Original Article

# Comparative Histological and Immunohistochemical Changes of Dry Type Cutaneous Leishmaniasis after Administration of Meglumine Antimoniate, Imiquimod or Combination Therapy

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**Abstract**

**Background:** This study compared histological and immunohistochemical changes of cutaneous leishmaniasis treated with meglumine antimoniate, imiquimod, and the combination of both therapies.

**Methods:** Single blind clinicopathological studies of fifteen patients with old world cutaneous leishmaniasis in Kerman, Iran were included. A total of four patients received a combination of imiquimod (5% cream) and intra-lesional meglumine antimoniate weekly for four weeks. Monotherapy with imiquimod was given to seven patients and four patients were treated with meglumine antimoniate intralesionally. Histological confirmation was performed before and during therapy. Semi-quantitative histological parameters such as numbers of mixed inflammatory cells (cells/mm$^2$) and percentages of Langerhans cells (CD1a+), T-cells (CD3+), B-cells (CD20+), and macrophages (CD68+) were calculated immunohistochemically in the dermis and adjacent epidermis.

**Results:** Topical imiquimod significantly reduced mean histiocytic cellular aggregation size ($P$<0.05). Meglumine antimoniate reduced parasite load and infected activated histiocytes in the dermis ($P$<0.05). Meglumine antimoniate therapy decreased epidermal CD3+ lymphocytes but increased them in the dermis, within the granulomas ($P$<0.05). During topical application of imiquimod a depletion of CD1a+ dendritic cells in the epidermis ($P$<0.05) and slight predominance of dendritic cells in the dermis were observed. Combined therapy and imiquimod monotherapy decreased CD68+ macrophages in the dermis ($P$<0.05).

**Conclusion:** Meglumine antimoniate decreases parasite load with considerable effect on up-regulation of T-cells, which demonstrates that meglumine antimoniate works as parasitocidal and immunomodulator, which could be as the first line of treatment. Imiquimod, accentuates the host immune response and reduces granuloma size which could be effective immunomodulator for combination therapy. Monotherapy of imiquimod is less effective than the two other regimens in decreasing parasite load, inflammation and congestion at the inoculated site.

**Keywords:** CD3, CD20, CD1a, CD68, cutaneous leishmaniasis, imiquimod, meglumine antimoniate

## Introduction

Leishmania species are protozoa belonging to the family trypanosomatoidae. They are obligated intracellular parasites transmitted to the mammalian host by bites of infected sand flies. Leishmaniasis refers to a diverse group of diseases depending on the infecting species.[1] World Health Organization subdivides leishmaniasis into two types, New World and Old World. Ninety percent of Old world cutaneous leishmaniasis (CL) occurs in Algeria, Syria, Saudi Arabia, Iraq, Iran, and Afghanistan.[2]

Cutaneous leishmaniasis (CL), as a dry type, caused by *L. tropica* is one of the most common parasitic diseases in Kerman Province, southeastern Iran.[3,4]

There are different types of treatments for CL, systemic and localized. Systemic type includes parenteral (I.V. or I.M.) pentavalent antimonials and oral agents (fluconazol, zinc sulfate, and azithromycin). Localized therapy includes intra-lesional injections of pentavalents, topical drugs (imiquimod and parmomycin) and physical methods (cryotherapy, $CO_2$ laser, topical heat, photodynamic therapy, and surgical excision of the lesion).[5,6]

Other modalities used for treatment are combination therapies. In recent years combination therapies with intralesional or systemic meglamine antimoniate have been used for new world CL with topical drugs such as imiquimod.[6–8]

Meglumine antimoniate is usually effective but should be given in adequate doses for the complete time-frame, often for weeks or months. For many years, it has been the most effective treatment for CL.[1]

Imiquimod is a form of imidazoquinoline, which works as an anti-tumor, antiviral and immunomodulator that accentuates host immune response by activating Langerhans cells, increasing IFN-α, TNF, IL1, and IL12 cytokines, enriching Th1 cells and enhancing apoptosis.[9] Imiquimod was investigated as a modulator of immune response such as changes of Langerhans cells migration from skin to lymph nodes,[10] as well as decrease of CD1a cells in treatment of lentigo malignant melanoma[11] and change in population of macropahges during treatment of basal cell carcinoma.[12] Imiquimod releases nitric oxide which can activate macrophages to kill *Leishmania amastigote*.[13] It leads to polarization of the immune response toward the Th1 response that is needed to kill intracellular leishmania.[14]

