Introduction

- Apoptotic and necrotic cell death
- Clearance of dying cells
- Anti-inflammatory effects of apoptotic cells
- Systemic lupus erythematosus

Apoptotic cell clearance deficiency

- Accumulation of apoptotic cells in cultured PBMC of humans with SLE
- Accumulation of apoptotic cells in germinal centers of humans with SLE
- Apoptotic trash as antigen for the affinity maturation of anti-dsDNA antibodies
- Accumulation of apoptotic cells in the skin of humans with cutaneous lupus
- Humoral factors contribute to the clearance of apoptotic cells in several ways
- Clearance deficiency in stem cell-derived phagocytes
- Animal models for clearance deficiency

Conclusion

- Deficiency of the clearance of apoptotic cells is a risk factor for the development of SLE

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Introduction

Apoptotic and necrotic cell death

En masse apoptosis during development:
death of interdigital cells in the limb bud

The balance of life and death

- The human body contains about $1 \times 10^{14}$ cells - and more than $1 \times 10^6$ cells/sec regenerate
- Each day $90 \times 10^9$ neutrophils are produced in order to die after only 1 day (Cohen & Duke, 1992)
- Apoptosis is over 20 times faster than mitosis (Gerry Melino, The siren's song, Nature (2001))
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Inducers of apoptosis

- Cytokines (TNF, CD95L)
- Stress (UV, ROI)
- Growth factor withdrawal
- Prostaglandins
- Inhibition of kinases (Staurosporine)
- Inhibition of protein synthesis (CHX, ActD)
- Bacteria & viruses
- Lectin
- Anticancer drugs

Physiological or pathological
- Specific stimuli
- Genetically controlled
- Chromatin condensation
- DNA-laddering
- Sub-G1 DNA content
- Dye impermeable membranes
- ATP often consumed before lysis
- HMGB-1 frozen on chromatin
- Anti-inflammatory clearance

Primary necrosis
- Pathological
- Toxic extraneous cause
- Not genetically controlled
- No chromatin condensation
- High MW DNA fragments
- G1-S-G2 DNA content
- Loss of membrane integrity
- ATP often released
- HMGB-1 released
- Inflammatory clearance

Apoptosis

The sites of apoptosis

- "Constitutive" apoptosis:
  - In lymphoid tissues (e.g., thymus, lymph nodes, and spleen) a huge amount of non-selected lymphocytes die from apoptosis. These tissues contain many phagocytes, referred to as tingible body macrophages. Their main task is to instantly dispose autoreactive cells to avoid the stimulation of an auto-immune response.
  - Some of the cells dying during normal inflammation-free tissue turnover are phagocytosed by immature dendritic cells, which use their constituents as antigens for the establishment of peripheral tolerance.
  - "Expected" apoptosis:
    - During development many tissues undergo massive periods of remodelling. The transiently appearing high amounts of apoptotic cells are often cleared by both neighboring cells and professional phagocytes. This cooperation safely disposes dying cells to avoid secondary necrosis and inflammation.
  - "Accidental" apoptosis:
    - In the skin, mucosa, and other tissues exposed to the environment, apoptosis may be induced under certain pathogenic conditions (UV-irradiation, pathogens). In case of massive apoptosis, monocytes and granulocytes are recruited to clear the dying cells and to resolve inflammation.

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Exposure during apoptosis of phosphatidylserine
There are only few papers on apoptosis in the absence of PS exposure.
PS exposure is dependent on extracellular Ca²⁺
occurs downstream of the activation of caspases
is induced by NEM (inhibitor of amino-PL translocase)
in the absence of other indices of apoptosis
induced by NEM is sufficient to trigger removal by macrophages
Amino-PL translocase is sensitive to oxidative/reductive modification of its SH groups
ROS may also play a role in inhibition of the amino-PL-translocase

Exposure on viable cells of phosphatidylserine
PS colocalizes with lipid rafts in the outer membranes of activated, viable B cells.
Mature B cells expose PS on their surfaces, colocalizing with Ag-receptors.
Viable skeletal/heart muscles transiently expose PS during development.
PS is essential for myotube formation in mice.
Normal macrophages expose PS on their surfaces, which is required for phagocytosis of PS-exposing targets.
However, apoptotic HL-60 and Jurkat cells externalized up to 280-fold more PS than non-apoptotic controls.

