Post Kala-azar Dermal Leishmaniasis (PKDL) from the field to the cellular and the subcellular levels

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Introduction

• PKDL is a VL related skin condition characterized by macules, papules, nodules and a combination of these
• It occurs mainly in areas where VL is caused by *L donovani* and very rarely seen with other parasites: *L infantum* and *chagasi*.
Introduction

- The frequency of PKDL in Sudan is 58% in patients treated for VL. It is a reservoir for *L. donovani*. Parasite transmitted by the sand fly *P. orientalis*.
- In the majority of patients it occurs within the first two months following treatment of VL.
- Spontaneous healing occurs in the majority of cases.
- Lesions that do not heal in 6 moths become chronic and may continue for years. It requires drug treatment for 2-4 months.
- We developed a vaccine (L Major+ BCG adjuvent0 and used it with Pentostam and reduced the treatment of chronic cases to 40 days.
• The problem in PKDL is that it occurs in VL patients who are cured by drug treatment
• In other words the parasite is eliminated from the spleen, lymph nodes and bone marrow after treatment of VL.
• It appear in the skin. Reason and mechanism are poorly understood
• This presentation addresses the immunopathogenesis of PKDL and tries to unravel the conundrum of this intriguing condition
Clinical features

- Lesions of PKDL are papules, nodules, macules and a combination of these occurring in the exposed parts of the body. The face is always affected.

- This patient shows a papulonodular form of PKDL.
Distribution of lesions in PKDL

- PKDL often mirrors the clothing habits of the patient, being confined to or most severe in the sun-exposed parts of the skin.
Distribution of lesions in PKDL

- This patient, like many conservative females, has part of her face covered whenever she goes out.
- Her PKDL lesions were almost exclusively in the uncovered part of the face.
Distribution of lesions in PKDL

- The distribution of lesions in the face of the previous patient. Note the covered part of the face is free of lesions.
Clinical features of PKDL

• In grade 3 (most severe form) all of the body surface is affected
• This occurs in children who run about naked in the villages
Distribution of lesions in PKDL

- The parents of this child were better off than others. The child was always dressed in the traditional dress which leaves the face and part of the chest uncovered.
- The lower part of the rash marks the upper margin of the dress.
Diagnosis of PKDL

• In the past the diagnosis of PKDL was mainly on clinical features, the past history of VL and positive serology to leishmania infection
• This is because the parasite is rarely found in smears or sections from lesions
• Nowadays we use PCR for the definitive diagnosis of PKDL. The problem is that the test is not available except in specialized labs and is not available for the worker in the field
Two main observations to explain

• Why there is inflammation in the lesion when parasites are usually scanty and lesions persist for months or years
• Why lesions are more common or restricted to areas exposed to the sun exposed parts of the skin
• To answer these questions we had to link field observations with investigations at the cellular and subcellular levels. Hence the title of the presentation
Two Hypotheses were made

• Hypothesis 1: There is paucity of parasites but there is persistence of antigen in lesions. This requires cytotoxic T cells not cytokines to resolve.

• Since we have shown before and in the present study that cytotoxic T cells are present in lesion we argued that they are inactivated probably by Treg cells, known deactivator of CD 8 in autoimmunity and chronic infections
Hypothesis 2

• If Treg cells are involved in the pathology and since lesions are in sun-exposed skin and because UVB light damages Langerhans cells and such cells present antigen to Treg cells only we studied the morphology of Langerhans cells and the Treg cells to see if the cells show features of the effect of UVL.
Definition of a case of PKDL

- The following criteria are used to define a case of PKDL
- Patient should have the characteristic PKDL rash. The face must be involved
- Demonstration of parasite in lesion by microscopy, PCR and/or leishmania antigen in sections using *anti-L donovani* antibody.
Hypothesis # 1

• We first consider the first hypothesis
• In the next slides we show the histopathology of the of PKDL patients
• There are mainly 2 types of reaction:
  1. The first is characterized by a diffuse infiltrate composed mainly of macrophages, loose epithelioid granulomas and lymphocytes
  2. The second consists mainly of compact epithelioid granulomas.
• Parasites are usually undetectable in H&E stained sections in both types of reaction
Histopathology of the PKDL lesion

