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Determination of the Role of T-Helper 17 and 22 vs T-reg Cells in Patients with Molluscum Contagiosum

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Abstract:

Molluscum contagiosum is skin disease and well known by dermatologists, the causative agent is a pox virus. Infection, usually self-limited within few months in individuals with a normal health and immune system. From 205 case females recorded 95(46.3%) and 110(53.7%) males. The results showed that there were a highly significant increment in concentration of IL-17A (132.66 ± 24.7pg/ml) compared with control group which was (42.59±13.4pg/ml). Results also showed that there is highly significant increment in concentrations of IL-23P19 (112.33 ±17pg/ml) compared with control group which was (21.11±9.6pg/ml). Also documented data declared a highly significant increment in concentrations of IL-22 (77.11±8.8pg/ml) in comparison to control group which was (18.11±12pg/ml). The relative expression of the gene FoxP3 gene showed increment in patients group (17.723 ± 1.227) compared with control group which was (2.133±1.234), results of NFAT gene showed increment in patients group (13.676±2.223) compared with control group which was (2.743±1.133) and result of AP1gene showed increment in patients group (3.7883±1.234) compared with control group which was (1.8766±0.889). These findings indicate the potent immune response against the virus infection which belongs to Th-17 and 22 and the controlling mechanisms through the regulatory T-cells which maintain the response in the normal levels.

Keyword: Molluscum Contagiosum, T Helper 17, T Helper 22, Regulatory T Cell

1. Introduction

Molluscum contagiosum is one of the viral skin infections that resulted from the infection with poxvirus and occur mainly in young, children and sometimes adults. The disease is usually self-limited, except in case of patients with immune problems such as immune suppression or deficiency. But in normal individuals last in few months. In some cases Molluscum can persist for long period of time ; and that's may lead to certain complications especially in epidermis in which viral replication occur, immune competent cells usually play an important role [1]. Physicians usually didn't recommend chemotherapy due to spontaneous resolving of infection which will not leave any sign on skin in normal individuals [2]. Interestingly there is no effective specific drug against Molluscum, and only the applicable treatment is painful removal of infected skin which may leave scares, hypopigmentation [3]. The main step in development of Molluscum infection and the development of

disease is the effective immune response which is principally depend on Immune effector cells, especially CD4 cells have critical function in management of infection, including the induction of B cells and regulation of the cooperation of CD8 cells and phagocytes towards microbes [4].Depending on immunological action and function CD4 cells appeared as many linages, according to the expression of many transcription factors and , cytokines, and some cells receptors. Such lineages of effector cells, responsible of the protection against pathogenic microbes, another type of cells with regulatory function is Treg which responsible for controlling the level of immune response which may be very aggressive and harmful to host, as in some autoimmune diseases. naïve CD4+ T cells mainly differentiate into a specific phenotypes depending on the cytokine profile and composition. The current knowledge of CD4+ T cell differentiation include at least nine phenotypically different types: T helper type 1(Th1) and Th2, Th9, Th17, Th22 Follicular T helper cells (Tfh), induced regulatory T cells (Treg) and type 1 regulatory cells (Tr1) [6-9]. Th17 cells is a third subset of T helper cells changed the classical Th1/ Th2 example of T helper cell differentiation and Th17 cells are CD4+ T cells which are responsible for the production of interleukin-17A (IL-17A) [10]. IL-17A is produced usually by Th17cells and represents the important mediator in many autoimmune diseases, as in rheumatoid arthritis, Th17 frequency and existence in joints is strongly associated with disease severity.

Th22 cells are a unique subtype of $CD4^+$ T cells characterized by secretion of both IL-22 and TNF-α as well as expression of C-C chemokine Receptor type4 (CCR4), CCR6 and CCR10.Th22 also able to express other mediators such as fibroblast growth factor (FGF) isoforms which are important in tissue re-modeling. Recently, it is suggested that IL-22 and TNF-α produced by Th22 cells, synergistically balancing immune modulatory mediators [12]. Th22 are characterized by production of a set of cytokines, including IL-22, IL-13, and tumor necrosis factor (TNF)-α. Like Th17 cells, Th22 cells express IL-22, CCR4, and CCR6[13]. Th22 lymphocytes do not secret IFN-γ, IL-4 (Th2 marker) as well as IL-17, while IL-23 receptor, CCL20, CD161 (Th17 markers). Moreover, Th22 cells are distinct from Th17 cells because of high degree of multi- functionality and low expression of CD161 [14] All these cells are effector types, from the other hand there is a regulatory T cells (Treg) which have crucial function in controlling and directing the immune response away from self-antigens and pathogens . Foxp3 is transcription factor that is remarkable for this type of cell lineage. [15].

