

## Different localisation of cystatin C in immature and mature dendritic cells

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**Background.** Limited antigen degradation by proteolytic enzymes and their control by protease inhibitors represent a crucial step in generating antigenic peptides inside the endocytic pathway of antigen-presenting cells such as dendritic cells.

**Methods.** Human dendritic cells were used as a cell model in which quantitative immunogold electron microscopy was applied in order to study endogenous protease inhibitor cystatin C. Ultrathin cryosections were prepared from immature and mature dendritic cells and labelled with specific antibody. Under the transmission electron microscope gold particles, bound to specific probe (antibody), pointed the exact localization of labelled inhibitor.

**Results.** Quantification of immunogold labelling and further statistical analysis by chi-squared test emphasized the differences in cystatin C content in different cell compartments.

**Conclusions.** Statistically significant differences in intracellular distribution of cystatin C have been determined between immature and mature dendritic cell population.

*Key words:* dendritic cells; cryoultramicrotomy; microscopy, electron; immunohistochemistry

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### Introduction

Following the processes of internalization, different particles, parts of apoptotic and necrotic cells, pathogens (bacteria, viruses) and other foreign proteins are taken up into the endocytic pathway of immune cells such as macrophages and dendritic cells (DC).<sup>1</sup> As antigen-presenting cells (APC) these cells play a pivotal role in the adaptive immunity by initiation of efficient T-cell mediated immune response.<sup>2</sup> DC circulate through tissues like veiled scavengers. Their maturation

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Abbreviations: Ag - antigen, APC - antigen-presenting cells, DC - dendritic cells, MHC II - major histocompatibility complex class II molecules, TNF- $\alpha$  - tumour necrosis factor-alpha, TEM - transmission electron microscopy

occurs as they migrate from peripheral tissues, where they search for antigen (Ag), to lymphoid organs, where they present captured and processed Ag to specific T cell receptors. Limited Ag degradation performed by proteolytic enzymes (cathepsins) and their control by intracellular protease inhibitors represent a crucial step in generating antigenic peptides.<sup>3,4</sup>

Due to the increased research interest in events taking place inside the endocytic pathway<sup>5</sup> of immune cells, a high-resolution technique has been applied to observe cell organelles and search for specific intracellular proteins. Differentiation of monocytes to immature DC and their further maturation with TNF- $\alpha$  to mature DC population<sup>6,7</sup> was applied as a model system for studying transport pathway of endogenous protease inhibitor cystatin C in immune cells. Described method consists of preparation of ultrathin cryosections from frozen cells,<sup>8</sup> their immunogold labelling,<sup>9</sup> quantification of attached gold particles by transmission electron microscopy (TEM) and statistical analysis of obtained data.<sup>10</sup>

### Material and methods

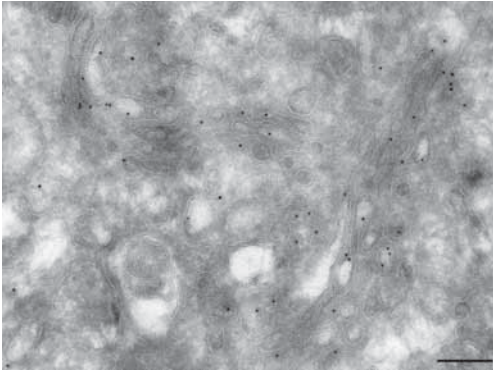
Immature DC were generated from isolated blood monocytes and further stimulated with TNF- $\alpha$  as described.<sup>6,7</sup> Cells were fixed with 4% paraformaldehyde (Sigma-Aldrich). They were embedded into 10% gelatin (Merck), infused with 2.3 M sucrose (Merck) and cut to ultrathin cryosections (70 – 80 nm) at minus 120 °C<sup>8,9</sup> using Leica EM FC6 ultramicrotome for cryosectioning. Sections were thawed in a droplet of 1:1 mixture of 2.3 M sucrose (Merck) and 2% methyl cellulose (Sigma-Aldrich). Retrieved ultrathin sections were put on carbon-coated formvar films (TAAB Lab.) and spread over hexagonal Cu/Pd grids (Agar). Sections were labelled with rabbit anti-hu-

man cystatin C antibody<sup>11</sup> or rabbit anti-human cathepsin S antibody.<sup>12</sup> Their specificity and cross-reactivity against recombinant human antigens<sup>13,14</sup> was checked as reported<sup>7,11,12,15</sup> prior to described immunogold labelling experiments. After the removal of an excess of primary antibody thin sections of DC were incubated with Protein A-gold conjugate with 10-nm gold particles (Cell Microscopy Centre, University Medical Centre Utrecht). Sections were contrasted with uranyl acetate (Aurion) and viewed with Philips CM120 BioTWIN transmission electron microscope. KeenView digital camera and iTEM software were used for taking pictures.