Use of the combination therapy of imiquimod with systemic meglu-

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mine antimoniate was successful for treatment of resistant new world CL and seems to be a good compromise between efficacy and toxicity.[7,8]

Based on the author's pilot study of a combination of imiquimod and intralesional meglumin antimoniate administered weekly for six weeks with a beneficial effect,[15] we attempted to study the histopathology and immunohistochemical changes of inflammatory cells, too. A later study in another center showed no beneficial clinical effect from the combination of a four week course of treatment with 5% imiquimod cream and a two week course of systemic meglumine antimoniate in patients with CL.[16]

There is no histopathogical study of CL, which evaluates the population and performance of host's defense cell response, both before and during therapy with meglumine antimoniate, imiquimod, and the combination of both. The current study is a morphometric and topographic study at the immunologic and histologic levels of acute and chronic inflammatory cells before and during treatment with imiquimod, meglumine antimoniate, and the combination of both medications.

## Materials and Methods

We did histopathologic and immunohistochemical studies on fifteen biopsies out of ninety cases whom had a comparative clinical study of the efficacy of combined imiquimod 5% cream and intralesional meglumine antimoniate vs. imiquimod 5% cream and intralesional meglumine antimoniate alone for the treatment of CL, *L. tropica*.[15]

After obtaining written consent from patients and, if needed, from guardians, punch, and incisional biopsies were taken both before and during treatment from 15 patients (8 female and 7 male). Patients underwent treatment for five weeks and were randomly assigned to three different treatment groups: i) imiquimod (7), ii) imiquimod with meglumine antimoniate (4), and iii) meglumine antimoniate (4). Imiquimod 5% (Aldara, 3M) as a topical was administered nightly for 4 – 8 weeks and intralesional meglamine antimoniate (0.5 – 1 ml/cm²) on a weekly basis.

On all biopsies, in addition to hematoxylin and eosin (H&E) staining, four immunohistochemical markers studies were done. The markers were monoclonal antibodies purchased from DAKO Company as CD3 (code m 7254 moaHu, clone F7.2.38; dilution 1/100), CD20 (code m9755 moaHu, clone L26; dilution 1/200), CD1a (code m3571 moaHu, clone O10; dilution 1/50), and CD68 (code m0814 moaHu, clone KP1; dilution 1/100).

Numerous epidermal and dermal histologic findings on H&E stain were defined by a visual semi-quantitative scale as: absent (0), subtle (1+), mild (2+), moderate (3+), and severe (4+). These parameters were based on previous studies of the histopathology of CL which were: inflammation, congestion, hyperkeratosis, parakeratosis, ulceration, acanthosis, flattening, exocytosis, abscess formation, spongiosis, apoptotic body, atrophy, fibrosis, pigment incontinency, pseudoepitheliomatous hyperplasia, and perineural lymphocytic infiltration.

Leishman body (parasite load) was confirmed in H&E sections with micrometer as the following semi-quantitative scale: absent (0), scattered visible only in oil immersion (+), some visible in high power field [400×, (2+)], many visible in mid-power field (3+).

Mean granuloma size was determined by measurement of histiocyte aggregation diameters by Ziess ocular micrometer. The semi-quantitative scale of granuloma size was: no granuloma (0), small sized granuloma <100 $\mu^2$ (1+), mid-sized granuloma 100-200 $\mu^2$ (2+), and large granuloma >200 $\mu^2$ (3+).

Numbers of different inflammatory cells (cells/mm$^2$) were determined by an ocular micrometer and manual hematology cell counter. Totally, 320 fields of H&E sections that included $6.5\times10^3$ inflammatory cells were counted by micrometer.

From a immunohistochemical point of view, the study was performed by microscopic ocular micrometer. Topography and percentages of Langerhans cells (CD1a), T-cells (CD3), B-cells (CD20), and macrophages (CD68) were calculated in total population of inflammatory cells inside and outside of the granuloma. Epidermal percentage of CD1a+ measured, compared to other cells in at least ten high power fields. Approximately 1200 fields of IHC sections were reviewed by micometer and counting performed within a population of $1.1\times10^5$ cells.

For data analysis, the correlations between variables were performed using SPSS computer program version 11.0 (SPSS, Chicago, IL) and hypothesis testing as paired *t*-test, ANOVA, and the Wilcoxin test. The significant level was $P<0.05$.