Oxidation of phosphatidylserine
A preferential oxidation of PS is typical for apoptosis induced by oxidants.
There is a strong correlation between PS peroxidation and its externalization.
The radical scavenger, PMC completely protected phospholipids (but not PS) against oxidation.
In 1BuOOH induced apoptosis PS oxidation is highest in the plasma membrane.
These effects were suppressed by inhibitors of the NADPH oxidase, DPI, or by staurosporine.
zNAD-fmk protects PS (but not other phospholipids) against PMA-induced oxidation.
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**Cyt c as a catalyst for selective oxidation of PS during apoptosis**

- Release from mitochondria into cytosol of cyt c is a common event in apoptosis.
- In the cytosol, cyt c participates in the activation of the apoptosome.
- Positively charged (pI 10.3) cyt c unfolds and interacts electrostatically with acidic phospholipids (e.g., PS).
- Prooxidant activity of heme-containing cyt c is selectively directed toward PS.
- Superoxide and H₂O₂ produced by disrupted mitochondrial electron transport facilitates formation of ROS (e.g., o xo-ferryl) in the immediate vicinity of PS.
- PSox is externalized and may enhance co-externalization of PS.

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**Endogeneous pro- and anti-inflammatory molecules in activated cells**

- Non-activated
  - Uric acid
  - PS
  - HMGB-1
  - ATP

---

**Endogeneous pro- and anti-inflammatory molecules in early apoptotic cells**

- Early
  - Uric acid
  - PS
  - PS
  - HMGB-1
  - ATP
### Endogeneous pro- and anti-inflammatory molecules in late apoptotic cells

**Early**
- Uric acid
- PS
- HMGB-1
- ATP
- Uric acid crystals

**Secondary necrosis**
- Uric acid
- PS
- HMGB-1
- ATP
- Uric acid crystals

### Endogeneous pro- and anti-inflammatory molecules in necrotic cells

**Non-activated**
- Uric acid
- PS
- HMGB-1
- ATP
- Uric acid crystals

**Primary necrosis**
- Uric acid
- PS
- HMGB-1
- ATP
- Uric acid crystals

### Endogeneous pro- and anti-inflammatory molecules in apoptotic cells

<table>
<thead>
<tr>
<th>Viable cell not activated</th>
<th>Viable cell activated</th>
<th>Early apoptotic cell</th>
<th>Late apoptotic cell</th>
<th>Secondary necrotic cell</th>
<th>Primary necrotic cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low content and release</td>
<td>Intermediate content and release</td>
<td>Intermediate content low release</td>
<td>High content low release</td>
<td>High content low release</td>
<td>Low content sudden release</td>
</tr>
<tr>
<td>Sequestered inside</td>
<td>Low exposure no phagocytosis</td>
<td>Low exposure no phagocytosis</td>
<td>High exposure phagocytosis anti-inflammatory</td>
<td>High exposure phagocytosis anti-inflammatory</td>
<td>High exposure phagocytosis anti-inflammatory</td>
</tr>
<tr>
<td>Intermediate content</td>
<td>Low exposure limited secretion</td>
<td>Intermediate content Low release</td>
<td>Frozen to chromatin low release</td>
<td>Frozen to chromatin low release</td>
<td>Very low content sudden release</td>
</tr>
<tr>
<td>Sequestered inside no release</td>
<td>Frozen to chromatin low release</td>
<td>Frozen to chromatin low release</td>
<td>Frozen to chromatin low release</td>
<td>Frozen to chromatin low release</td>
<td>Frozen to chromatin low release</td>
</tr>
</tbody>
</table>

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Introduction

Clearance of dying cells

The communication network of apoptosis

Killer (cells, parasites, viruses, physico-chemical insult) Apoptotic cell Phagocyte

Fas, TNF-R

Fas, TNF-R

Caspases, CAD

Caspases, CAD

LPC

LPC

PS, PSbox, immature sugar

PS, PSbox, immature sugar

GST-R

GST-R

Integrins, Scavenger R, CD91

Integrins, Scavenger R, CD91

IL-10, TGFβ

IL-10, TGFβ

Apoptotic cell

Cleared by neighbouring cells

No inflammation

Cells get digested immediately

No cytokines

No immune reaction

Tolerance

Cleared by macrophages

Anti-inflammation

Cells get digested immediately

No IL-12, TNF but IL-10, TGFβ

Some cells get access to dendritic cells

Presentation of antigen without co-stimulation

No IL-12 ...

No immune reaction

Tolerance

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Necrotic cell

<table>
<thead>
<tr>
<th>Cleared by neighbouring cells</th>
<th>Cleared by macrophages</th>
<th>Some cells get access to dendritic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not described</td>
<td>Inflammation</td>
<td>Presentation of antigens with co-stimulation</td>
</tr>
<tr>
<td></td>
<td>Cells get digested immediately</td>
<td>IL-12 ...</td>
</tr>
<tr>
<td></td>
<td>No IL-10, TGFβ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>but IL-12, TNF</td>
<td></td>
</tr>
</tbody>
</table>

Immune reaction

- Cells get digested immediately
- No IL-10, TGFβ but IL-12, TNF
- Some cells get access to dendritic cells
- Presentation of antigens with co-stimulation
- IL-12...