The formation of Treg by sub immunogenic activation and the differentiation from naïve cells, is resulted from negative effects of the strong costimulation, which lead to assembly of Fos and Jun driving AP-1 to interfere with NFAT resulting in the blocking the formation of FOXp3-NFAT complexes which is necessary for Treg [17]. The NFAT transcription factors family include four calcium-/calcineurin-regulated members, NFAT1-4, and NFAT5 [18] In activated T cells, the NFAT

usually forms crucial cooperative groups with the AP-1 (Fos-Jun) proteins on composite NFAT: AP-1 DNA elements [19]. This work was aimed to focus on some kinetics of T-cell subsets through determining the activity of T-helper 17 and 22 vs T-reg cells in patients with Molluscum contagiosum.

2. Materials and Methods

Skin biopsies were collected from 205 patients with Molluscum contagiosum and 40 healthy control individuals in Al-Qadisyia city/Iraq, from the period between 1/2/2014-30/9/2015. Serum samples were obtained by taking 5 ml of venous blood and were collected by sterile tubes and then allowed to stand for 20 min at room temperature then centrifuged at 1000 rpm, sera were immediately separated and stored at -20 C in three aliquots to avoid multiple thawing until the time of assay.

Virus detection :All samples were tested for the presence of the virus by using Elisa technique according to manufacture (Biolegened, USA) .

Primers: Primers that used in this work, *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) gene primers used as (Housekeeping gene), the tested genes ;NFAT gene, FoxP3 gene and AP1gene primers used as target genes for gene expression. These primers were previously designed by using NCBI-Gene Bank data base and Primer 3 design, and primers that used in the quantification of the gene expression using qRT-PCR techniques based SYBER Green DNA binding dye, and imported by (Bioneer, Korea). As in (Table -1).

Table 1. The Primers, sequences, gene bank accession number, and references.

Primer	Sequence		Reference
NFAT Gene	F	GT TGGGGAGT TGGCACTAGC	In this study
	R	GACCCGGGCT T TCTACTGG	
AP1 Gene	F	GGTGGGATAAGACCCCCTCA	In this study
	R	TCCTGCCTGCATAGCAATAGG	
FoxP3 Gene	F	TGTGCTAGGGCGGTATGAGA	In this study
	R	GCTGGGGTGCAACTATGGG	
GAPDH	F	ACGACCACTTTCTCAAGCTC	[20]
	R	T TCCTCT TGTGCTCT TGCTG	

2.1. Quantitative Reverse Transcription Real-Time PCR (RT-qPCR)

Quantitative Reveres Transcription Real-Time PCR technique was performed as assessment of comparative quantification (gene expression analysis of FOXP3, NFAT & AP-1). This technique was done according with the described method by [21]. The following thermo cycler protocol in the following (Table -2):

Table 2. Thermocycler protocol.

qPCR step	Temperature	Time	Repeat cycle
Initial Denaturation	95°C	3 min	1
Denaturation	95°C	20 sec	45
Annealing\Extension Detection(scan)	60°C	30 sec	
Melting	60-95°C	0.5 sec	1

2.2. Data Analysis of qRT-PCR

The data of results of qRT-PCR for target and housekeeping genes were analyzed according to the relative quantification gene expression levels (fold change). Reference method that described by[22].

2.3. Cytokines Assay

Serum levels of IL-17A, IL-23P19, IL-22 and TNF-α were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (KOMA biotech / Korea) according to the manufacturer's instructions.

2.4. Statistical Analysis

Obtained data were analyzed by SPSS version 10.0. Parameters that had been investigated were presented as mean± standard error (S.E). Least significant differences were done by ANOVA table (Variance analysis) depending on LSD(least significant difference) by finding differences among means. The difference was considered significant when the probability (*P*) value was ≤ 0.05 [23].

3. Results

Recorded results of this study showed that total recorded cases of infection is 205, from which 95(46.3%) were females and 110(53.7%) males, young adults patients (1-20) years old (56.1%) and (21-40) years old 60 (29.3%) years old constituted the highest ratio of infection as showed in (table-3).

