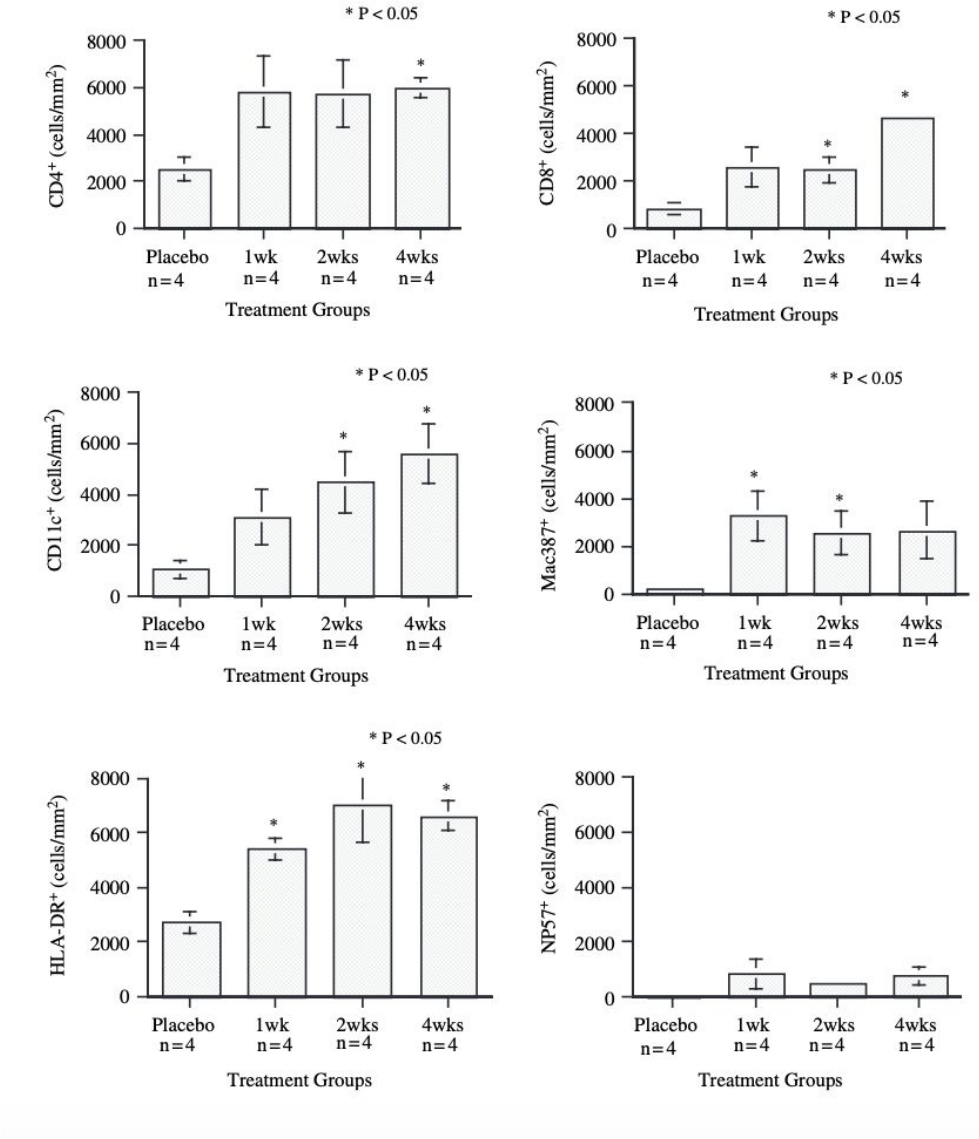

Figure 11. Imiquimod recruits cellular infiltrates in BCC cells over different time courses. Biopsies were taken at week 1, 2, and 4 and stained by IHC for CD4, CD8, CD11c, Mac387, HLA-DR, or NP57 (Barnetson et al., 2004).

It has been determined that imiquimod induces apoptosis, directly inhibits the hedgehog pathway, and recruits cellular infiltrates for effective cancer clearance.

Figuring out the specific proteins and routes of action involved in imiquimod's mechanism of action has been of interest since it is still a topic of conjecture. The hedgehog pathway provides specific proteins and one mechanism of action for how imiquimod carries out its antitumor effects. To research more into additional routes of action and the possible role other proteins may play, Vidal, Matias-Guiu, & Alomar, (2004) sought out the roles of Bcl-2, Ki67, and p53, as these proteins are specific to apoptosis, cell proliferation, and cell cycle regulation. Bcl-2 is a proto-oncogene that encodes an anti-apoptotic protein, and overexpression of this protein can result in cancerous features promoting tumor progression (Vidal et al., 2004). Ki67 is used as a marker for cell proliferation and is detected in the nucleus in G1, S, G2, and M of the cell cycle, but not G0. The protein p53 is a tumor suppressor gene involved in cell regulation and stability, and mutations in this gene can inhibit DNA repair. It mediates cell cycle arrest; so damaged DNA has time to repair if necessary, as well as triggering apoptosis when DNA damage is non-reparable. Researchers performed a double-blind randomized, clinical study on 30 patients diagnosed with basal cell carcinoma. There were 24 patients treated with 5% imiquimod either three times or five times a week. The remaining six patients were treated with imiquimod excipient, having no medicinal properties and used solely to facilitate physiological drug absorption. A 4mm punch biopsy was performed prior to treatment and at day 8 and day 15 during treatment for immunohistochemical analysis. Apoptosis was observed using TUNEL technique and immunohistochemical staining using monoclonal antibodies for the proteins (Vidal et al., 2004). TUNEL technique allows for observation of apoptosis through DNA fragmentation. It uses an

enzyme to label the 3' hydroxyl termini of DNA strand breaks in order to observe cells undergoing apoptosis.