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Summary
the clearance of apoptotic cells

In living tissues, the steady state of apoptotic cells is very low.
Most of them have already been taken up by neighbouring cells or by specialized phagocytes.
Apoptotic cells that are not cleared duly undergo secondary necrosis, disintegrate, and release toxic internal compounds.
Secondary necrotic cells exert mild pro-inflammatory effects.
They have been discussed to be sources for autoantigens that trigger and maintain autoimmunity.

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Putative model of phagocyte attraction

Activation of the Ca\textsuperscript{2+}-independent iPLA\textsubscript{2} induces the release of LPC

Model of caspase and iPLA\textsubscript{2} mediated attraction of phagocytes

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**Introduction**

Anti-inflammatory effects of apoptotic cells

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**Model of caspase and iPLA₂ mediated attraction of phagocytes**

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**Apoptotic cells exert an anti-inflammatory effect on monocytes activated with LPS**

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Voll et al., Nature 1997

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AxV skews cytokine secretion of apoptotic cell clearance towards inflammation

- No AxV
- With AxV

Benign

Blocking their clearance restores the Ig response against apoptotic cells

- Blocking AxV
- No blocking AxV

Stach et al., CDD 2000

P < 0.0001
P = 0.036
P = 0.0015
P = 0.044

Bacteria and viruses

IFNαβ
PAMP
LPS
CpG
Inflammation

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**Early apoptotic cells**

- Anti-inflammation

**Late apoptotic cells**

- Secondary necrosis

**Introduction**

Systemic lupus erythematosus

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Systemic lupus erythematosus SLE

- Genetic predisposition
- Environmental impact
- Racial influences
- Higher incidence in females
- UV sensitivity

SLE clinical characteristics

- Diagnosis made according to the actual ACR criteria
- Systemic autoimmune disease
- Virtually all organs can be affected
- Renal involvement
- Cardiovascular disease
- Reduced life expectancy

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SLE etiology

- Deficiencies of the early components of the classical complement pathway: C1q and C4
- MBL alleles with low MBL plasma levels (e.g., exon 1 codon 54 B, promoter-550 L, and -221 X)
- Associated with the HLA alleles DRB1*03 and B*08 (general propensity for autoimmunity)
- Associated with HLA-DR3 and with the extended haplotype DQB1*0201; DRB1*0301; TNF2; LTA2
- SLE patients with anti-Ro in the absence of anti-La are associated with the extended haplotype DQB1*0602; DRB1*1501; TNF1; LTA1
- Negatively associated with the DR7 extended haplotype DQB1*0303; DRB1*0701/2; TNF5; LTA3

SLE pathology

- Kidney deposition of immune complexes
- Atherosclerosis
- Cardiovascular disease
- Higher incidence of lymphoma

Apoptotic cell clearance deficiency

Accumulation of apoptotic cells in cultured PBMC of humans with SLE
The clearance of apoptotic cells
is impaired in MoMa of patients with SLE

NHD  SLE

Diseases with tissue accumulation
of apoptotic cells
Cystic fibrosis, an inflammatory lung disease, is characterized by influx and release of proteases of inflammatory cells into the airways.
The inflammation is persistent and necrotic/postapoptotic cells accumulate in the airways.
App. 50% of patients with SLE show accumulation of apoptotic cells in various tissues (lymph nodes, skin).
App. 50% of patients with CLE show accumulation of apoptotic cells in the skin.
Many apoptotic cells can be found in the circulation of patients with AIDS.
In a model of Chagas’ disease, a debilitating cardiac illness caused by Trypanosoma cruzi, the infection is accompanied by and dependent on intense lymphocyte apoptosis.

Apoptotic cell clearance deficiency
Accumulation of apoptotic cells
in germinal centers of humans with SLE
Apoptotic Cell Clearance Deficiency
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Uningested apoptotic nuclei can be found in germinal centers of some SLE patients

SLE RM
SLE KR
FL 75532
Non SLE 3278

Impaired clearance of apoptotic nuclei in germinal centers of SLE patients

Total apoptotic nuclei / mm²
P = 0.372

Free apoptotic nuclei / mm²
P < 0.01

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Impaired clearance of apoptotic nuclei in germinal centers of SLE patients