Table 3. The patients of Molluscum contagiosum in relation to age and gender.

Gender	Age (year)				Healthy
	(1-20)	(21-40)	(41->60)	total	human control
Female	50(43.5%)	35(58.3%)	10(33.3%)	95(46.3%)	20
Male	65(56.5%)	25(41.7%)	20(66.7%)	110(53.7%)	20
Total	115(56.1%)	60(29.3%)	30(14.6%)	205(100%)	40

Interleukin-17A(IL-17A): The results indicated that there were a highly significant increment p≤0.05 in concentration of IL-17A(132.66±24.7pg/ml) compared with control group which was (42.59±13.4pg/ml). as shown in table 4.

Interleukin- 23P19 (IL-23P19): The documented data showed that there were a highly significant increment p≤0.05 in concentration of IL-23P19 (112.33±17pg/ml) compared with control group which was (21.11±9.6pg/ml). as shown in table 4.

Interleukin- IL-22 (IL-22): The obtained results of this study found that there were a highly significant increment p≤0.05 in concentration of IL-22 (77.11±8.8pg/ml) compared with control group which was (18.11±12pg/ml). as shown in table 4.

Table: 4. The results of IL-17A, IL-23P19 and IL-22 by ELISA.

No.	cytokine	patients	control
1	IL-17A	132.66±24.7pg/ml	42.59±13.4pg/ml
2	IL-23P19	112.33±17pg/ml	21.11±9.6pg/ml
3	IL-22	77.11±8.8pg/ml	18.11±12pg/ml

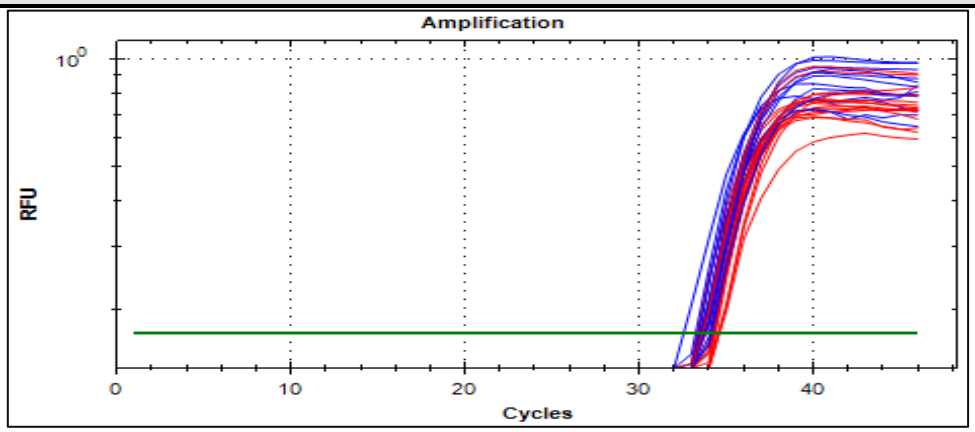

Figure 1. Real-Time PCR amplification plot of GAPDH gene in patients and healthy control groups. Where, blue plot patients samples and red healthy control samples.

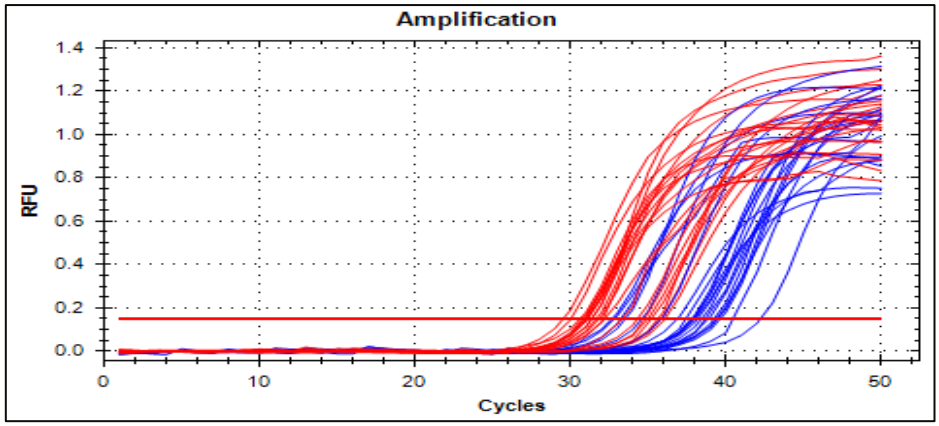

Figure 2. Real-Time PCR amplification plot of FoxP3 gene patients group samples (red plot) and healthy control group samples (blue plot).