**Table 1.** Clinical response in three different regimen groups.

| Treatment group | Clinical findings | | | Number of patients |
|---|---|---|---|---|
| | Cure | Partial response | No response | |
| Imiquimod | 5 | 0 | 2 | 7 |
| Meglumine antimoniate | 2 | 1 | 1 | 4 |
| Combined | 3 | 0 | 1 | 4 |
| Total | 10 | 1 | 4 | 15 |

**Table 2.** Mean number of major inflammatory cells (cells/mm2) in dermal infiltrate before and during treatment.

| Treatment Groups | Before treatment | | | | During treatment | | | |
|---|---|---|---|---|---|---|---|---|
| | Epithelioid histiocyte | Active histiocyte | Lymphocyte | Neutrophils | Epithelioid histiocyte | Active histiocyte | Lymphocyte | Neutrophils |
| Imiquimod | 1893±9.84† | 757±145.45* | 3712±325.75 | 166±20.43* | 2272± 19.69 | 378± 87.87* | 4621±141.36 | 50± 7.45* |
| Meglumine antimoniate | 2575±81.81* | 606±187.88 | 3731±83.33* | 75± 8.42 | 1212±21.81* | 530± 12.88 | 4924±71.21* | 114±15.37 |
| Combined | 2384±358.33 | 454± 98.48 | 3939±477.27 | 23± 4.13 | 1515±495.45 | 681±355.30 | 4603±186.36 | 61± 5.22 |

†Values are mean±SD, *Difference between groups before and during treatment with $P<0.05$ are significant.

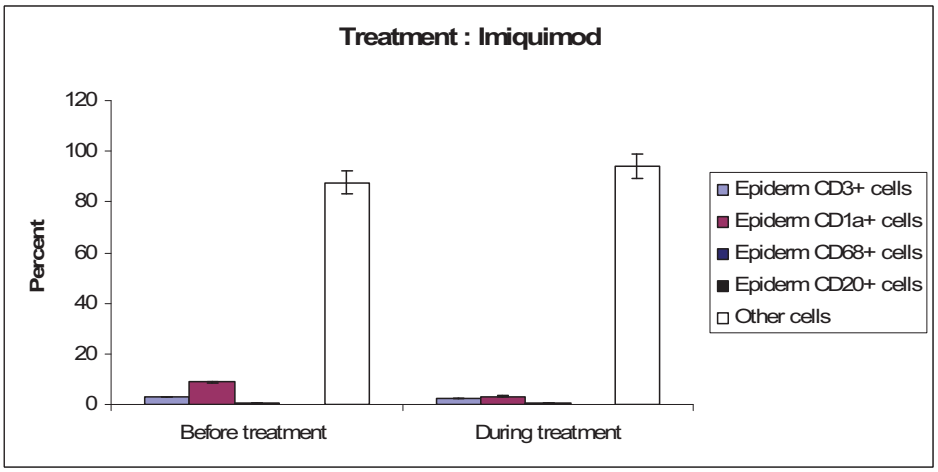


**Figure 1.** Comparing percents of CD3+, CD1a+, CD68+, and CD20 + cells in the epidermis before and during treatment with imiquimod.

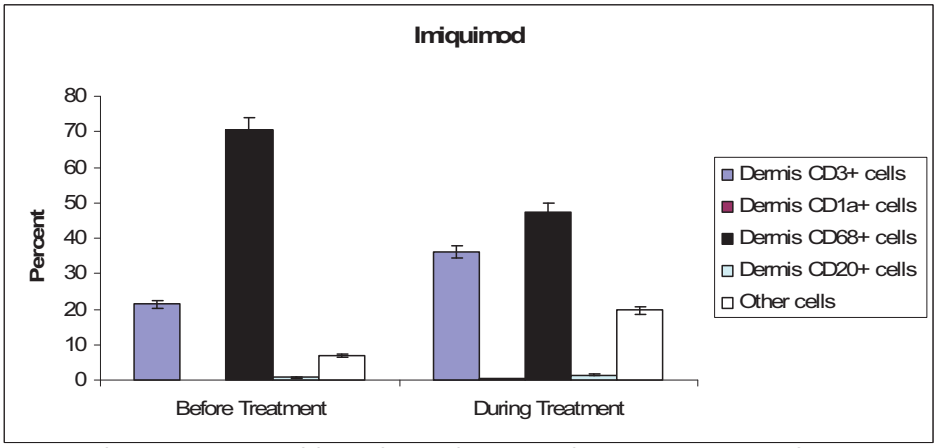


**Figure 2.** Comparing percents of CD3+, CD1a+, CD68+, and CD20+ cells in dermis before and during treatment with imiquimod.