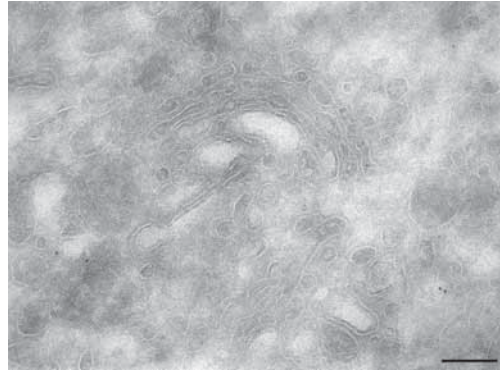
Quantification of immunogold labelling patterns in the same sets of compartments (or organelles) in different groups of cells (immature and mature DC) was performed for protease inhibitor cystatin C. Random sampling of specimen was followed by counting of approximately 200 gold particles on two labelled grids. The distributions of gold particles in different compartments was compared by contingency table analysis with statistical degrees of freedom for chi-squared values being determined by the number of compartments and the number of experimental groups of cells. Compartmental chi-squared values making substantial contributions to the total chi-squared values identified where the main differences between groups resided.<sup>10</sup>

### Results

Strong immunogold labelling of Golgi was observed in immature DC (Figure 1) whereas in mature DC Golgi remained practically unlabelled after incubation with specific antibody (Figure 2). Gold counts and results of calculations for Golgi and other compartments (organelles) are provided in Figure 3 and Table 1. High number of gold



**Figure 1.** Strong immunogold labelling of protease inhibitor cystatin C in Golgi apparatus of immature dendritic cells. Gold particles are showing the exact localization of cystatin C. Bar: 200 nm.

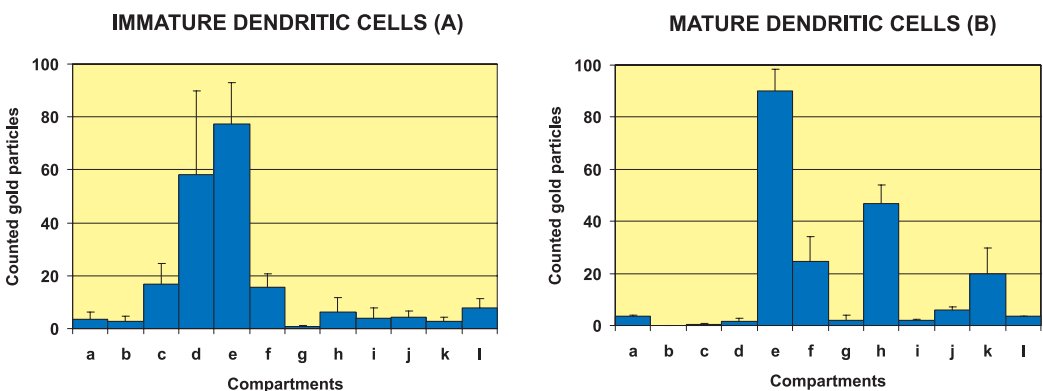


**Figure 2.** Golgi apparatus in human mature dendritic cells is not labelled for cystatin C. Bar: 200 nm.

particles associated with Golgi (Figure 1, Figure 3A), endoplasmic reticulum (Figure 3A) and nuclear membrane (Figure 3A) indicated high expression of cystatin C in immature DC. In mature DC number of gold particles increased in multivesicular bodies and small vesicles near the plasma membrane (Figure 3B).