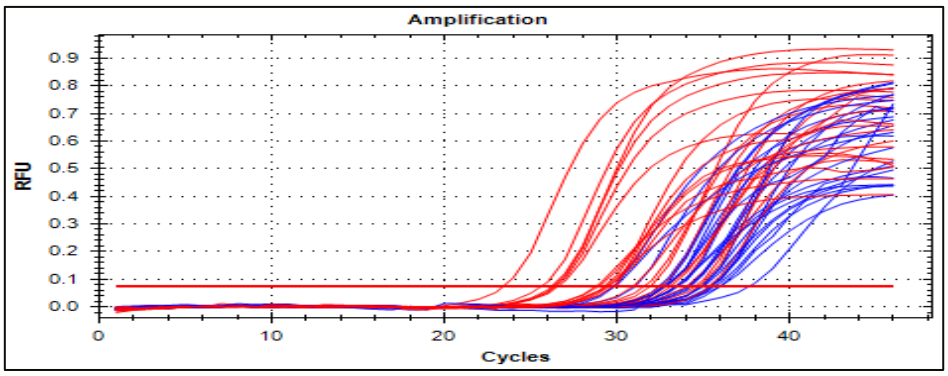

Figure 3. Real-Time PCR amplification plot of NFAT gene in patients group samples (red plot) and healthy control group samples (blue plot).

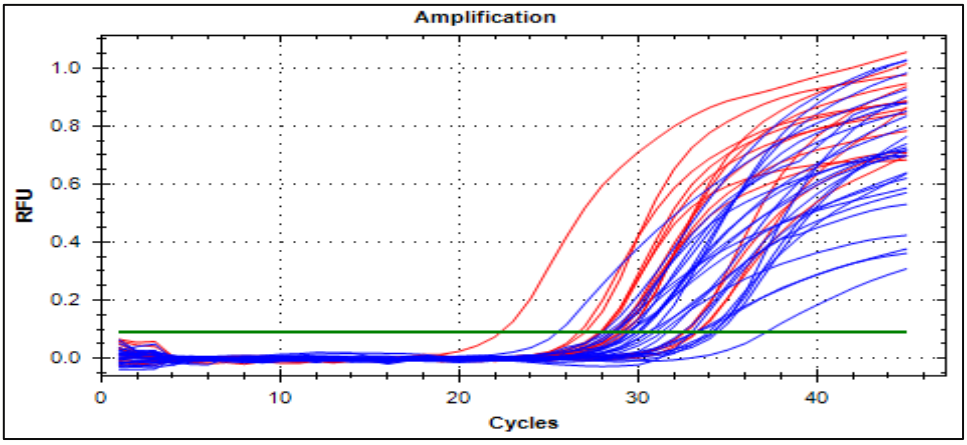

Figure 4. Real-Time PCR amplification plot of API gene in patients group samples (red plot) and healthy control group samples (blue plot).

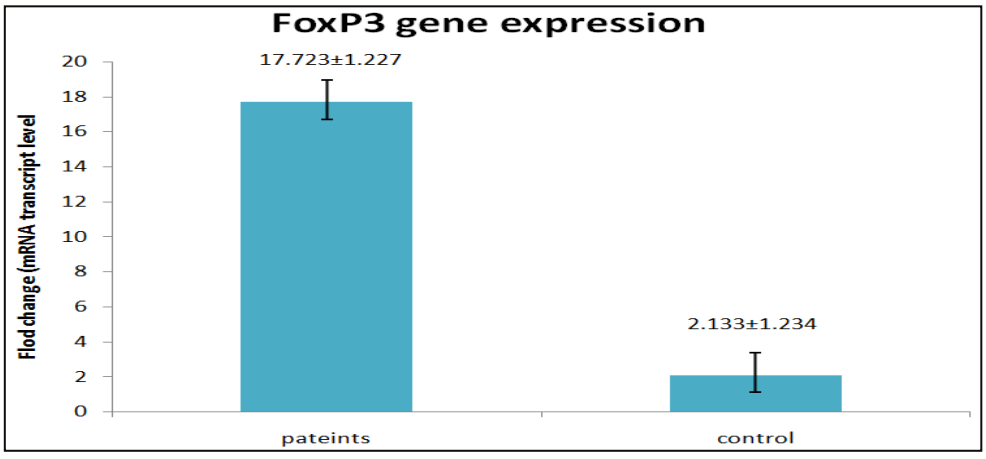

Figure 5. Gene expression of FoxP3 gene.