• We studied the pathology of PKDL lesions in a total of 40 cases

• After identifying the pathological changes we used several immunological and molecular techniques to explain the pathology in tissues and peripheral blood
Histopathology of a nodular PKDL lesion. Dense inflammation undetectable parasites. (H&E)

- Infiltrate in dermis composed of macrophages and small lymphocytes. Note degeneration of cells in basal layer of epidermis
Pathology

- In some patients the lesions consist mainly of epithelioid granulomas. These are found in long standing lesions.
- Parasites are scanty or not detectable.
• Leishmania parasites are few or apparently absent in legions
• In all cases macrophages and epithelioid cells are positive for leishmania antigen
• Patients who have compact granuloma have persistent PKDL usually for years when compared with those without compact granuloma. They require treatment with daily Pentostam for 2-4 months
• We developed a vaccine (Autoclaved *L. major* + BCG as adjuvant) and used it to treat cases along with Pentostam (Immunochemotherapy) and reduced treatment period to 40 days.
Five cases of PKDL showing leishmania antigen in macrophages. The red arrow shows antigen in the basal layer of the epidermis. Slide 3 shows amastigotes and antigen (Black arrow). Normal mouse serum and immune serum absorbed with promastigotes showed a negative results. Immunoperoxidase stain x40)
Demonstration of parasite and parasite load in lesions using real time PCR and conventional PCR

• We used the PrimerDesign genesig Kit for all leishmania species to determine the parasite DNA load in PKDL lesions.
• The method quantifies leishmania cytochrome b (cytb) kinetoplast gene in tissue relative to standard dilutions of a positive control of known copy numbers.
• the data were analyzed using MxPro QPCR software.
• We also used conventional PCR to demonstrate parasites
PCR and Real time PCR were used to detect Parasites. Both were positive for *L. donovani*.

Parasites were shown to be *L. donovani* by Real Time PCR and conventional PCR except in 2 cases where real time PCR was negative but conventional PCR was positive.
• All 15 cases except cases # 2 and 5 were positive for the cytochrome b gene of *L donovani*

• Cases 2 and 5 were positive for the leishmania antigen.

• Both cases were positive for *L donovani* by PCR
Cases 2 and 5 were positive for leishmania antigen.
Changes in Langerhans cells in PKDL are consistent with effects of UVB light.

Normal Langerhans cells.

Langerhans cells in PKDL: note depletion of the cells in the upper layers of epidermis.
Effect of UV light on epidermal Langerhans cells

- UVB light

Normal epidermal Langerhans cells

UVB light

DNA damaged cells Few or no dendrites.
Pick up Leishmania antigen in dermis

Home to regional lymph node

Stimulate Treg cells

Leishmania specific T reg home to the dermis where their specific antigen is
There they secrete IL-10 causing suppression locally
Langerhans cells in PKDL

Dermal infiltrate of PKDL lesion stained for CD1a

Cells are round and have few or no dendrites
Trafficking of Langerhans cells in PKDL

• In a previous study and the present one we showed that Langerhans cells are positive for leishmania antigen
• We traced them to the regional lymph nodes where they interact with T cells in the paracortex
• It is now known that the damaged Langerhans cells present antigen to Treg cells only. The Treg cells produce IL-10 and cause immunosuppression specific for the antigen that evokes them.
Cells in the PKDL lesion

- The majority of the cells in this lesion are CD3 positive T cells
Other cells in the PKDL lesion

- CD 8 cells form the majority of T cells
- Some are in basal layer
- Note lack of melanin in the basal layer (Arrow)
Other cells in PKDL lesions are Treg cells

- intact epithelioid granuloma with many Treg cells within the granuloma.
- Treg cells are recognized by their dark nuclei (Immunoperoxidase stain x 40).
Treg cells

• T reg cells are a subset of CD4 cells that suppress immune reaction in an antigen specific fashion
• They are CD4+CD25+Fox3+
• They are important for prevention of autoimmune disease
• They dampen the immune response in chronic inflammation to avoid tissue damage
Other cells in the PKDL lesion

- CD 68 showing many histiocytes; some have dendrites
Other cells in the PKDL lesion

• B cells are absent or scanty. This is unlike CL due to *L major*
Trafficking of Treg cells