Of 204 gold particles counted in immature DC 59 were associated with Golgi (Figure 3A, Table 1). In contrast, in mature DC 203 gold particles were distributed so

that only 2 were observed on Golgi (Figure 3B, Table 1). By contingency table analysis, the predicted number of gold particles on a given compartment in a given cell group is given by  $[(\text{column total} \times \text{row total})/\text{grand total}]$  where the grand total is the sum of the column (or row) totals.<sup>10</sup> Consequently, the predicted number of Golgi gold particles in immature DC, for example, is calculated to be  $[(61 \times 204)/407]$  or roughly 30.6 (Table 1). For each compartment and cell type, the partial chi-squared value is given



**Figure 3.** Comparison of immunogold labelling of inhibitor cystatin C in immature (A) and mature (B) dendritic cells. Gold particles were counted on two grids. Distributions of app. 200 gold particles (+ SD) are shown. Compartments: (a) nucleus, (b) nuclear membrane, (c) rough endoplasmic reticulum, (d) Golgi apparatus, (e) small vesicles, (f) multivesicular bodies, (g) multilamellar bodies, (h) vesicles near plasma membrane, (i) plasma membrane, (j) cytoplasm, (k) mitochondria, (l) unknown structures, other.

Compartments	Immature dendritic cells	Mature dendritic cells	Rows total	Chi-squared values	
				Immature dendritic cells	Mature dendritic cells
nucleus (a)	4 (4.0)	4 (4.0)	8	<0.01	<0.01
nuclear membrane (b)	3 (1.5)	0 (1.5)	3	1.49	1.50
endoplasmic reticulum (c)	17 (9.0)	1 (9.0)	18	7.06	7.09
Golgi apparatus (d)	59 (30.6)	2 (30.4)	61	26.43	26.56
small vesicles (e)	78 (84.2)	90 (83.8)	168	0.46	0.46
multivesicular bodies (f)	16 (20.6)	25 (20.5)	41	1.01	1.01
multilamellar bodies (g)	1 (1.5)	2 (1.5)	3	0.17	0.17
vesicles near plasma membrane (h)	6 (26.6)	47 (26.4)	53	15.92	16.00
plasma membrane (i)	4 (3.0)	2 (3.0)	6	0.33	0.33
cytoplasm (j)	5 (5.5)	6 (5.5)	11	0.05	0.05
mitochondria (k)	3 (11.5)	20 (11.5)	23	6.31	6.34
unknown structures, other (l)	2 (6.0)	4 (6.0)	12	0.66	0.66
<b>Columns total</b>	<b>204</b>	<b>203</b>	<b>407</b>	<b>120.02</b>	

**Table 1.** Labelling distributions of inhibitor cystatin C and chi-squared test. Values represent observed and expected (*in brackets*) numbers of 10-nm gold particles in immature and mature dendritic cells. For a total chi-squared of 120.02 and 11 degrees of freedom,  $P < 0.001$ . These two labelling distributions are significantly different.

by  $[(\text{observed golds} - \text{expected golds})^2 / \text{expected golds}]^{10}$ . Therefore, the partial chi-squared value for Golgi in immature DC is equivalent to  $[(59 - 30.6)^2 / 30.6]$  or 26.4. Table 1 shows that the total chi-squared value is 120.02. For 11 degrees of freedom [(2 - 1 columns) by (12 - 1 rows)] this indicates that the null hypothesis of no difference between groups must be rejected ( $P < 0.001$ ).

Therefore, the distribution of cystatin C is significantly different in immature and mature DC and the gold counts are consistent with a shift towards greater-than-predicted labelling of Golgi in immature DC (Table 1).

Furthermore, the partial (or compartmental) chi-squared values indicate that immature DC have more-than-expected gold particles on Golgi, endoplasmic reticulum and nuclear membrane (Table 1). On the contrary, partial chi-squared values indicate that mature DC have fewer-than-expected gold particles on Golgi but more-than-expected gold particles on the vesicles near the plasma membrane (within

a distance of 200 nm), small vesicles and multivesicular bodies (Table 1).

Population of multivesicular bodies (in addition to some other organelles) has also been labelled with anti-cathepsin S antibody and gold in both, immature and mature, DC population (data not shown).

## Discussion

Antigen processing by MHC class II molecules (MHC II) is tightly linked to the activity and stability of proteolytic enzymes present inside the endocytic pathway of DC.<sup>16</sup> Lysosomal cysteine proteases (cathepsins), which constitute a major portion of this proteolytic system, have an essential role in both Ag processing and maturation of MHC II.<sup>17</sup> Among the regulatory molecules of cathepsins a low molecular weight (MW 13.000) type II inhibitor cystatin C was suggested to have a role in controlling the proteolytic activity of cathepsin S inside the endocytic route of mouse DC.<sup>18,19</sup> By binding to cathepsin S during

DC maturation cystatin C was suggested to compromise the step-wise degradation of MHC II-associated chaperone molecule (invariant chain).