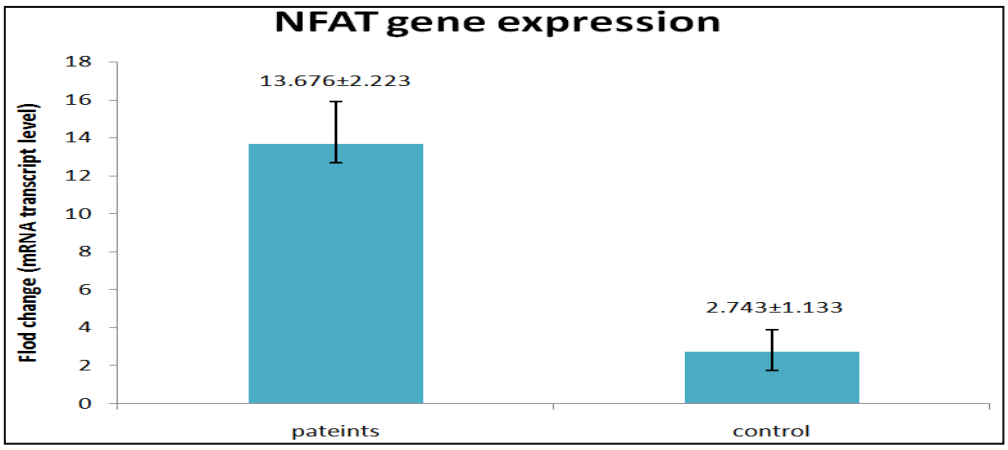

Figure 6. Gene expression of NFAT gene.

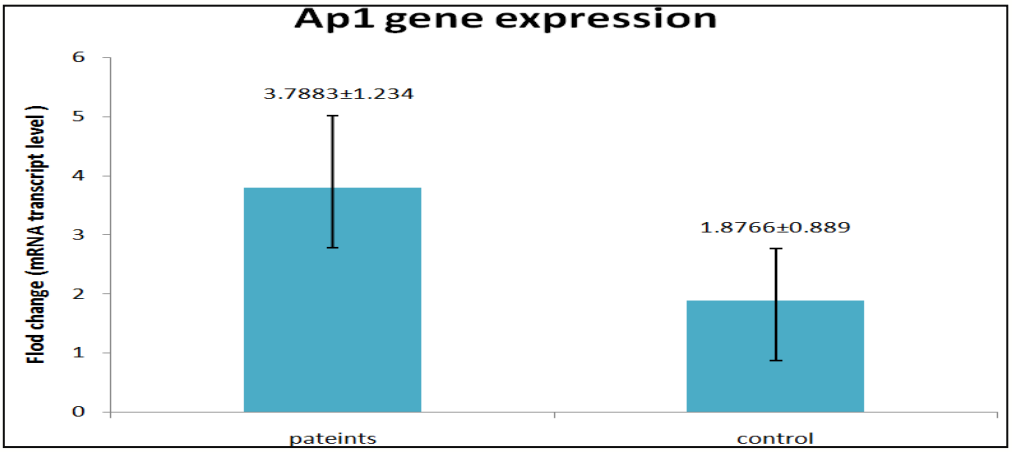

Figure 7. Gene expression of AP1 gene.

Quantitative Reverse Transcriptase Real- Time PCR: (RT-qPCR) was carried for the assaying of relative gene expression quantity for NFAT gene, FoxP3 gene and AP1.Gene expression values were monitored according to housekeeping gene expression (GAPDH) ,which was based on values threshold cycle numbers (CT) of amplification plot of target genes and housekeeping gene. Where the results of Real-Time PCR amplification plot of housekeeping gene GAPDH gene appeared no difference in CT value, where the control group (CT=27) while the treated groups which also appeared (CT=27) (Figure-1) While, The results of Real-Time PCR amplification plot of target genes (NFAT gene, FoxP3 gene and AP1gene) showed differences in CT value between control and treatment groups (Figure-2), (Figure-3) and (Figure-4).