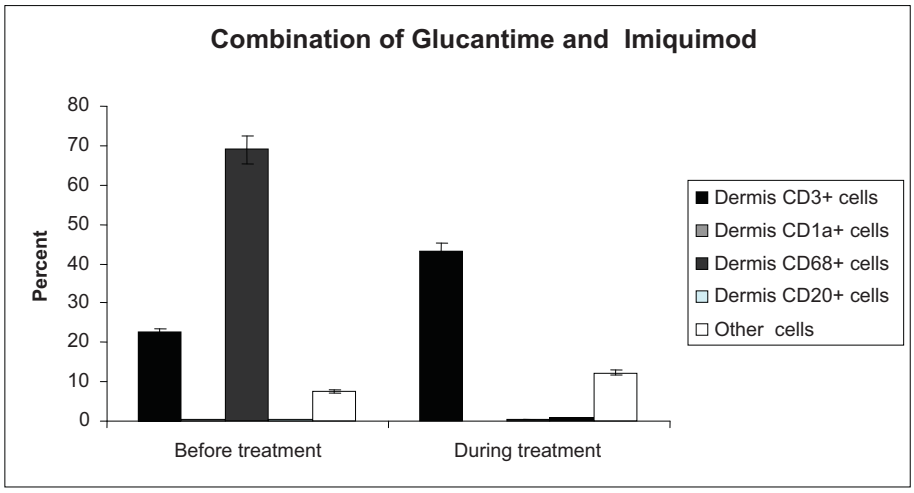


**Figure 3.** Comparing percents of CD3+, CD1a+, CD68+, and CD20+ In meglumine antimoniate monotherapy cells in the dermis before and during treatment with combination therapy.

## Results

A total of fifteen patients (eight female, seven male) participated in the study with an age distribution of 8 – 65 years old. Table 1 summarizes the clinical responses in these groups.

Results of H&E staining revealed monotherapy treatment with imiquimod significantly decreased the mean number of histiocytic cellular aggregation size from 2.75 to 1.4 ($P$<0.05). In the combination group, histiocytic cellular aggregation sizes also decreased.

Monotherapy with imiquimod decreased active histiocytes from 757 to 378 cells/mm², as seen in Table 2. Imiquimod decreased the mean number of neutrophils in the dermis from 166 to 50 cells/mm².

In meglumine antimoniate monotherapy, the mean numbers of parasite load considerably decreased from 1.5 to 0.75 ($P$=0.06). Monotherapy with meglumine antimoniate decreased mean num-