We have confirmed that in human immature DC cystatin C content was highly elevated compared to mature DC population as well as to their precursors – monocytes.<sup>7</sup> But with this study, cystatin C extremely high content in Golgi apparatus as well as its presence in endoplasmic reticulum and nuclear membrane, continuous with the endoplasmic reticulum, was quantified for the first time in human immature DC. Furthermore, the transport of cystatin C was shown from Golgi towards the cell membrane, supported by the decrease of cystatin C in Golgi and endoplasmic reticulum and increase in labelling of different populations of transport vesicles (small vesicles, vesicles near plasma membrane) and multivesicular bodies.

MHC II-loading compartments (multivesicular bodies) were positive for cathepsin S<sup>13,14</sup> (proposed target enzyme of cystatin C, involved in maturation of MHC II) in both, immature and mature DC (Zavašnik-Bergant, unpublished data). Labelling of multivesicular bodies for cystatin C was higher-than-expected in mature but not immature DC indicating higher content of cystatin C inside Ag-loading compartments in mature DC compared to immature population. Differentiation and maturation dependence of endogenous cystatin C supports its intracellular regulatory potential in human DC<sup>7</sup>, as well as it further suggests its new role in Golgi apparatus of immature DC.

In conclusion, preparation of cryosections of human DC, their immunogold labelling with specific anti-cystatin C antibody and transmission electron microscopy were successfully applied to quantify protease inhibitor cystatin C in different organelles of immature and mature DC.

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## Zaščita plodnosti pri bolnicah z rakom

Del Pup L, Campagnutta E, Giorda G, De Piero G, Sopracordevole F, Sisto R

**Izhodišča.** Biološko starševstvo je pomembno tudi za bolnike z rakom, zato vedno pogosteje že pred pričetkom onkološkega zdravljenja zaščitimo njihovo plodno sposobnost. Naloga onkologov je bolnike seznaniti z možnostjo, da bo onkološko zdravljenje lahko trajno okvarilo njihovo plodnost in kakšne so možnosti, da bi to jatrogeno okvaro zmanjšali ali preprečili. Metode zaščite plodnosti se zelo hitro razvijajo, čeprav o njih v strokovni literaturi ne poročajo velikokrat. Da bi onkologom pomagali pri svetovanju bolnicam z rakom, v članku navajamo indikacije, kontraindikacije, omejitve in kontraverznosti različnih zaščit plodnosti.

**Zaključki.** Ko onkolog obravnava bolnice z rakom, ki so v rodnem obdobju, naj bolnice seznanjeni z možnimi posledicami zdravljenja in z načini, da bi bolnica ohranila plodno sposobnost. Priporočamo, da bolnico čim prej napoti na posvet k strokovnjaku, ki ima izkušnje z zdravljenjem plodnosti. Če se bolnice odločijo za eno od metod zaščite plodnosti, predlagamo vključitve v študijske raziskave, ki bodo lahko pokazale učinkovitost posamičnih metod.

## Različna lokalizacija cistatina C v nezrelh in zrelih dendritičnih celicah

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**Izhodišča.** Razgradnja antigenov s proteolitskimi encimi (proteazami) v endocitozni poti antigena predstavitevni celic (dendritičnih celic) ter njihova regulacija z inhibitorji proteaz predstavlja pomemben korak pri nastanku antigenskih peptidov.

**Metode.** Človeške dendritične celice so bile uporabljene kot celični model za študij proteaznega inhibitorja cistatina C. Pripravljene so bile tanke zamrznjene rezine nezrelh in zrelih dendritičnih celic ter označene s specifičnimi protitelesi za kvantitativno elektronsko mikroskopijo. Pod transmisijskim elektronskim mikroskopom so zrna koloidnega zlata, vezana na specifične sonde (protitelesa), pokazala natančno lokalizacijo označenega inhibitorja.

**Rezultati.** Ovrednotenje označenih celic s statističnim testom Hi-kvadrat je potrdilo razlike v vsebnosti cistatina C v različnih celičnih organelih.

**Zaključki.** Potrjena je bila statistično značilna razlika v znotrajcelični porazdelitvi cistatina C med populacijama nezrelh in zrelih dendritičnih celic.