Relative gene expression: The relative expression of (NFAT gene, FoxP3 gene and AP1gene) in patients' blood samples was estimated depending on Livak Method (2^-ΔΔCT) which based on normalization of RT-qPCR (CT values) of tested genes with (GAPDH) as reference gene in control and treatment groups. The results of relative gene expression in FoxP3 gene showed increment in patients group (17.723±1.227) compared with control group which was (2.133±1.234) as shown in

figure5 results of NFAT gene showed increment in patients group (13.676±2.223) compared with control group which was (2.743±1.133) as shown in figure-6 and result of AP1gene showed increment in patients group (3.7883±1.234) compared with control group which was (1.8766±0.889) as shown in figure-7. The statistical analysis of relative gene expression in FoxP3 gene, NFAT gene and AP1gene were found significant changes in patients groups in comparison with control groups at level ($P \leq 0.05$).

4. Discussion:

Molluscum infection observed in all parts of the world especially in the countries with warm climates, such as; Fiji, Papua New Guinea , and Congo. Infection often occurs In ages from1-5 years old and sometimes in infants. [24]. In the data that collected in this study it was obvious that the females were less than males in the infection ratio 95(46.3%) and 110(53.7%), Which may be due to the social and personal usual life style in Iraq, because Iraqis used to warm welcoming each other by hands and cheeks kissing, and the young adults age groups are the most affected groups. In any immune response, naïve CD4 cells differentiation occur resulting in production of many subsets such as effector cells (Th1, Th2, Th9, and Th17)and another type with regulatory function Treg. These cells work in orchestrating manner during immune response by keeping this response within certain frame [25].Protective but not harmful excessive response in which deleterious inflammatory response or autoimmune disease could occur.

Th17 cells are responsible for the secretion of IL-17, IL-17F, IL-23P19, IL-21, IL-22, and CCL20 [26]. The Results of IL-17A Showed increment in patients of Molluscum contagiosum. IL‑17 is produced by the Th17 cells, as well as CD8 T cells and innate cells, such as NK cells, lymphoid tissue inducer cells, innate lymphoid cells and γδ-T cells[27] and Th17 cells can induce the secretion of other important cytokines with variable properties, including IL-6 and IL-23, its noted that reinforcing the destructive function of Th17 subset in oral lesions [28] therefore, the results of IL-23P19 showed an increment in patients. T-helper 22 or (Th22) cell is a novel lineage of CD4+ T cells which produce interleukin (IL)-22 but not interferon-γ or IL-17. Th22 is very distinct from Th17 and other linages of CD4 cells function [29]. The Results of IL-22 Showed increment in patients of Molluscum contagiosum. One of the most effector molecules that produced by Th22 is IL-22 , it can work synergistically with IL-17 and TNF[30]. During the virus infection , the increased activity of cells such as Th-22 and Th-17 represent an indicator of the potency of the immune response against virus spread and pathogenicity.

Also results sowed increment in Foxp3 gene, NFAT gene and AP1gene by RT-qPCR. NFAT is an transcription factor which represent the key regulator that activate the immune cells upon immune response, by forming a complex with Ap-1 and the associated genes with naturally active after the

antigen stimulation [17]. Therefore, FOXP3 has a remarkable role in regulation the development and function of regulatory T cells. [31,32]. Activator protein 1(AP-1) is critical regulator of inflammatory responses, also proliferation and differentiation of T-cells (33, 34). It was found that the (Treg) regulatory T cell function is achieved by parallel cooperative binding of NFAT with the factor FOXP3 forkhead transcription (a lineage specification factor for Tregs). So in a case such as molluscum infectivity usually encountered completely by a strong effective immune response, and this response should be controlled by regulatory mechanism to reduce the tissue damages and any other deleterious effects may result from the immune response.

5. Conclusion

The findings of this study indicate that, Molluscum contagiosum is a well-known skin infection in Iraq, caused by a pox virus. The disease disappear within months in people with a normal health status. The potent immune response against the virus infection which occurs by Th-17 and 22 and the controlling mechanisms through the regulatory T-cells are very important in managing the virus pathogenicity and disease process development.

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