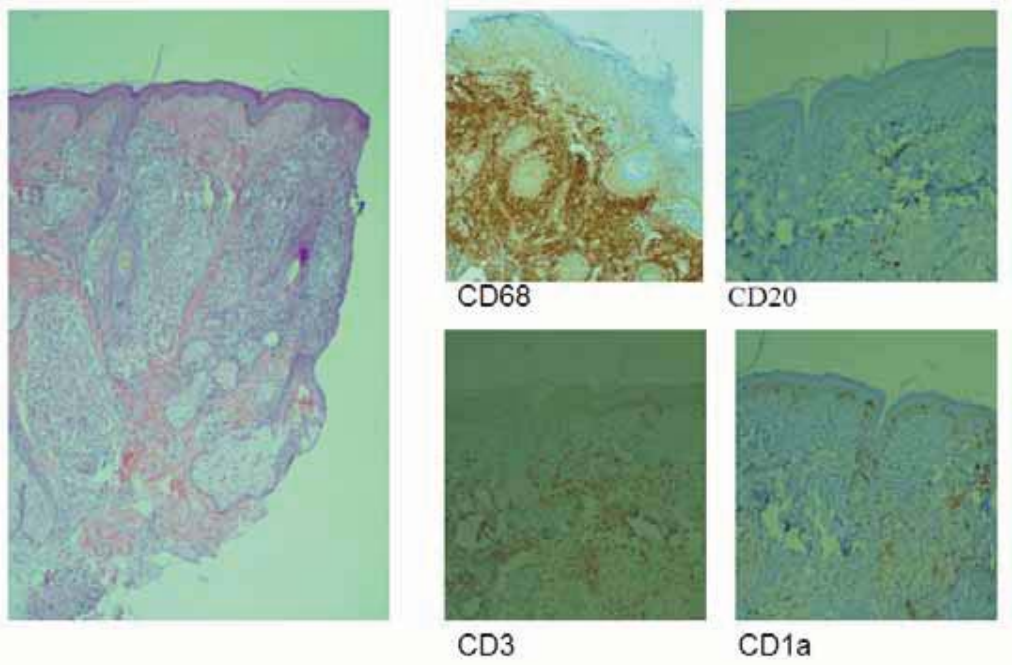


**Figure 4.** Histopathology(H&E, 50×) and immunohistochemistry(100×) of case no. 23 before combination treatment noting histiocytic cellular aggregations.

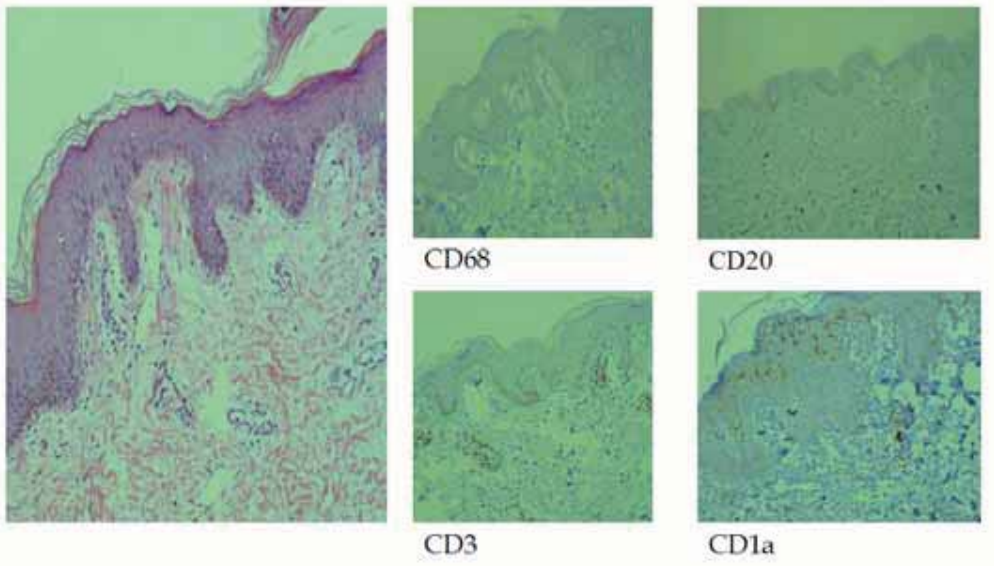


**Figure 5.** Histopathology (H&E, 100×) and immunohistochemistry (100×) of case no. 23 during combination treatment. Note decrease in all inflammatory cells.

bers of epithelioid histiocytes from 2575 to 1212 cells/mm² (Table 2).

Meglumine antimoniate monotherapy demonstrated a considerable increase in the number of lymphocytes (cells/mm²) during treatment, in comparison to the other groups.

In our study, measurements of the sixteen different histological parameters mentioned in the materials and methods showed no significant alterations between the three regimens, with the exception of meglumine antimoniate monotherapy, which reduced lymphocytic epidermal exocytosis ($P$<0.05).

### Immunohistochemical results

Imiquimod monotherapy decreased the percent of epidermal CD1a+ cells from 8.79 to 3.27% ($P$<0.05; Figure 1). Imiquimod increased CD1a+ cells from 0.19 to 0.5% in the inflammatory population of the dermis ($P$=0.08). There was considerable depletion of dermal CD68+ cells (macrophages) from 70.61 to 47.44% ($P$<0.05) in monotherapy with topical imiquimod (Figure 2).

Meglumine antimoniate monotherapy reduced epidermal CD3+ lymphocytes, but increased them within the dermis and intra-granuloma areas from 21.45 to 36.17% ($P$= 0.07). A slight depletion of epidermal CD1a+ cells and dermal CD68+ cells in meglumine antimoniate monotherapy were observed. A subtle increase of CD20+ cells in the dermis was noted ($P$=0.06).