• They suppress cell mediated reactions by secreting IL-10 when they come in contact with antigen they had been primed against.
• Thus they act only where the antigen is.
• In other words their action is not generalized in the body but is restricted to the area where the antigen resides.
To sum up so far

• We therefore explained the two hypotheses we made:
  
  • That parasites are scanty in lesions but all cases show leishmania antigen which is responsible for the inflammation. This needs CD\(^8\) cells to be eliminated from tissues
  
  • That Treg cells are generated in PKDL and that they specifically suppress cytotoxic CD\(^8\) cells and are generated in response to UVB damaged Langerhans cells
• After demonstrating Treg cells in lesions we studied these and other cell phenotypes in the peripheral blood
FACS analysis PBMC in PKDL in PKDL and endemic controls in Gedaref State

• We examined the cell phenotypes in the peripheral blood of PKDL patients before and after treatment or spontaneous regression:

We were particularly interested in the following:

1. Total lymphocyte count
2. CD 4
3. CD 8
4. Treg cells
Example: Cell phenotypes in peripheral blood of a PKDL patient: FACS analysis

- Up at the top are CD 4 and CD 8 gated cells
- Bottom is T reg cells and CD 4 T cells
- T reg form 33% of total CD 4 cells
- We repeated the tests after treatment as will be discussed later
PKDL. Case # 3 Left at presentation: Right after treatment or spontaneous healing

T reg cells 31%  

T reg cells 7%
Patient # 1

March/2011

June 2011

Sept/2011
Recurrence of PKDLS

- Patient was treated with Pentostam.
- She improved and her Treg cells returned to normal.
- When she relapsed Treg increased significantly.
PKDL patient relapsed after treatment

Treg dropped significantly at healing and rose sharply at relapse
Peripheral blood mononuclear cell counts compared to endemic controls

- **CD**4 & **CD**8 are significantly higher in PKDL compared to endemic controls: P values: 0.04, 0.01 respectively.
- **Treg** are significantly higher in patients compared to endemic controls: P value: 0.0000002
- There is no significant difference in **CD**4 & **CD**8 counts before and after healing: P values: 0.2, 0.08 respectively
  - **Treg** are significantly lower at healing: P value: 0.002.
- There is no difference in **CD**4, **CD**8 and **Treg** counts in healed PKDL and endemic controls: P values: 0.3, 0.4 and 0.2 respectively.
Cytokines in PKDL lesions

IL-10 positive cells in the dermal infiltrate. IL-4 was also found (not shown).

IFN-γ positive cells in the infiltrate of PKDL lesion.

Lesions show mixed Th1 and Th2 patterns. The same cytokine pattern was observed in the peripheral blood. The interferon-gamma is produced by T cells arriving from the spleen where they were produced as a result of treatment of VL.
Are there other causes contributing to the immunosuppression in PKDL

• And the answer is yes
• UVB induces several cytokines and some molecules some of which are immunosuppressant
Mechanism of immunosuppression: effect on keratinocytes. Urocanic acid the natural sunscreen in the stratum corneum

UVB

Keratinocyte

Isomerization of urocanic acid from trans to cis by UVB

TNF-α  IL-10

Langerhans cell

Presentation of antigen to Th2 cells
Cytokines in the epidermis and dermis
PKDL lesions

TGF-β in keratinocytes and dermis

TNF-α in keratinocytes and dermis

Another cytokine in keratinocytes is IL-12 (Not shown). All these cytokines are inducible by UVB.
What does all this mean?

• We showed that UVB induced Treg cells are major players in the pathogenesis of PKDL
• Persistent antigen is responsible for the persistence of inflammation in lesion. This requires CD cells to be eliminated
• CD 8 cells are present in lesions but they are suppressed by Treg cells
What is the implication of this?

- Since UVB is major factor in the pathogenesis of PKDL through stimulation of Treg cells and its effects on the keratinocytes we suggest that it may be possible to prevent PKDL by sun screens.
- This will be our next project.
- Patients who respond by a compact granuloma are unlikely to heal lesions and should be treated.
Contributors to this work

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• This work is by a team consisting of clinicians, a general practitioner, technicians, immunologist, molecular biologists using state of the art methodology

• The work was ongoing for the last 21 years!
Thank you