Biopsies on day 15 showed that only 18 of the 30 original patients still had basal cell carcinoma. The expressions of Bcl-2, Ki67, p53, and apoptosis are shown in table 2 with p-values determined by a two-tailed student t-test. For both of the imiquimod treatment groups, Bcl-2 expression decreased from 88.7% on day 0 to 72.1% on day 8 to 61.4% on day 15 giving a significant p-value of 0.01. Apoptotic index increased from 0.53% on day 0 to 0.92% on day 8 to 1.66% on day 15 giving a significant p-value of 0.002. Ki67 and p53 had no significant changes over the treatment course. For the imiquimod treatment group three times weekly, there were no significant changes even though all three proteins decreased in expression. There was a significant increase however in the apoptotic index giving a p-value of 0.03. For the Imiquimod five times weekly group, Bcl-2 expression decreased from 93.5% on day 0 to 66.4% on day 8 to 46.9% on day 15 producing a p-value of 0.03. The apoptotic index significantly increased from 0.57% on day 0 to 0.99% on day 8 to 1.54% on day 15 producing a p-value of 0.05. Ki67 and p53 did not have any significant changes over the course of treatment. For the imiquimod excipient group, there were no significant changes in protein expression or apoptotic index over the treatment course. These results suggest that imiquimod treatment three and five times weekly decreases Bcl-2 expression. It also suggests that Ki67 and p53 are not modified by imiquimod and do not play a role in Imiquimod's mechanism of action. The apoptotic index increased after treatment with Imiquimod three and five times a week, showing that this is also an important mechanism by how Imiquimod exerts its

function. Imiquimod decreases Bcl-2 expression thus causing BCC cells to be more susceptible to apoptosis, and this decrease could contribute to the significant changes in the apoptotic index (Vidal et al., 2004).

Table 2. Protein expression and apoptotic index at baseline, day 8, and day 15. P-values obtained using ANOVA test (Vidal et al., 2004).

	Expression (SD) (%)			
	Day 0	Day 8	Day 15	P^a
Imiquimod $n = 15$				
Bcl-2	88·7 (15·1)	72·1 (32·7)	61·4 (33·5)	0·01
Ki67	38·9 (17·2)	33·0 (17·0)	33·8 (15·3)	0·2
p53	45·1 (41·7)	32·1 (34·3)	31·9 (31·3)	0·1
Apoptosis	0·53 (0·13)	0·92 (0·66)	1·66 (1·05)	0·002
Imiquimod 3 days per week, $n = 8$				
Bcl-2	84·6 (19·7)	77·2 (30·0)	74·3 (27·8)	0·3
Ki67	37·5 (17·8)	29·1 (14·4)	33·0 (18·3)	0·3
p53	31·8 (38·8)	24·6 (34·5)	24·3 (34·1)	0·4
Apoptosis	0·51 (0·04)	0·86 (0·68)	1·77 (1·23)	0·03
Imiquimod 5 days per week, $n = 7$				
Bcl-2	93·5 (5·7)	66·4 (37·1)	46·9 (35·5)	0·03
Ki67	40·6 (17·9)	37·5 (19·9)	34·7 (12·7)	0·3
p53	60·3 (42·4)	40·8 (34·7)	40·7 (27·6)	0·2
Apoptosis	0·57 (0·19)	0·99 (0·69)	1·54 (0·90)	0·05
Excipient $n = 3$				
Bcl-2	65·6 (34·1)	67·0 (37·3)	65·1 (37·9)	0·7
Ki67	31·2 (18·2)	45·0 (22·5)	36·0 (12·0)	0·7
p53	27·8 (43·5)	19·5 (26·5)	24·7 (33·0)	0·4
Apoptosis	0·53 (0·05)	0·49 (0·01)	0·49 (0·01)	0·4

Imiquimod has proven its efficacy in treating superficial basal cell carcinomas. The size of superficial lesions have been assessed to determine chance of recurrence with significant values showing lesions measuring less than 0.40 mm can have complete,

effective clearance with imiquimod. Surgery still remains the gold standard for treatment of superficial basal cell carcinoma as it has recurrence rates of less than 2% (Raasch, 2009). Imiquimod has been shown to induce apoptosis and autophagy in BCC cells and does so in a cooperative manner. Both processes work together for more effective cancer clearance and compensate when one another is inhibited. Imiquimod directly inhibits the hedgehog-signaling pathway by binding to ADORA receptors and activating protein kinase A to phosphorylate GLI, a glioma-associated oncogene. Mutations in this pathway are responsible for more than 90% of basal cell carcinomas. It acts as a key repressive step in inhibiting this pathway, thus turning off transcription factors responsible for cancer cell survival and proliferation. Imiquimod also induces tumor regression by recruiting various immune system cells that operate over different time courses. It recruits early infiltration of CD4 T cells and dendritic cells as well as later infiltration of CD8 T cells, consistent with an immune-mediated mechanism. Imiquimod induced apoptosis has been linked to decreased expression of anti-apoptotic gene Bcl-2. This linkage contributes to the apoptotic effect imiquimod induces on basal cell carcinomas. Imiquimod is a cost effective, non-invasive, therapeutic method that can be used effectively in superficial basal cell carcinomas. It can be utilized when tumors extend past margins in cosmetically important areas, or for patients where surgery is not an option. It is underused even though it has proven its significance throughout hundreds of studies. Imiquimod offers an effective choice to patients, to stimulate ones own immune system as a way of fighting cancer. Even though surgery remains the “gold standard”, patients

and physicians should be educated on the treatment regimens available, in order to make an educated decision concerning their own bodies.

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