Combination treatment decreased CD1a+ cells in the dermis from 0.43 to 0.10% ($P$<0.05) and reduced CD1a+ cells in the epidermis. This type of treatment significantly ($P$<0.05) depleted CD68+ cells in dermis infiltrates and granulomas from 68.98 to 43.1% ($P$<0.05; Figures 3 – 5).

## Discussion

The important findings from this study were a reduction in aggregations of histiocytes, decreased cellular parasitic load and increased numbers of lymphocytes in response to treatment.

These changes were seen more in the combination treatment when compared with monotherapy. Immunohistochemical studies demonstrated decreased epidermal CD1a+ cells and increased CD1a+ cells in the dermis.

Also noted were an increased number of CD3+ lymphocytes and decreased CD68+ macrophage populations in the dermis. These changes confirmed microscopic findings of routine H&E staining.

In this study, meglumine antimoniate significantly increased lymphocytes in the dermis as seen with H&E stain. CD3+ lymphocytes increased, which was the same as other similar studies.[17–19] A similar study by Pasteur Institute in Iran revealed an increase in the percentage of CD3+ cells in peripheral blood after treatment with meglumine antimoniate. They confirmed that meglumine antimoniate had a reverse effect on reduction of gamma-delta T-cells; it also increased CD3+ lymphocytes as confirmed by a flow cytometry study on blood.[17]

In another study there was an increase in the percentage of TC1 cells in patients with leishmaniasis under treatment with meglumine antimoniate.[19] There were some reports of HLA-DR changes on keratinocytes in acute CL under treatment with meglumine antimoniate and its role in host immune responses.[18] Histopathological and immunohistochemical changes in this study indicated that meglumine antimoniate effected the host immune response in addition to parasitocidal effects. Our study revealed decreased numbers of parasites according to a semi-quantitative scale in meglumine antimoniate treatment compared with the other two regimens.

Parasitocidal effects of meglumine antimoniate have been proven to reduce parasitic load in comparison with other drugs, via real-time PCR analysis.[20] The basic impact of this drug on parasites is by the topoisomerase I enzyme and a desirable effect is seen in decreasing the parasitic load in new world CL as confirmed by lominometric assay.[21]

In our study meglumine antimoniate significantly reduced active histiocytes and decreased lymphocytic exocytosis of the epidermis. Meglumine antimoniate increased the scattered population of dermal CD20+ cells. Until now, no study existed that compared the relation of CD20+ cells and meglumine antimoniate. Meglumine antimoniate effects both on cellular and humoral immunity.

Studies of new world CL revealed that the important mechanism for killing parasites by imiquimod is programmed cell death (apoptosis) of infected macrophages.[22] Imiquimod treatment for actinic keratosis is by apoptotic induction effects via the Fas ligand, BCL2 inhibition, and activation of caspases 3 and 9.[23]

Other histological findings in our study were inflammation, vessel congestion, and edema in patients treated with imiquimod. These findings confirmed common side effects of topical imiquimod such as erythema, edema, and ulceration.[24] Imiquimod activates nitric oxide synthetase enzyme, which increases nitric oxide (NO) production and consequently causes blood vessel dilatation, congestion, edema, wounds, and increasing immigration of white blood cells with inflammation.[25] We found that imiquimod significantly decreased neutrophilic cellular population in the dermis, which confirmed evidence noted in a literature review. On complete blood count from patients under treatment with imiquimod (13%), a decreased neutrophil absolute count was seen, which could be the reason for the drug's systemic effects.[26]

In the combination group, (imiquimod-meglumine antimoniate) a significant decrease in the number of dermal and epidermal CD1a+ cells and decreased CD68+ macrophages were suggestive of the synergistic effect of imiquimod with meglumine antimoniate. This combination also revealed clinical improvement in a pilot clinical study.[15]

Dermal CD20+ lymphocytes were seen as focal clusters in imiquimod monotherapy.

In one study where the numbers of CD3+, CD4+, CD8+, and CD20+ cells were estimated, scattered CD20+ cells were seen in the dermis, but they did not show a specific distribution.[27] In our study CD20+ lymphocytes were not as prominent as other cells.

The findings of this study based upon histological evidence suggested the importance of meglumine antimoniate as a basis for treatment in old world CL. The combination treatment of intralesional antimoniate with a new immunomodulator drug such as imiquimod can be more effective to enhance the immune response and reduce side effects of systemic antimoniate therapy.

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