<table>
<thead>
<tr>
<th>Size of CD68+ cells / µm</th>
<th>Macrophages / mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLE</td>
<td>non-SLE</td>
</tr>
<tr>
<td>15</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td>-</td>
</tr>
<tr>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td>35</td>
<td>-</td>
</tr>
<tr>
<td>40</td>
<td>-</td>
</tr>
</tbody>
</table>

P = 0.026
P = 0.17

P = 0.019
P < 0.01

CD68-PE TUNEL

non-SLE 40187
SLE BG

CD21-PE TUNEL

SLE RM
Summary
accumulation of apoptotic cells in germinal centers of humans with SLE
Scavenging of apoptotic cells in the germinal centers is insufficient
Non cleared apoptotic cells accumulate and undergo secondary necrosis
Chromatin of disintegrated apoptotic cells activates complement
Complement opsonized chromatin associates with the FDC

Apoptotic cell clearance deficiency
Apoptotic trash as antigen for the affinity maturation of anti-dsDNA antibodies

Are there implications of a phagocytic defect for the generation of anti-dsDNA autoantibodies?

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### Binding specificity of 33.C9 variants

<table>
<thead>
<tr>
<th>Method</th>
<th>Phenotype of Mutant</th>
<th>specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td>SPR</td>
<td>ELISA</td>
</tr>
<tr>
<td>33D TN/HE</td>
<td>o o o</td>
<td>o</td>
</tr>
<tr>
<td>33D TN</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>33D 100K</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>33D 10K</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>33D</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>33D 10K</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>33D 100K</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>33D 10K</td>
<td>o</td>
<td>o</td>
</tr>
</tbody>
</table>

### The 33.C9 Ab has gained its anti-dsDNA activity in the germinal center reaction

Binding analysis using surface plasmon resonance:
33.C9 mAb and binding mutants bind to dsDNA with similar $K_a$ and $K_d$ values.

- $K_d = 10^{-8}$

#### Sensograms

<table>
<thead>
<tr>
<th>Association</th>
<th>Dissociation</th>
</tr>
</thead>
<tbody>
<tr>
<td>10, 5, 2.5, 0.6</td>
<td>0</td>
</tr>
</tbody>
</table>

### Both analyzed high affinity DNA binding IgG autoAb

T. Winkler 2005
Apoptotic cell clearance deficiency

Accumulation of apoptotic cells in the skin of humans with cutaneous lupus

Accumulation after UV exposure of apoptotic cells in the skin of patients with CLE (act caspase 3)

Kuhn et al., 2004

Accumulation after UV exposure of apoptotic cells in the skin of patients with CLE (TUNEL)

p<0.0019

Relative apoptotic cell count [%]

controls CLE

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Apoptotic cell clearance deficiency

Humoral factors contribute to the clearance of apoptotic cells in several ways

Several components of the sera of NHD have been described to bind and opsonize dying and dead cells. These factors are heat labile and are strongly increasing the clearance activity of macrophages. The sera of some SLE patients contain factors which even more strongly increase the clearance by NHD-derived macrophages of dead cells. However, there are also SLE patients' sera that show no support of the clearance by NHD-derived macrophages.

Apoptotic cell clearance deficiency

Clearance deficiency in stem cell-derived phagocytes

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SLE and NHD-derived stem cells show similar proliferation *in vitro* V15.3.05

CD34 positive PBMC cultured 8 days in stem cell medium

NHD 100µm SLE 100µm

SLE and NHD-derived stem cells show similar proliferation *in vitro* V23.5.05

CD34 positive PBMC cultured 7 days in stem cell medium

NHD SLE

G1: 43% S: 48% G2: 9%

G1: 43% S: 48% G2: 9%

SLE stem cell-derived Mph show reduced uptake of IgG-coated beads V9.03

CD34 positive PBMC cultured 10 days in stem cell medium and 10 days in 10% FCS and GM-CSF

NHD 100µm SLE 100µm

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Summary

SLE and NHD-derived stem cells show similar proliferation in vitro.

The differentiation into adherent macrophages is reduced in SLE stem cell cultures.

Phagocytic activity of SLE stem cell-derived macrophages is partially reduced in vitro.

In certain SLE patients' macrophages there is an intrinsic defect of particle uptake and cell clearance.

Apoptotic cell clearance deficiency

Animal models for clearance deficiency

Gene targeted mice with SLE-like symptoms

- MER: Impaired phagocytosis of apoptotic cells
- DNase1: Impaired removal of chromatin, ANA, glomerular IC, glomerulonephritis
- SAP: Defective handling of chromatin, ANA, severe glomerulonephritis
- C1q: Deficient removal of apoptotic cells, autoantibodies, glomerulonephritis
Conclusion

The deficiency of the clearance of apoptotic cells is a risk factor for the development of SLE

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